Aus dem Institut für Virologie
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Novel insights on viral factors involved in the Marek's Disease virus' life cycle

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“Seja corajoso. Assuma riscos. Nada pode substituir a experiência.”

“Be brave. Take risks. Nothing can substitute experience.”

-Paulo Coelho.

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1. Table of Contents

2. List of Figures

3. List of Tables

4. List of Abbreviations

Components of this list are presented in alphabetical order

ƛ- Lambda

∆mTMR – Virus containing the deletion of the multiple telomeric repeats

AIDS – Acquired immune deficiency syndrome

ALT - Alternative telomeral lenghtening

ANHEJ - Alternative non-homologous end joining

APC - Antigen presenting cells

BAC – Bacteria artificial chromosome

BHV-1 - Bovine herpesvirus 1

CECs- Chicken embryo cells

CEFs- Chicken embryo fibroblasts

CR – *Carina* retina cells

DAPI- 4′,6-diamidino-2-phenylindole

DD - Destabilization domain

DHFR – Dihydrofolate reductase

DIG- Digoxin

DMEM- Dulbecco's Modified Eagle Media

DNA – Deoxyribonucleic acid

DSB - Double strand break

EBV - Epstein-Barr virus

ER - Endoplasmic reticulum

FACS - Fluorescence-activated cell sorting

FBS – Fetal bovine serum

FISH – Fluorescent *in situ* hybridization

FITC - Fluorescein isothiocyanate

GFP – Green fluorescent protein

HCMV - Human cytomegalovirus

HHV-6 – Human herpesvirus 6

HR - Homologous recombination

HSV-1 – Herpes simplex virus 1

HVEM – Herpesvirus entry mediator

HVT – Herpesvirus of Turkeys

ICP8 - Infected cell protein 8

ICP4 – Infected cell protein 4

ICTV – International Committee on Taxonomy of Viruses

IE – Immediate early gene

iNOS - Inducible nitric oxide synthase (iNOS

IRL - Internal repeat long

IRs- Internal repeat short

MD - Marek's disease

MDV – Marek’s disease virus

MEM – Minimum essential medium

mTMR - Multiple telomeric repeats

NF - Non-functional

NHEJ - Non homologous end joining

NLS - Nuclear localization signal

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

qPCR – Quantitative PCR

PRV -Pseudorabies virus

RFLP – Restriction fragment length polymorphism

RNA – Ribonucleic Acid

RT – Room temperature

SDS – Sodium dodecyl sulfate

shRNA – Short-hairpin RNA

SSA – Single strand annealing

SSB - Single stranded DNA binding protein

sTMR - Short telomeric repeats

TERT - Catalytic subunit of reverse transcriptase

THF - 5,6,7,8-tetrahydrofolate

TMP - Trimethoprim

TR_L - Terminal repeat long

TR_S - Terminal repeat short

U_L - Unique long

U_S - Unique short

VZV – Varicella zoster virus

5. Introduction

Viruses are microorganisms that comprise a massive proportion of our environment. Basically, all living things encounter billions of different virus particles, every day. Considering that viruses are obligate intracellular parasites and dependent on their host cells for all aspects of the viral life cycle, the human and the animal bodies are essentially reservoirs for viruses that reside in the respiratory and gastrointestinal tracts, as an example. Every cell in the human and animal body contains viral DNA. As example, retroviruses that are endogenous to humans and their elements make up about 5-8% of the human DNA [1]. However, this DNA is most of the time inactive, being a remnant fossil from infections of germ cells that have been occurring over millions of years during human and animal evolution.

Considering the constant exposure of humans and animals to viruses throughout their existence, it is nothing short of amazing that the vast majority of viruses that infect them have a minimal impact on health and welfare of the host. Vertebrates owe such relative safety to their complex immune defence systems, which, together with viruses, evolved to fight potential infections. Nevertheless, despite such elaborated defences, some of the most devastating diseases have been or still are caused by viruses, such as influenza, rabies, yellow fever and AIDS. Viruses are still responsible for approximately 20% of the human cancer burden [2], whilst viral infections of the respiratory and gastrointestinal tracts kill over millions of individuals in the developing world, yearly [3]. These facts show that the biomedical importance of viruses and its impact in human and animal lives is indeed unquestionable.

Because viruses are fully dependent on their hosts for survival and propagation, ground-breaking studies of different animal viruses as models for human viral diseases established many fundamental principles of virus infection, disease onset and development and outbreak predictions [4–6]. One of the biggest revelations of the modern century virology was the genetic basis of cancer transforming viruses, indicating that more than ever, studies of viruses are of indispensable matter for the promotion of human and animal health.

The impact of viruses on highly complex multicellular systems such as humans and animals justifies indeed the expansion of new research projects to better understand these fascinating, minimized, yet imperative microorganisms.

5.1 Herpesviridae

Herpesviruses are highly spread throughout different environments in nature. Over 200 vertebrate and two invertebrate species have been identified as possible hosts for herpesviruses so far [7–10]. The International Committee on Taxonomy of Viruses (ICTV) has updated the taxonomy of herpesviruses in 2009, where the former *Herpesviridae* family has been split into three families that have been incorporated into the new order *Herpesvirales* [9]. The revised family *Herpesviridae* maintains the mammal, bird and reptile viruses, whilst the new family *Alloherpesviridae* incorporates the fish and frog viruses and the *Malacoherpesviridae* comprises two identified herpesviruses of invertebrates, one of oysters and one of another edible mollusk, the abalone.

The *Herpesviridae* family is further divided in three different subfamilies: the *Alpha-, Beta-* and *Gammaherpesvirinae*, which are based initially on their common genetic and biological characteristics [11]. The typical morphology of all herpesviruses known today can be attributed to years of evolution, where common characteristics of herpesvirus virions emerged from an ancestor virus that existed prior to the first vertebrates [8]. The typical anatomy of a herpes virion comprises the following structures: envelope, outer tegument, inner tegument, capsid and a double stranded (ds) DNA genome (Figure 1) [9].

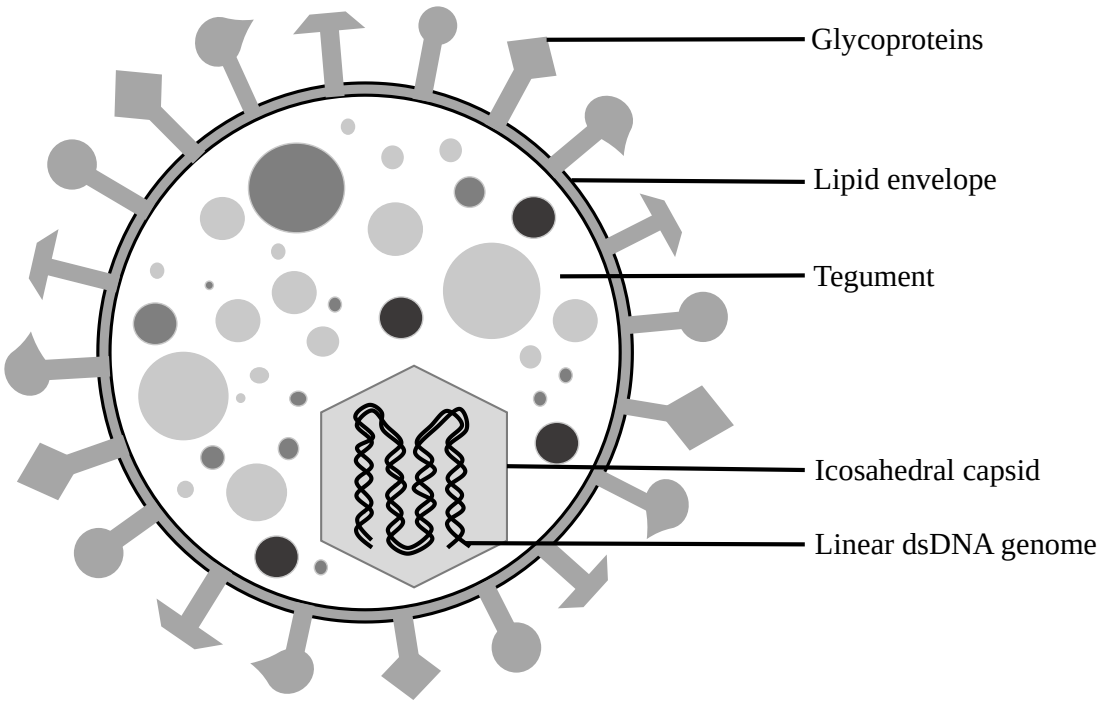

Figure 1. Schematic representation of a typical herpesvirus virion morphology. Each virion contains a liner, double-stranded DNA genome of approximately 108-290 kilo base pairs (kb) that is located inside an icosahedral capsid. This capsid containing the dsDNA is present

in the tegument, which is a proteinaceous matrix of different thickness delimited by a lipid bilayer envelope obtained through the process of viral budding from cellular membranes of the host.

5.1.1 Subfamily *Alphaherpesvirinae*

Members of this subfamily are divided into five different genera. Some prototypic examples of each genus are the human herpesvirus 1 (herpes simplex virus 1 (HSV-1); genus *Simplexvirus*), human herpesvirus 3 (varicella-zoster virus (VZV); genus *Varicellovirus*), gallid herpesvirus 2 (Marek's disease virus (MDV); genus *Mardivirus*), gallid herpesvirus 1 (infectious laryngotracheitis virus; genus *Iltovirus*) and chelonid herpesvirus 5 (genus *Scutavirus*) [12]. Alphaherpesviruses are classified together due to their common biological properties. Most viruses in this subfamily have a fast lytic cycle followed by lysis of infected cells and establishment of latent infections initially in sensory ganglia or mononuclear blood cells [12]. In such aspect, alphaherpesviruses differ from betaherpesviruses, mainly because members of the betaherpesvirinae subfamily usually have a slow replicative cycle with delayed cell lysis. Moreover, betaherpesviruses usually remain latent in secretory glands, kidneys and lymphnodes, but can also be kept in other tissues [13].

Despite the differences between the biological dynamics of alpha and beta herpesviruses, both still share common biological processes during latent infection, such as viral integration into the host telomeres. Examples of this are the MDV, an alphaherpesvirus, and human herpesvirus 6 (HHV-6), a betaherpesvirus.

5.2 The replication and life cycle of alphaherpesviruses

Unless otherwise stated, the descriptions that are to follow are based on the studies performed in Herpes simplex virus type 1 (HSV-1), a virus who is the prototype member of alphaherpesviruses.

5.2.1 Attachment and entry of herpesviruses

Once a herpesvirus enters an organism, it will search for target host cells to infect and initiate the process of virus replication. The initial process when the herpesvirus attaches to the surface of a host cell is called attachment. Here, viruses reach the target cells and approach them via pedesis or Brownian motion, followed by the association with the cell membrane.

[14]. Following this first approach, there is the interaction of the herpesvirus with the target host cells' receptor, which is a more specific interaction. Here, cellular receptors interact with the virion membrane's integrated glycoproteins allowing a strong binding. There are two herpesvirus glycoproteins that binds to glycosaminoglycans (GAGs) and mediate the actual entry process of alphaherpesviruses: glycoprotein (g)C and glycoprotein (g)B. [15–17]. Despite these, there are other glycoproteins such as gD, gH and gL who supposedly will form a complex with gB and induce a conformational change in the fusogenic gB [16] in a process that has not yet been fully understood. Importantly, gB is the glycoprotein that then allows the cellular and viral membranes to merge. There are suggestions that the exact location where the membrane fusion event occurs may vary since HSV-1 [18,19] has been proposed for direct membrane fusion on the cell surface. After the envelope has been left behind and the viral particle has been successfully released into the cytoplasm, the viral capsid is taken via the microtubule network to the nucleus [20]. When the capsid approaches the nucleus, virus DNA is "injected" into it, where it circularizes, in the case of HSV-1, or it integrates into the host chromosomes, in the case of Marek's disease virus (MDV), and can proceed with gene expression and further DNA replication [13,21].

5.2.2 Lytic replication

A defining feature of herpesviruses is the ability to switch from lytic to latent infection. The virus initially multiplies during the lytic stage in specific types of cells and generates new virions. Consequently, herpesviruses use lytic replication as a process to increase the viral progeny massively, and it happens concomitantly with the expression of a complete set of genes in a cascade-like structured way, another hallmark of herpesvirus replication (Figure 2) [13,21]. The earliest viral proteins present in the infected cell, however, are those brought in by the tegument [22]. The first genes that will be expressed are the immediate-early genes (IE), who will have its expression induced by viral transcriptional activators, without any *de novo* translation needed. These IE genes in most of the cases encode additional sets of transcriptional regulators, which are essential to trigger the next step in the cascade: the expression of early genes (E), whose proteins initiate DNA replication. Next, the DNA replication must switch to a rolling circle mechanism so it can be packed into capsids in order to generate the necessary concatemers that can be cleaved into full-length genomes [23–25] (Figure 3). After this, late viral genes (L) can be transcribed and translated with the onset of DNA replication. L gene encoded-proteins primarily set the components needed to form viral structures, such as capsid, tegument, and glycoproteins [25]. Upon completion of the whole

process, the virus has completely taken over the molecular processes of the cell and used it to produce more virions, which can be evidenced by the consequent cytotoxic effects.

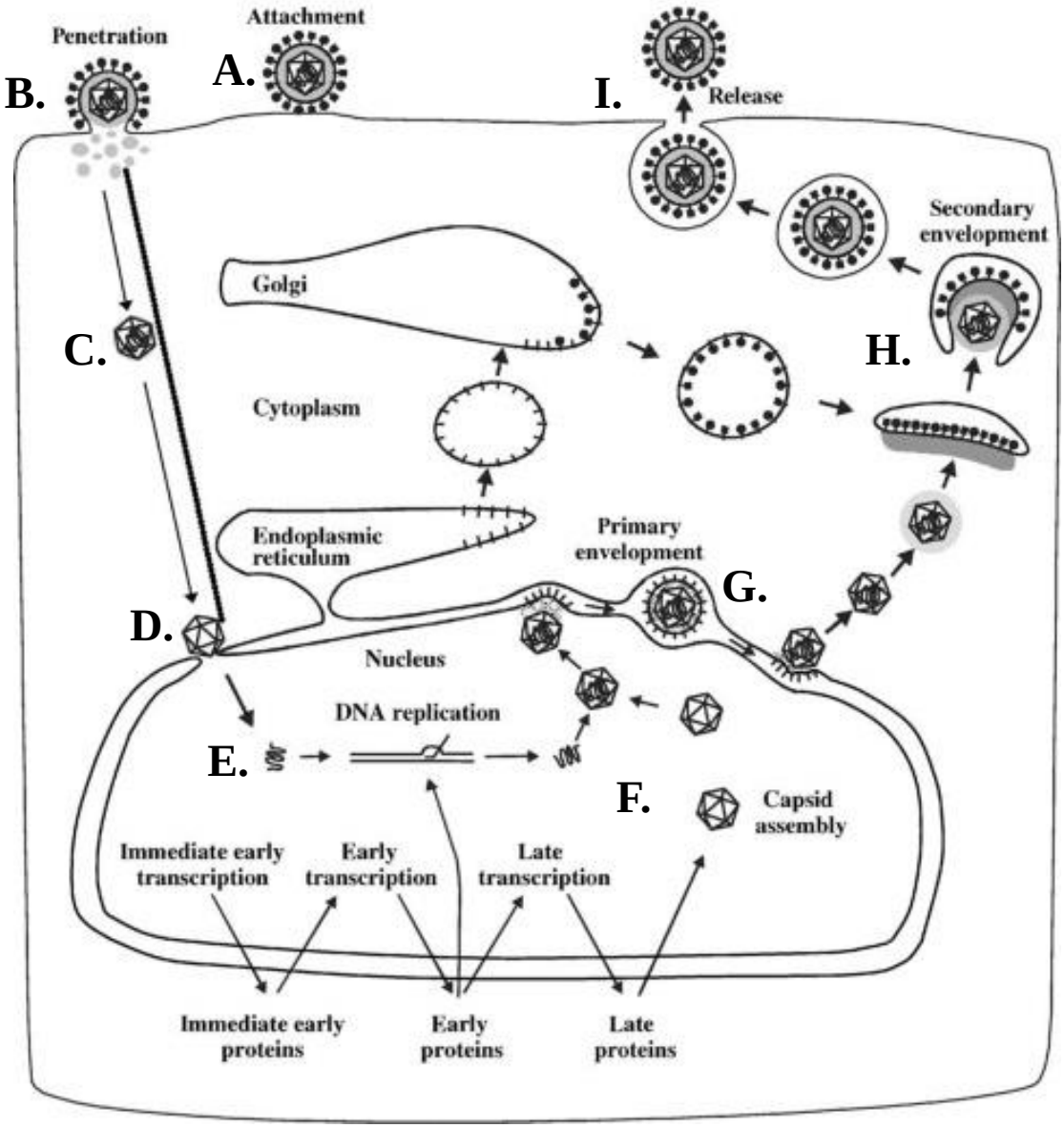

Figure 2. The replication cycle of a herpesvirus. A) Virus attachment and fusion; B) capsid release and tegument dissociation; C) transportation of incoming capsid to the nuclear pore and release of viral DNA into the nucleus; D) transcription, DNA replication and nucleocapsids assembly; E) envelopment-deenvelopment process at the nuclear membrane; F) tegumentation and envelope acquisition at the Golgi compartment; G) viral particles release through the cellular membrane. Modified from Mettenleiter, 2004 [26].

HSV-1 as a representative example of alphaherpesvirus lytic replication encodes seven proteins necessary for viral DNA synthesis: UL9 (origin-binding protein), ICP8 (single-strand

DNA [ssDNA]-binding protein), UL30/UL42 (polymerase), and UL5/UL8/UL52 (helicase/primase) [27]. Among all herpesviruses, there are seven essential replication proteins that are conserved. From these genes, six of them play crucial roles at replication: UL30 and its subunit UL42, UL29, and the helicase/primase complex formed by UL5, UL8 and UL52 gene products, who together works in unwinding dsDNA (double stranded DNA) and produces the lagging strand using RNA primers. [28]. In addition to the core replication proteins, herpesviruses also encode an origin-binding protein (UL9), which is the protein responsible for targeting the initial point of replication. Although the overall strategy of lytic viral replication appears to be preserved in all herpesviruses, the mechanism and control of viral synthesis initiation may differ between the three subfamilies of herpesviruses [27].

The production of concatemeric herpesvirus DNA is a critical step in progeny generation because the packaging machinery must recognize concatemers during encapsidation [27]. Inspite of the claims that herpesvirus genome first circularizes and follows the rolling circle replication to produce concatemers (Figure 3), some lines of evidence suggest that herpesvirus DNA replication is much more complex and can also involve recombinant replication [27].

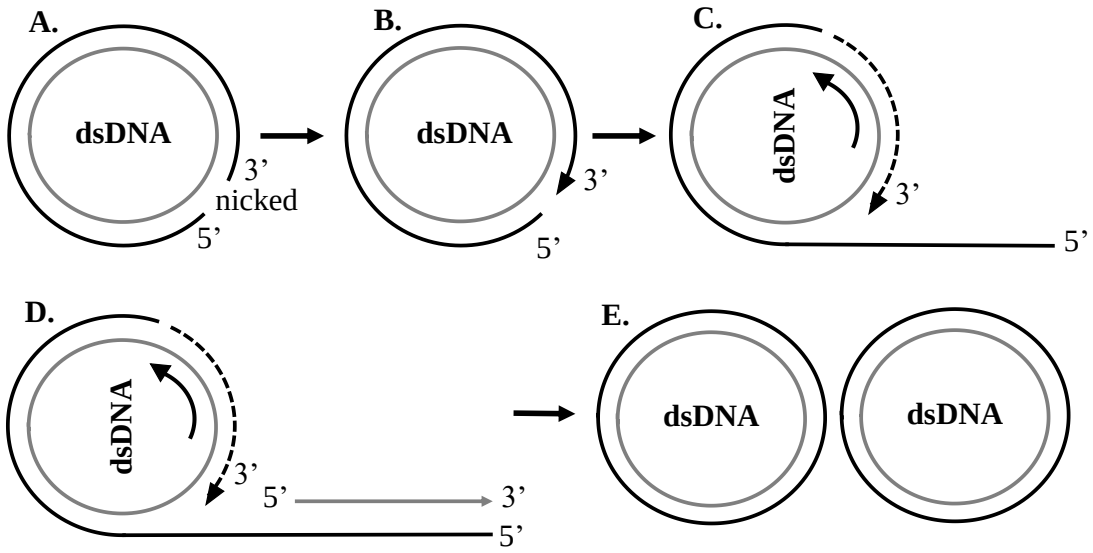

Figure 3. The proposed rolling circle mechanism for DNA replication of alphaherpesviruses. (A) The circular dsDNA is initially nicked, and the 3’ end is then elongated using the intact DNA as leading template strand (B). (C) The 5’ end then becomes a lagging strand and is made double stranded via the addition of a series of Okazaki fragments (approximately 150-200 nucleotides) (D). The final step consists of the circularization of the unnicked and the new DNA fragments (E).

The most effective way of producing active virion particles is through lytic replication. Herpesviruses, nevertheless, can also have its DNA replicated thought another process that does not produce virus particles and hardly leads to any signs of infection.

5.2.3 Latency

By definition, latency is a viral genomic persistence state in a host cell without new virions being produced [17]. A big advantage for herpesviruses that infect vertebrates is the fact that latency offers the possibility of having the viral genome maintained for life in the host cell with passive replication happening concomitantly to the hosts' cellular replication. Also, because herpesviruses in latency have less expressed proteins, which results in fewer epitopes being presented on the cellular surface, a more effective evade of the immune system upon infection is possible. There are different ways that the viral DNA can exist in the host cell during latency, either through the form of a circular episome or through direct integration into the hosts' chromosomes. An advantage of tying the herpesvirus DNA to host chromosomes is the possibility of transferring episomal DNA during cell division from old to new young cells. [12]. The Marek's disease virus and the Human herpesvirus 6 (HHV-6) are examples of herpesviruses that are to integrate their genome into host chromosomes while ensuring viral DNA replication similarly to a provirus [29]. When a virus goes latent in the host cell it enters this well-balanced quiescent state, where the DNA can persist in the host cell for long periods without being noticed or activating the immune system. Despite the protection and convenient situation that latency provides to a virus, herpesviruses do not produce virions and infect other targets during this stage. Herpesviruses need to reactivate from latency and return to the lytic replication cycle in order to leave the host cell and progress with further infections. Some factors including physical or psychological stress, reduction of physiological functions or processes linked with ageing or primary infections can be enough to disturb the balance between viral activity and immune response and interrupt latency. These factors impact the balance between viral activity and immune system, leading to immunosuppression, compromising the quality of immune surveillance, which creates the trigger for herpersvirus reactivation [30].

5.2.4 Maturation and virion egress

Following lytic viral DNA replication, virion assembly starts once there are enough amounts of replicated DNA and capsid components (mainly encoded by late genes) available. The capsid, once formed, it is filled with a single genomic viral DNA unit, that is determined using a set of

enzymes, including the terminase complex and the pac sequences, which are the DNA signal sequences [31,32]. After the capsid is complete with the viral DNA, theories point that there is an envelope-de-envelopment egress process, which capsids bud from the inner nuclear membrane into the perinuclear space, thus acquiring a primary envelope. Next, the capsid buds into the cytoplasm from the endoplasmic reticulum (ER) in a second step, thus losing its primary envelope [33]. The tegumentation process and the secondary envelopment step take place within the cytoplasm at the Golgi apparatus membrane network [26,33].

5.3 Marek's disease virus

Marek's disease (MD) is a lymphoproliferative disease of chickens (*Gallus gallus domesticus*) caused by an oncogenic herpesvirus, the Marek's disease virus (MDV). MDV, also called Gallid Herpesvirus 2 (GaHV-2), is classified in the genus *Mardivirus*, from the family *Herpesviridae* and subfamily *Alphaherpersviridae* based on its sequence homology with other viruses in this subfamily. There are currently three strains of MDV, whilst only strain 1 (MDV-1), the MDV type strain for the genus *Mardivirus*, is pathogenic. Within the genus, two other viruses are recognized, the apathogenic gallid herpes virus type 3 (GaHV-3) (formerly MDV-2) and the meleagrid herpesvirus type 1 (herpesvirus of turkey, HVT) [8,11]. The MDV genome is composed of a double strand of linear DNA, with the size of approximately 160 to 180 kilobase pairs (kbp), which carries 70 to 80 genes and encodes 103 proteins [34]. MDV is also a cell-associated herpesvirus [35], establishes latency in lymphocytes [36], contains an oncogene named Meq in its genome [37], as well as the ability to induce lymphomas [38–40].

Because herpesvirus DNA replication happens in the nucleus, viral particles (virions) are initially generated and mostly found in the nucleus, and more rarely in the cytoplasm and spaces extracellular. MDV causes T-cell lymphomas and mononuclear infiltrate of the peripheral nerves in animals susceptible after approximately 2 weeks post-infection [40]. Among all herpesviruses, MDV has the highest degree of affinity with T-CD4 cells, in which latency and transformation occurs. Lytic infection happens mainly in B lymphocytes, which are present mainly in the cloacal sac and the bursa of Fabricius [40–42].

5.3.1 History

The veterinarian and researcher, József Marek, first reported MD in chickens in Hungary in 1907. The disease was then characterized by polyneuritis and paralysis resulting from the infiltration of lymphoid tumours and inflammation of peripheral nerves [43,44]. However, the disease became more important during the 20th century when industrial poultry disease

outbreaks were recognized in the United States of America, and subsequently in New Zealand, Great Britain, the Netherlands and several other countries [41]. Some years later polyneuritis was associated with the development of tumours in visceral organs [45] and consequently, MD became known as neurolymphomatosis gallinarum or neurolymphomatosis of chickens [45]. Since then, MD has been the subject of extensive research due to its oncogenic, neurotropic and integration potential leading to the animal's death, whilst been reported in different countries, given its economic and scientific importance.

5.3.2 Genomic structure

Similarly to HSV-1, MDV possess a class E genome that comprises a unique long (U_L) and a unique short (U_S) sequences, which are flanked by the short and long terminal repeat (TR_S & TR_L) and short and long internal repeat (IR_S & IR_L) regions (Figure 4).

Functional and structural proteins are encoded by both UL and US regions, whilst virus-specific genes are found in both repeated regions. Similarly to HSV-1, the MDV genome contains homologous regions named as a-like sequences, where all the necessary packaging signals and cleavage sites are found. The a-like sequences also contain two classes of telomeric repeats (TMRs), the short telomeric repeats (sTMR) and the multiple telomeric repeats (mTMR). Both repeats are present at the ends of MDV's linear genome with a variable number of up to 100 repeats, mTMR and with a fixed number of 6 repeats, sTMR, respectively (Figure 4).

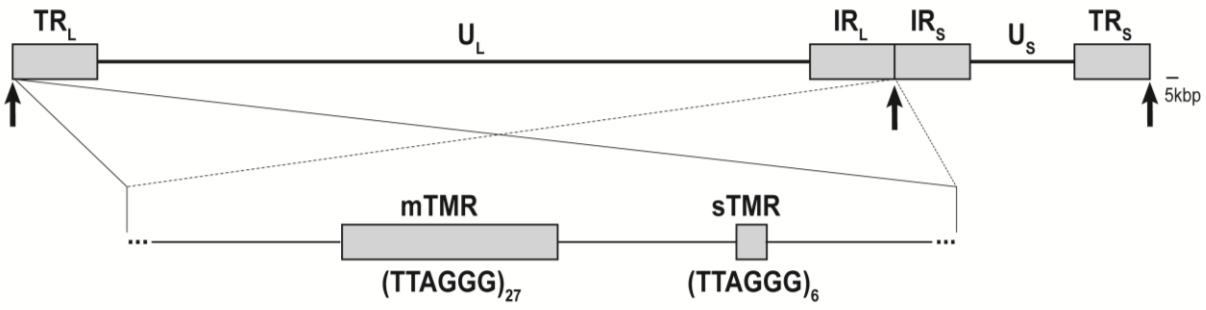

Figure 4. Schematic representation of the organization of the MDV genome, focusing on the a-like sequence. The MDV genome organization comprises a unique long (U_L) and a unique short (U_S) sequences, which are flanked by repeated regions, the terminal long and short (TR_L, TR_S) and the internal long and short (IR_L and IR_S) repeats, respectively. The a-like sequences present in the genome are pointed by arrows. A focus on the a-like sequence is shown, containing both telomeric repeats (mTMR and sTMR).

Genomically, MDV can also be divided in three categories according to the serotypes, as mentioned previously: MDV-1 (RB-1B and RISPENS CVI988); MDV-2 (SB-1); and MDV-3, which is also called turkey herpesvirus type 1 (HVT). Only MDV-1 is capable of inducing disease in chickens, while MDV-2 and MDV-3 are avirulent and have been extensively used as vaccines [41]. The MDV genome encodes more than two hundred genes, and among these, the Meq gene, which encodes a leucine-zipper protein named meq (Marek's EcoRI-Q-encoded protein), is the hallmark of a MDV gene associated with oncogenicity [37]. Between the viral determinants of oncogenicity, Meq is considered to be the most important and studied [40]. Meq is expressed lately and abundantly in MDV-infected cells and in MD tumor cells, participating as a transcriptional activator, with important role in the transformation process of cells by MDV [46,47]. Nevertheless, other MDV genes also play an important role in the development of lymphomas as for example the herpesvirus telomerase RNA (vTR) gene [48–50], the viral interleukin-8 (vIL-8) gene [51,52] and the phosphoprotein 38 (pp38) [53,54] (Table 1). Also, there are evidences that both sTMR and mTMR regions play a role in tumour formation [55,56]. During MDV latent infection, some T cells infected may transforms forming generalized T-cell lymphomas in chickens, suggesting that latency is a precursor event to tumorigenesis.

Table 1. MDV strains and genetic structures involved in oncogenicity.

Serotype	Strain	Virulence level	Gene vTR	Gene vIL8	Gene pp38	Meq oncogene	TMRs
1	GA	Virulent	+	+	+	+	+
1	Md5	Very virulent	+	+	+	+	+
1	Md11	Very virulent	+	+	+	+	+
1	RISPENS/CVI988	Attenuated	+	+/NF	+/NF	+/NF	+
2	SB-1	Non-oncogenic	-	+/NF	+/NF	-	+
3	HVT	Non-oncogenic	-	-	-	-	+

Legend: vTR: Viral telomerase; vIL8: viral interleukin 8; pp38: phosphoprotein 38; TMRs; telomeric repeats; +: present; -absent; NF: present but not functional. Adapted from McPherson, 2016 [57].

During latency, gene expression inducer of lytic replication is suppressed and host apoptosis is blocked, and the oncogene of Meq plays a critical role in the activation of transformation/latency genes, as well as in the repression of lytic genes [40]. Despite having

a large majority of factors involved in oncogenesis already revealed, the precise relationships between the stages of latency and tumour development are yet unknown.

5.3.3 MDV infection and replication cycle

In vitro, MDV has very slow replication kinetics mainly due to the fact that it is highly cell-associated. After infection, plaque formation typically occurs after several days. Nevertheless, *in vivo*, MDV is highly lymphotropic, infecting both B and T cells [58]. Despite its lymphocytic tropism, the knowledge on the precise sequence of events and its details of MDV infection from the uptake of the pathogen in the environment to the final shedding of cell-free virus through the feather follicles still contains unanswered questions.

The model accepted nowadays for the *in vivo* lifecycle is the “Cornell model”, which suggests that the infectious life cycle begins with MDV having access to the lung of the bird by inhalation of contaminated fomites in the environment (Figure 5). Once the virus entered the lungs, antigen-presenting cells (APC), such as macrophages or dendritic cells will supposedly be the first line of cells that will be infected with the virus during the initial phase of the cycle. Next, infected APCs will migrated to secondary lymphoid tissues, where MDV will be transmitted to B cells, where the first event of massive lytic replication and production of virions will occur, following by subsequent infection of T cells. Nevertheless, a recent study by Bertzbach and colleagues (2018) showed that MDV is capable of infecting T cells directly in birds independently from an initial infection of B cells, suggesting that B and T cells can be infected concomitantly rather than sequentially [59]. T and B cells infected will act as reservoirs where the viral latency phase will be triggered approximately 7 days post infection (dpi). Additionally, T cells infected with MDV are able to transport the virus to the feather follicle epithelium in the dermis, where it will shed cell-free viral particles protected in a sheath of keratin, which happens approximately two weeks post infection. Lastly, transformation of individual T cells can lead to the formation of solid lymphomas in basically all internal organs, characterizing this stage as the hallmark of the MDV infection, followed by the animal’s death [60].

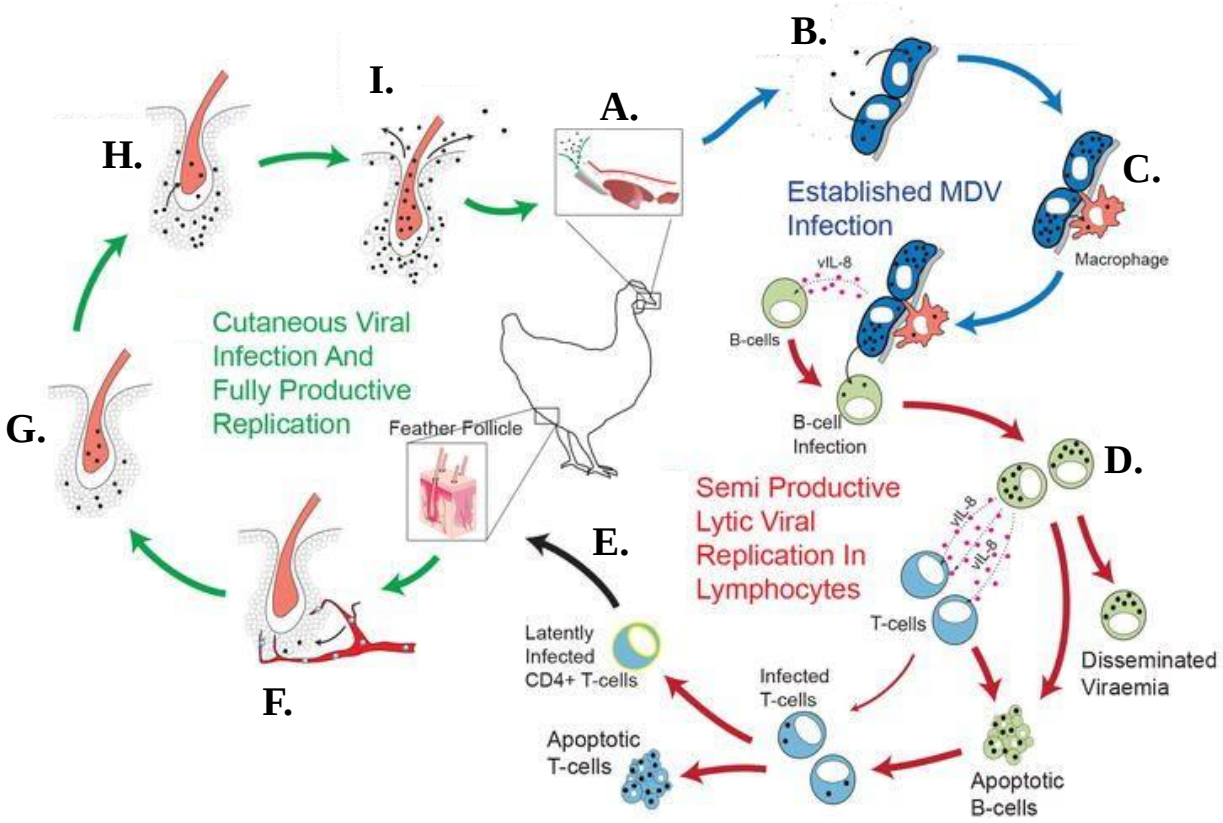

Figure 5. Model of MDV life cycle *in vivo*. (A) MDV infection begins with inhalation of dust or skin dander, encapsulated with respiratory viral particles. (B) Primary infections happens when virus particles are able to break the mucosal defense and enter epithelial cells. Local viral replication establishes infection and initiates the transcription and translation of viral immediately early genes. (C) The inflammatory response generated in the tissue will trigger the recruitment of cells from the innate immune system, resulting in the uptake of infectious virus particles by macrophages. Infiltration of lymphocytes via action of viral interleukin 8 (vIL-8) occurs, resulting in the infection of B cells with MDV. Viral B cell replication causes lytic infection and progression of the disease. (D) MDV infected B secret cells vIL-8, which acts as a chemical factor for T-cells access. (E) MDV-infected, resistant cells lead the virus into latent infection while certain T-CD4 + cells are subjected to apoptosis. (F) T-CD4+ latently infected cells merge to the feather follicles, which are the cutaneous sites of virus replication. (G) Feather follicle epithelium infection hosts productive viral replication. (H) Viral replication results in syncytia formation. (I) Feather epithelium infection leads to the secretion of mature virions in skin dander and dust, as the main source of infectious materials. Horizontal transmission of virions present in cellular sheathes or dust is the only accepted form of environmental persistence and infection. Modified from Boodhoo et al (2016) [61].

5.3.4 Clinical importance

The lymphoproliferative process, which occurs in a bird infected with MDV, may involve most organs and tissues, including peripheral nerves [41,43,62]. MDV infections in chickens can result in a wide variety of clinical forms, but the acute form is one of the most virulent, affecting birds that are three to four weeks old [41,43,62]. However, according to Payne and Venugopal (2000) [63], the acute form of MD can occur more commonly between three and six months of age.

Marek's disease in its classical form causes lymphoid tumour infiltration of the peripheral nerves, especially sciatic nerve, leading to paralysis. Lymphoma infiltration happens in several organs as well as, for example, gonads, liver, spleen, lungs, skin, heart and kidneys (Figure 6). In the acute form, visceral tumours can lead to sudden death in two to five days without the presence of paralysis and without the lymphoma evolution being detectable microscopically. The affected animals die between seven and twenty days after the appearance of clinical signs, mainly due to insufficient dietary cachexia and multiple organ failure. MD presents a major economic problem with regard to poultry industry, due to the fast spread and presence of lethal cutaneous or visceral tumours [41,62].

This is a disease that exists in practically all chickens raised intensively throughout the world, besides being considered the disease with the greatest impact on subsistence poultry farming. The exposure to the etiological agent can occur soon after hatching of the chick, and the disease can occur during the whole life of hens, as well as in the form of acute outbreaks where more than 50% of the birds can succumb within a few weeks [64].

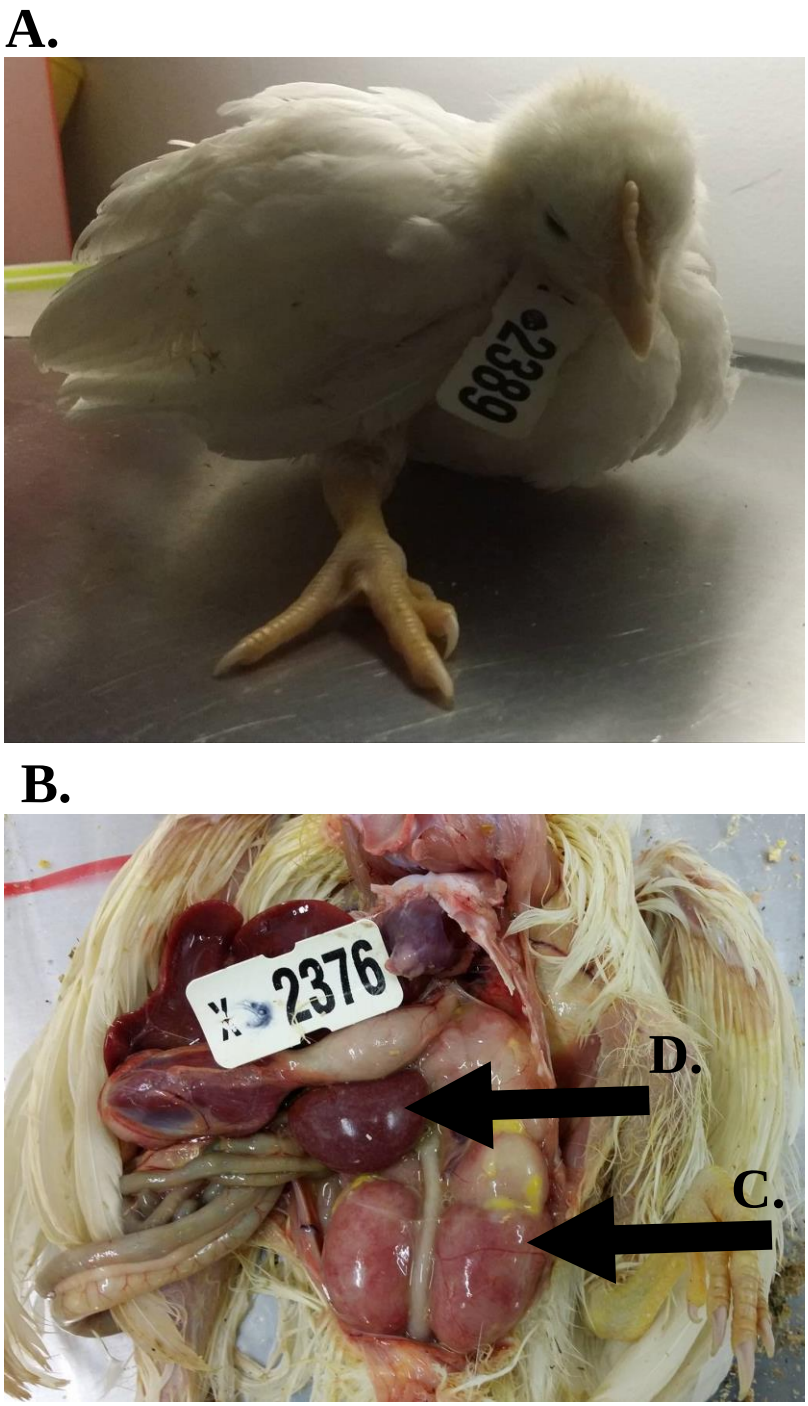

Figure 6. Clinical manifestations of MD. (A) The infiltration of tumor cells in organs and tissues compress the nerves of the limbs and the animal becomes unable to stand up due to the virus-associated paralysis. (B) Enlargement of internal organs upon necropsy. Arrows indicates enlargement of kidney (C) and spleen (D) with infiltration of transformed cells leading to tumor formation.

5.4 MDV integration

5.4.1 Telomere structures

By definition, telomeres are biological structures formed by repetitive regions present at the terminal area of linear chromosomes [65]. Telomeres have been a subject of study, mainly because it is present in a variety of species, consisting of a double stranded DNA with the tandem repeated sequence TTAGGG [66], which, for example, in humans correspond to 10-15 kb approximately. These repeats are directly bound by telomere repeat binding factors 1 and 2 (TRF1 and TRF2) that recruit other cellular factors and form a specialized complex called shelterin [65,67]. The Shelterin complex, also called telosome, is a complex formed by different proteins that caps and protects the telomeres from DNA repair mechanisms in different eukaryotic cells. It also regulates telomerase activity in conjunction with other protein complexes by controlling access to the single-strand overhang. In the absence of the Shelterin complex, telomeres would remain uncapped and exposed to the risk of activating damage-signaling pathways that could possibly lead to non-homologous end joining (NHEJ), homologous recombination (HR), end-to-end fusions, genomic instability and even apoptosis. [66].

The biggest barrier in the replication of DNA is the "end-replication issue" [68]. This is an event, when the DNA polymerase cannot properly replicate the sequences at the chromosome termini, resulting in a progressive shortening of chromosomes proposed as a molecular time clock and a major molecular aging mechanism [65], limiting somatic cell lifespan. Excessive telomere shortening or capping dysfunction activates the DNA damage response (DDR), which recognizes chromosome ends as double strand breaks [68]. Telomerase is added to counter the loss of telomeric sequence. Telomerase is an enzymatic complex made up of two major molecular components: the RNA component of telomerase (TERC or TR), which works as a template, and the catalytic subunit of reverse transcriptase enzyme (TERT) [65]. Telomerase's action is only active in certain types of cells such as germ line and stem cells [69]. Telomerases, however, are inactive in differentiated human cells and shows limited activity in stem cells, which as a result, telomeric DNA is eroded in most cells as a result of incomplete lagging-strand synthesis and end-processing that restores single-strand overhangs [70]. Disruption of the telomere capping function or regulation of telomere length may result in tumourigenesis caused by genome instability, and the presence of a few short telomeres [33,55] induces senescence that has known roles in ageing. An alternative telomeral lengthening (ALT) has been described in some telomerase-negative tumor cells [71]. To maintain their length, ALT uses homologous recombination between telomeres [72]. Immortalized ALT cells display high inter-and intra-teleomeric recombination levels [73].

Two models for the homologous recombination on telomeres were proposed. The first model involves the formation of one daughter cell with long telomeres and another with shortened telomeres through the unequal exchange of genetic material between two identical sister chromatids [72]. In the second, recombination is thought to be mediated as a copy template by synthesizing new telomeric DNA using existing telomeric sequence from an adjacent chromosomal telomer or t-loop formation [72]. In both cases, DNA repair protein involvement and protein shelter is necessary in ALT cells for telomer maintenance [73].

5.4.2 The chicken telomere

Chickens have a diploid number of 78 chromosomes, or 38 autosomal pairs, that could be divided into three groups: 5 macro-; 5 intermediate and 28 micro-chromosomes [74]. Although the genome of chicken is quite compact, it has a higher amount of telomeric sequence than the genomes of humans or mouse [75]. Three classes of telomeres were found on chicken chromosomes: class I (0.5 to 10 kb) within the chromosome (interstitial) rather than terminal; class II (10-40 kb) similar in structure to mammalian telomeres and evidence of telomere shortening; and class III (40 kb-~2Mb) which are extremely variable and specific to microchromosomes [75]. During chicken's development, telomerase activity in chicken tissue is regulated similarly to that observed in mammals, with high activity in the early stage of chicken embryos and gradual down-regulation in tissue-specific patterns [75]. Up to date, it is known that the strong selection pressure for rapid tissue growth and high reproductive rates in poultry production stocks may affect chromosome stability, but the mechanisms underlying it have not yet been understood [75].

5.4.3 Telomeric repeat sequences and integration

The majority of herpesviruses retain their genome as a circular DNA molecule called an episome in the nucleus of the host cells [17]. It has been shown that MDV and human herpesvirus 6 (HHV-6) integrate their genome into host chromosomes, but the underlying mechanisms are poorly understood [29]. Integration is not an impasse for these viruses, as the viruses can easily reactivate and mobilize the genome from the integrated state, but rather represents a means of maintaining their genome during latency [76]. A common feature between MDV and HHV-6 is that both have telomeric repeats in their genomes [55,76]. The genome of HHV-6 was found to be integrated into host telomeres [76,77], suggesting involvement of the telomeric repeat in the integration of the viral genome, but telomeric repeats alone do not seem to support the integration process alone [29]. Studies have showed that

MDV can be integrated into T-cell chromosome, close to the telomeric region, with rather no preference for a specific chromosome [55,78].

5.5. Herpesvirus pUL12/ICP8 recombinase complex

MDV harbors, in addition to the herpesvirus telomeric repeats, other factors that could be involved in integrating the virus genome into host telomeres. MDV encodes, like all herpesviruses, a putative recombinase complex consisting of two core components UL12 and ICP8, which resemble well-characterized recombination systems such as the Red recombination system encoded with bacteriophage λ [27,79–81]. The pUL12/ICP8 complex is believed to be involved in the replication of herpesvirus DNA, initially associated with the high degree of homologous recombination [23,24]. In addition, it was suggested that the complex could help to resolve branches of DNA that occur during branched rolling circle replication and thus enable unit-length genome packaging into preformed capsids [82,83]. The first structure part of the recombinase complex is the pUL12 protein, which acts as a 5'-3 'exonuclease, preferring to digest dual stranded DNA and generating 3' single strand DNA (ssDNA) overhangs [79,81] (Fig. 7). A number of herpesviruses including HSV-1, Epstein-barr virus (EBV), bovine herpesvirus type 1 (BHV-1), pseudorabies virus (PRV) and human Cytomegalovirus (HCMV) have shown exonuclease activity with a catalytic optimum at alkaline pH, similarly [84]. Additionally, MDV's UL12 protein and other herpesviruses harbor all seven preserved motifs in alkaline nucleases, which confirms the protein's preserved nature [84]. In HSV-1, pUL12 is not essential for virus replication. Nevertheless, the abrogation of the expression of UL12 resulted in a 100-1.000-fold reduction in viral titers later on, which indicates that it is not essential, but yet plays an important role for virus replication [85]. In, HSV-1 UL12 also creates two separately promoted 3 ' coterminal mRNAs that encode separate but related proteins: a full-length UL12 and UL12.5, an amino-terminally truncated form that starts at UL12 codon 127. Because UL12's exonuclease function is involved in genomic recombination, its full - length UL12 locates from larger precursors to the nucleus where it promotes the generation of mature viral genomes. UL12.5, the truncated form, on the other hand, is predominantly mitochondrial and acts early during infection to trigger degradation of the mitochondrial genome [86].

The infected-cell protein 8 (ICP8), a single - stranded DNA binding protein (SSB) encoded by the gene UL29 is the second component of the putative recombination system. HSV-1's ICP8 tightly binds ssDNA and removes its secondary structures, resulting in an extended configuration of protein-DNA filaments [87]. HSV-1’s ICP8 facilitates re-annealing upon binding to complementary single-stranded DNAs, a hallmark of all known SSBs [88]. ICP8 is

essential for replication of HSV-1's DNA [89], but the role in replication of MDV remained yet unknown. It has previously been shown that ICP8 and alkaline nuclease pUL12 of HSV-1 interact with each other and have in vitro strand exchange activity, a prerequisite for homologous recombination [81]. Electron microscopy showed that strand reaction products are generated between linear double-stranded DNA and circular single-stranded DNA and are consistent with the expected shapes of the joint molecule [79]. As the integration of MDV into host telomeres is suggested to be facilitated by the homologous recombination of virus and host telomeric repeats, the integration process is likely to involve the virus encoded UL12/UL29 putative recombinase complex (Figure 7).

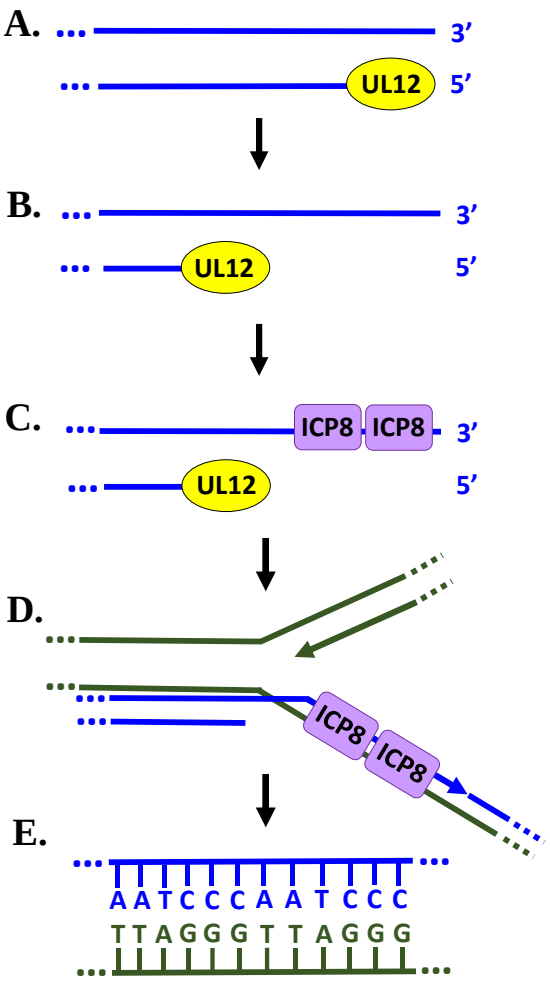

Figure 7. Schematic representation of a possible integration event mediated by pUL12/ICP8. (A) pUL12, an exonuclease of 5'-3, binds DNA to a double - strand break and produces a single stranded 3′ overhang (B). ICP8 binds and protects single-stranded DNA against pUL12 degradation (C). It is then thought that the single DNA strand bound by ICP8 invades the strand of a replication event (D), where it serves as a template for lagging strand

replication, which potentially could be a pathway leading to DNA integration in the telomeres through homologous recombination (E).

5.6 DNA repair and virus integration

In order to maintain chromosomal stability and prevent genetic loss, repairing DNA damage such as chromosomal double strand breaks (DSBs) is essential.

Eukaryotic cells have developed at least four distinct repair pathways for DSB repair, three requiring some degree of genomic homology: homologous recombination (HR); single strand annealing (SSA) and alternative non-homologous end joining (ANHEJ); and one requiring no level of homology whatsoever; the classic non-homologous end joining (NHEJ) [90,91]. It is known that avian and mammalian cells are capable of encoding two central recombinases that are involved in homologous recombination, Rad51 and DCMC1. The first is active in somatic cells and essential for DNA damage repair by HR, whilst the second is only restricted to meiotic cells [92–95]. Additionally, DNA repair can be also mediated by another cellular factor named Rad52, which is a DSB protein involved in repair of homologous sequences by SSA [96]. Knowing these cellular factors involved in herpesvirus integration can shed some light to the processes that contribute to herpesvirus integration.

In HSV-1, it has been long suggested that the U_L and U_S regions invert relative to one another during replication, and that rates of DNA recombination are high between co-infecting HSV viruses [11,24,27]. Despite the high level of recombination during infection, little is known about the proteins involved in herpesviruses recombination mechanisms and the importance of this event during replication. In HSV-1, UL12 and UL29, as a recombination complex, were thought to aid these recombination events due to their exonuclease and single-strand annealing properties [80]. Additionally, studies have showed that in HSV-1, UL12 can stimulate SSA and that SSA is the homology-mediated repair pathway used during HSV infection [80], but yet nothing of this nature has been addressed for MDV.

It has been proven that herpesvirus integration into host telomeres is an event dependent on homologous sequences between the host telomeres and the virus TMRs [55,56,77,97]. Also, it is suggested that integration is an event likely dependent on viral or cellular proteins capable of exhibiting recombination activity [29]. Nonetheless, it is still unrevealed if herpesvirus integration into the host chromosomes happens through SSA, HR, ANHEJ, NHEJ or any other recombination mechanism. As mentioned previously, UL12 and UL29 are preserved genes among herpesviruses [79,81,83], and these genes in HSV-1 facilitates DNA strand exchange [80], a pre-requisite for homologous recombination. Considering that viral integration possibly

occurs being mediated by homologus recombination [29,55], facts indicate that both UL12 and UL29 genes could be involved in virus integration into the host chromosomes.

5.7 Dihydrofolate reductase

Dihydrofolate Reductase (DHFR) is a very important enzyme encoded by the DHFR gene, and present in both eukaryotic and prokaryotic organisms. The DHFR gene is mainly involved in the production of cofactors that are necessary for DNA synthesis [98]. Specifically, DHFR catalyzes the reduction of folate and 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF) [99]. Because it is found in all organisms, including herpesviruses [100], the gene and protein DHFR have a crucial role in regulating the amount of tetrahydrofolate in the cell [99,101], which together with its derivatives are essential for cell proliferation and growth [98].

The first destabilisation domain (DD) technology was developed based on the human FKBP12 protein bearing a cavity-forming F36V mutation, as a family of FKBP ligands containing corresponding synthetic “bumps” has already been developed and found to be non-toxic in cultured cells and animals [102].

Like the FKBP-derived DDs, the *E.coli* DHFR-derived DDs (ecDHFR) reported in previous studies [103–105] confer instability to a fused protein, resulting in rapid degradation of the protein upon withdrawal of the ligand trimethoprim (TMP) (Figure 8). Characterization of the ecDHFR-derived DDs revealed that the DDs behave very similarly to the FKBP-derived DDs, and the two DD systems work orthogonally as demonstrated in mammalian cells [104]. However, the main notable differences between the two systems were noted. Firstly, the c-terminal ecDHFR-derived DDs have been showed to be more effective at destabilizing the fusion protein than the FKBP-derived DD, for example [104]. Secondly, the ligand to the DD DHFR, TMP, is an antibiotic listed on the World Health Organisation’s list of Essential Medicines [106], for being a safe and potent medicine and significantly inexpensive. Thirdly, the DD DHFR can be used successfully *in vivo* as TMP crosses the blood-brain barrier [107] and the placenta [108], facilitating the study and inducible regulation of proteins in the central nervous system and in the womb, respectively.

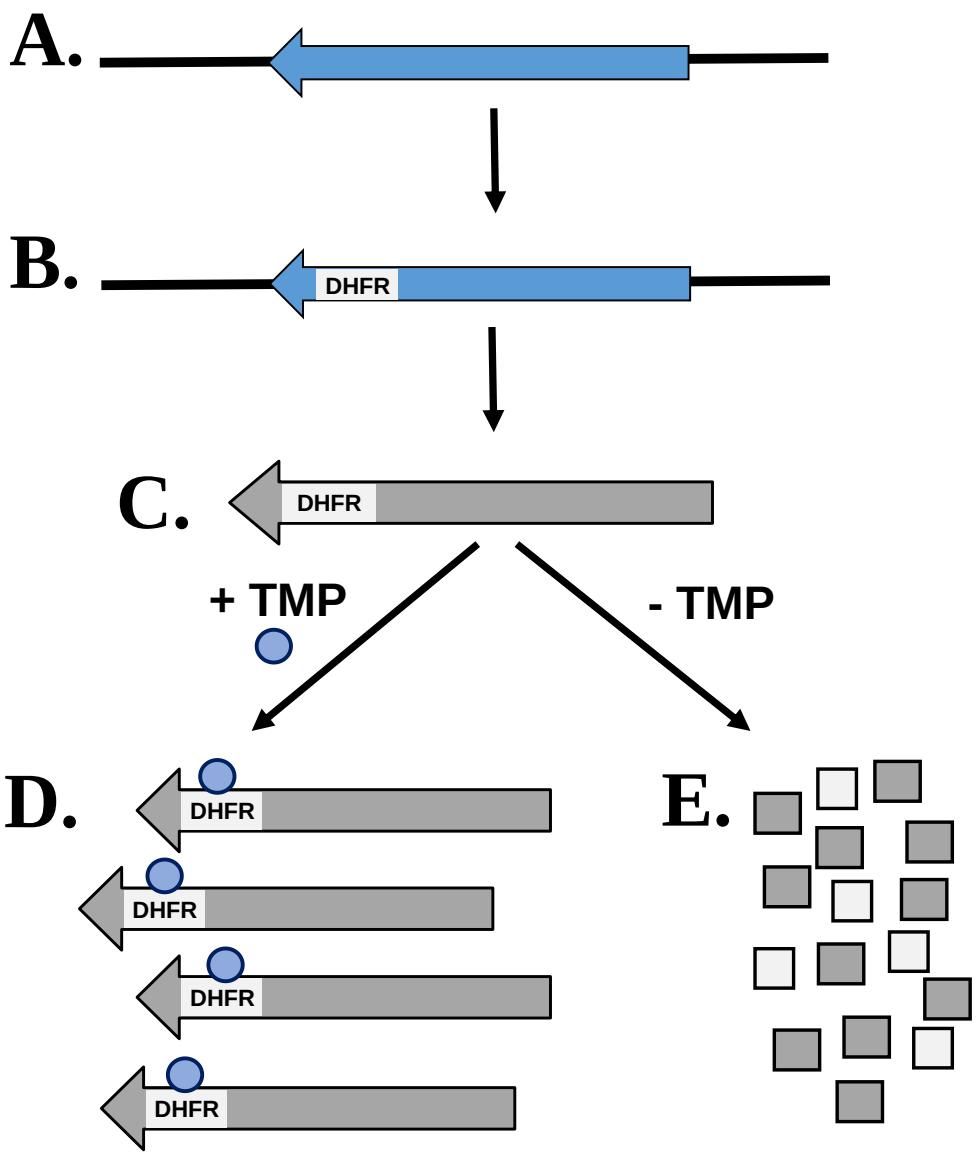

Figure 8. Schematic representation of the DD DHFR system. (A) A target gene sequence in the DNA is identified for the insertion of the destabilization domain. (B) The DHFR sequence is introduced in frame with the gene. (C) A protein encoded by the target gene is translated through an mRNA, containing the ecDHFR DD active in the cytoplasm. (D) When present, molecules of the ligand TMP bind to the ecDHFR DD region of the protein and stabilizes the protein, whilst in the absence of the ligand (E), there is no binding and the protein becomes unstable, being rapidly degraded by proteasomes in the cellular cytoplasm.

6. Project introduction

6.1 Project 1

The role of Marek's Disease Virus UL12 and UL29 in DNA recombination and the virus lifecycle

Studies have showed that MDV can integrate its genome directly into the telomeres of latently infected cells and tumour cells. This integration process has been shown to be similar to the integration of other herpesviruses in host chromossomes, including human human herpesvirus 6A (HHV-6A), 6B (HHV 6A), and 7 (HHV-7) [55,56,76,109,110]. An interesting common feature between MDV and these other herpesviruses is that they all harbor telomeric repeat arrays (TMRs) at the end of their genomes. These TMRs are formed by the nucleotide sequence of TTAGGG, which are identical to host telomere sequence [97,111]. It has been solidly evidenced that these TMR arrays facilitate integration of MDV and HHV-6A [55,56,77]. Although these telomeric repeat sequences are well characterized and showed to facilitate MDV integration events, the viral proteins that facilitate the process remain still unknown. In a very similar manner to HSV-1, MDV contains two genes, UL12 and UL29, which encrypts two proteins, pUL12 and ICP8, that are conserved among herpes viruses. HSV-1's pUL12 possess a 5'-3'exonuclease activity and is known to facilitate DNA recombination through single-strand annealing during virus replication [80,81], a process that could potentially facilitate MDV integration. The other component of this complex, is UL29, which is also known as infected cell protein 8 (ICP8) and performs like a single strand DNA-binding activity [17,79–81], a function that plays a crucial role in many recombination processes. HSV-1 UL12 and UL29 interact with each other and facilitate DNA strand exchange *in vitro*, an event that could conceivably initiate MDV DNA's integration. Even though UL12 and UL29 are present in all members of the *Herpesviridae* family, they are not highly conserved on the sequence level. UL12 and UL29 of MDV and HSV-1 only have a sequence homology of 36.2% and 43.7% respectively, suggesting that they may not have the same functions for MDV. While both genes are well characterized for HSV-1, the functions of these orthologue genes in MDV's replication, recombination, and integration remains still unknown.

The specific aims of this project were (i) to determine the role of UL12 and UL29 in the MDV replication cycle, (ii) to verify if MDV UL12 and UL29 are involved in DNA repair, a process that could facilitate MDV integration, and finally, to address if (iii) MDV's UL12 and UL29 facilitate viral integration and the maintenance of the latent virus genome in the host's cells.

6.2 Project 2

Investigating the role of Marek's Disease Virus DNA polymerase in the establishment of latency in reticuloendotheliosis-transformed T-cells.

As it has been previously discussed, there are solid evidences of essential intrinsic factors involved in MDV's integration process into telomeres of host cells, such as the mTMR and sTMR regions [55,56,77]. Moreover, studies showed that MDV DNA has been found in transformed T cells of chickens, suggesting that integration occurs majorly in those cells [36,49,55–58,63,78,109,112]. Albeit, the entire integration process still contains undiscovered variables involved in this process from both, the virus and the host sides. All strains of MDV produce a conserved viral DNA polymerase, which is encoded by an early gene named UL30 [113,114]. Considering the preserved characteristics of the main viral DNA polymerase among MDV's strains, the UL30 gene has been the target of important studies that investigated the evolutionary process of the virus throughout time and space [115]. In HHV-6, integration is thought to happen in a similar manner to MDV, and it has been showed that HHV-6's genome is lost during non-productive viral infection [116]. Also, studies on temporal analysis of MDV infection suggested that virus replication either happens before or together with integration on B and T cells, and that these events occur rather on an early time point of the infection [109] .

It is known that after an infection, MDV either triggers a lytic replication event with multiplication of viral genome and production of new virions, or, in resistant hosts, it evades the immune system by shielding its DNA through integration into the hosts' telomeres ensuring a lifelong viral infection [42,60,61]. Additionally, concatameric forms of MDV's genome has been found integrated in tumors cells, suggesting that herpesvirus replication preceded integration in this case [55]. These evidences suggest that for a successful short or long infection onset, viral DNA must initially be replicated, suggesting that UL30 may play an active role throughout MDV's lifecycle.

The aims of this project are therefore to (i) address the role of UL30 in the integration process into reticuloendotheliosis-transformed T-cells and (ii) to elucidate if replication is an essential event prior to integration.

7. Material and Methods

All the materials presented below were used in accordance with the instructions and guidelines of the manufacturers.

7.1 Materials

7.1.1 Chemicals, consumables and equipment

Chemicals

Name	**Type/ Cat. No.**	**Company**
Acetic acid	Cat.No. 20103.295	VWR, Dresden
Agar (agar bacteriological)	Cat. No. 2266.2	Carl-Roth, Karlsruhe
Agarose- Standard Roti® grade	Cat. No.3810.4	Carl-Roth, Karlsruhe
Ampicillin Na-salt	Cat. No.K029.2	Carl-Roth, Karlsruhe
Arabinose L (+)	Cat. No. A11921	Alfa Aesar, Karlsruhe
BSA (albumin bovine fraction V)	Cat. No. A6588.0100	Applichem, Darmstadt
$CaCl_2$ (calcium chloride) dihydrate	Cat. No. T885,2	Carl-Roth, Karlsruhe
CDP-Star Ready-to-use	Cat. No. 12041677001	Roche, Mannheim
CH_3COOH (acetic acid)	Cat. No. A3686, 2500	Applichem, Darmstadt
Chloramphenicol	Cat. No. 3886.1	Carl-Roth, Karlsruhe
Chloroform	Cat. No. 411 K3944831	Merck, Darmstadt
KaryoMAX® Colcemid™ Solution in PBS	Cat. No. 15212-012	Gibco, life Technologies, Darmstadt
Dextran Sulphate Sodium salt	Cat. No. 17-0340-01	Pharmacia Biotech, Uppsala
Dimethyl sulfoxide (DMSO).	Cat. No. 1.02952.2500	Merck, Darmstadt
dNTP Mix (10mM total)	Cat. No. BIO-39053	Bioline, Luckenwalde
EDTA (ethylendiamine tetraacetic acid)	Cat. No. A2937, 1000	Applichem, Darmstadt
Ethidium bromide 1%	Cat. No. 2218.2	Carl-Roth, Karlsruhe
$EtOH_{den.}$ Absolute	Cat. No. A1613	Applichem, Darmstadt
FACS Rinse	Cat.No. 340346	BD, San Jose
FACS Clean	Cat No. 340345	BD, San Jose
Formamide deionized Molecular biology grade	Cat. No. A2156	Applichem, Darmstadt
Glycerol	Cat. No. A2926,2500	Applichem, Darmstadt
HCl 37% (hydrochloric acid)	Cat. No. 4625.2	Carl-Roth, Karlsruhe
Hexadimethrine bromide	Cat. No. 28728-55-4	Sigma-Aldrich, St. Louis

Isopropyl alcohol (2-propanol)	Cat. No. A0892	Applichem, Darmstadt
Kanamycin sulphate	Cat. No. T832.2	Carl-Roth, Karlsruhe
KCH_3CO_2 (potassium acetate)	Cat. No. A4279,0100	Applichem, Darmstadt
Lipofectamine 2000	Cat.No. 11668027	Life Technologies, Carlsbad
β-mercaptoethanol (2-mercaptoethanol)	Cat. No.28625	Serva, Heidelberg
Maleic Acid	Cat. No. A1841	Applichem, Darmstadt
Methanol	Cat. No. 20847.320	VWR, Dresden
$MgCl_2$ (magnesium chloride hexahydrate)	Cat. No. 5833.025	Merck, Darmstadt
Mounting Medium Vectashield with DAPI	Cat. No: H-1200	Vector Laboratories Inc, Burlingame
NaCl (sodium chloride)	Cat. No. A3597,5000	Applichem, Darmstadt
NaOH (sodium hydroxide)	Cat. No. 1.06462	Merck, Darmstadt
Nonfat dried milk powder	Cat. No. A0830	Applichem, Darmstadt
OptiMEM	Cat. No. 31985062	Life Technologies, Carlsbad
Paraformaldehyde	Cat. No. P6148	Sigma-Aldrich, St. Louis
Pepsine from porcine gastric mucosa	Cat. No. P7012	Sigma-Aldrich, St. Louis
Peptone/Tryptone	Cat. No. A2210,0250	Applichem, Darmstadt
Phenol/Chloroform	Cat. No. A0889,0500	Applichem, Darmstadt
Roti® Mount FluorCare DAPI	Cat.No. HP20.1	Roth, Karlsruhe
Roti™-Phenol	Cat. No.0038.3	Roth, Karlsruhe
Sodium butyrate	Cat.No. 286367-68-8	Sigma-Aldrich, St. Louis
SDS (sodium dodecyl sulfate)	Cat. No. 75746	Sigma-Aldrich, St Louis
Sodium Phosphate, monobasic, monohydrate	Cat. No. S9638	Sigma-Aldrich, St Louis
di-Sodium Hydrogenophsohate dodecahydrate	Cat . No. A3906	Applichem, Darmstadt
tri-Sodium Citrate dehydrate	Cat. No A1357	Applichem, Darmstadt
Trimethoprim (TMP)	Cat. No. 738-70-5	Sigma-Aldrich, St. Louis
Tris	Cat. No. A1086,5000	Applichem, Darmstadt
Triton X-100 detergent	Cat. No. 8603	Merck, Darmstadt
Tween-20	Cat. No. 9127.2	Roth, Karlsruhe
Water Molecular biology grade	Cat. No. A7398	Applichem, Darmstadt

Yeast extract granulated	Cat. No. 212750	Becton-Dickinson, Heidelberg

Consumables

Name	**Type/ Cat. No.**	**Company**
Cell culture dishes	6-well, 24-well, 96-well	Sarstedt, Nümbrecht
Cell culture flasks	25 ml, 75 ml	Sarstedt, Nümbrecht
Conical test tubes 17x120	15 ml	Sarstedt, Nümbrecht
Conical test tubes 30x115	50 ml, with and without feet	Sarstedt, Nümbrecht
Cryotubes	1.8 ml	Nunc, Roskilde
Eppendorf tubes 1.5 and 2 ml	1.5 and 2 ml	Sarstedt, Nümbrecht
Expendable cuvettes	1mm	Biodeal, Markkleeberg
Latex gloves	Size S	Unigloves, Troisdorf
Kimtech Science, Precision Wipes	Cat.No. 05511	Kimberly-Clark, Roswell
Microscope cover glasses	Cat.No. ECN631-1569	VWR, Sacramento
Nitrile gloves	Size S	Hansa-Medical 24, Hamburg
Nytran®SPC	Cat.No. 10416296	Whatman, Maidstone
Parafilm® M		Bems, Neenah
Pipettes for Pipetboy	5, 10, 25 ml	Sarstedt, Nümbrecht
Pipette tips	P1000, 200, 100 and 10	VWR International, West Chester
Pierce™ Concentrators, 150 MWCO, 20ml	Cat.No. 89921	ThermoFisher, Waltham
Petri dishes for cell culture	60 mm, 100 mm, 150 mm	Sarstedt, Nümbrecht
Petri dishes for bacteria		Sarstedt, Nümbrecht
Polystyrene round-bottom tube 5ml	Cat.No. 352063	VWR, Dresden
Polystyrene round-bottom tube with cellstrainer cap 5ml	Cat.No. 352235	VWR, Dresden
PVDF membrane	Cat.No. T830	Roth, Karlsruhe
Sterile syringe filters PVDF	0,45 µm	VWR International, West Chester

SuperFrost® Plus	Cat.No. J1800AMNZ	Menzel Glaser, Braunschweig
Transfection polypropylene tubes		TPP, Trasadingen
Whatman blotting paper	3MM	GE Healthcare, Freiburg

General equipment

Name	**Product info/ Cat. No.**	**Manufacturer**
Fast Real-time PCR system	ABI Prism 7500	Invitrogen Life Technologies, Grand Island
Bacterial incubator	07-26860	Binder, Turtlingen
Bacterial incubator shaker	Innova 44	New Brunswick Scientific, New Jersey
Bunsen burner	Type 1020	Usbeck, Radevormwald
Cell incubators	Excella ECO-1	New Brunswick Scientific, New Jersey
Centrifuge 5424	Rotor FA-45-24-11	Eppendorf, Hamburg
Centrifuge 5804R	Rotors A-4-44 and F45-30-11	Eppendorf, Hamburg
Centrifuge Sorvall RC 6+		Thermo Scientific, Dreieich
Cytospin3	Shandon	Dreieich
Imaging system	Chemismart 5100	Thermo Scientific, Dreieich Peqlab, Erlangen
Electroporator	Genepulser Xcell	Bio-Rad, München
Electrophoresis power supply		VWR International, West Chester
Flow cytometer	FACS Calibur	BD, San Jose
FACSsorter	FACS AriaIII	BD, San Jose
Freezer	-20°C	Liebherr, Bulle
Freezer	-80°C	GFL, Burgwedel
Mini centrifuge	Galaxy	VWR International, West Chester
Gel electrophoresis chamber		VWR International, West Chester
Gel electrophoresis chamber	SUB-Cell GT	Bio-Rad, München

Hybridization Oven	HB-1000	UVP Laboratory Product, Cambridge
Ice machine AF100	AF100	Scotsman, Vernon Hills
Pipetboy	INTEGRA	IBS Integrated Biosciences, Fernwald
Magnetic stirrer	RH basic KT/C	IKA, Staufen
Gel chambers	Mini Protean 2D	Bio-Rad, München
Protean Tetra Cell chambers		Bio-Rad, München
Nanodrop 1000		Peqlab, Erlangen
Newbauer counting chamber		Assistant, Sondheim/Rhön
Nitrogen tank	ARPEGE70	Air liquide, Düsseldorf
Nucleofector™ II		Lonza, Basel
Orbital shaker	0S-10	Peqlab, Erlangen
Pipets	P1000, P200, P100, P10	Eppendorf, Hamburg
Horizontal Maxi-Gel System	Perfect Blue™	Peqlab, Erlangen
pH-meter	RHBKT/C WTW pH level 1	Inolab, Weilheim
Sterile laminar flow	ScanLaf, Mars Safety Classe 2	LMS, Brigachtal
Sterile laminar flow		Bleymehl, Inden
Thermocycler	T-Gradient	Biometra, Göttingen
UV Transiluminator	Bio-Vision-3026	Peqlab, Erlangen
Transiluminator printer P93D	P93D	Mitsubishi, Rüsselsheim
Transiluminator	VL-4C, 1x4W-254 nm	Vilber-Lourmat, Eberhardzell
Vortex Genie 2™		Bender&Hobein AG, Zurich
Water baths	TW2 and TW12	Julabo, Seelbach
Water bath shaker	C76	New Brunswick Scientific, New Jersey

Microscopes

Name	**Product info/ Cat. No.**	**Manufacturer**
Fluorescence microscope	Axiovert S 100	Carl Zeiss MicroImaging GmbH, Jena

Fluorescence microscope	Axio Imager M1	Carl Zeiss MicroImaging GmbH, Jena
Microscope	AE31	Motic, Wetzlar

Softwares

Name	**Product info/ Cat. No.**	**Manufacturer**
Axiovision 4.8 software	-	Carl Zeiss MicroImaging GmbH, Jena
Chemi-Capt	-	Vilber-Lourmat, Eberhardzell
Graphpad Prism	Version 5	Graphpad Software Inc, La Jolla
Vector NTI	Version 9	Invitrogen Life Technologies, Grand Island
Vision-Capt	-	Vilber-Lourmat, Eberhardzell

7.1.2 Enzymes and markers

Name of enzyme	**Cat. No.**	**Manufacturer**
Antarctic phosphatase	Cat. No. M0289L	New England Biolabs, Ipswich
*Bam*HI	Cat.No. R0136	New England Biolabs, Ipswich
Benzonase	Cat.No. D00111784	Novagen, San Diego
*Dpn*I	Cat.No. ER1701	New England Biolabs, Ipswich
*Eco*RI	Cat. No. R0101	New England Biolabs, Ipswich
*Eco*RI HF	Cat. No. R3101	New England Biolabs, Ipswich
*Eco*RV	Cat. No. R0195	New England Biolabs, Ipswich
HaeIII	Cat. No. R0108S	New England Biolabs, Ipswich
*Hind*III	Cat. No. R0104	New England Biolabs, Ipswich
Hot FirePol® qPCR DNA Polymerase	Cat. No. 01-02-00500	Solis BioDyne, Tartu
Phusion Hot Start High-Fidelity DNA Polymerase	Cat.No. M0530S	Thermo Scientific, Rochester
Proteinase K	Cat.No. 7528.2	Roth, Karlsruhe

RNase A	Cat.No. 2326466	Applichem, Darmstadt
SacI	Cat. No. R0156S	New England Biolabs, Ipswich
T4 DNA Ligase	Cat.No. 01-1020	Peqlab, Erlangen
Taq DNA-Polymerase	Cat.No. 01-1020	Peqlab, Erlangen

Name of DNA Marker	**Cat. No.**	**Manufacturer**
Generuler ™ 1kb Plus DNA Ladder	Cat. No. SM0311	Fermentas, Mannheim

7.1.3 Antibodies

Name	**Dilution**	**Source**
Anti-β-Actin	1:1,000	Cell Signaling, Cambridge
Anti-DIG-FITC	1:1,000	Roche, Mannheim
Chicken anti MDV US2, polyclonal	1:1,000	[117]
Alexa goat anti-chicken IgG (H+L) 488	1:1,000	Invitrogen Life Technologies, Grand Island
Alexa goat anti-chicken IgG (H+L) 546	1:1,000	Invitrogen Life Technologies, Grand Island
Rabbit-anti Flag epitope	1:1,000	Sigma-Aldrich, St Louis
Mouse anti Flag-FITC labelled	1:1,000	Sigma-Aldrich, St Louis

7.1.4 Laboratory kits

Name	**Cat. No.**	**Company**
DIG High-Prime	Cat. No. 11585606910	Roche, Mannheim
DIG Wash and block Buffer set	Cat. No. 11585762001	Roche, Mannheim
Hi Yield Gel/PCR DNA Fragments Extraction Kit	Cat No. 30 HYDF100-1	SLG, Gauting
RTP® DNA/RNA Virus Mini Kit	Cat. No. 1040100300	STRATEC Molecular GmbH, Berlin
Qiagen Plasmid Midi kit	Cat. No. 12145	Qiagen, Hilden

7.1.5 Antibiotics

Name and Cat. No.	**Working concentration**	**Manufacturer**
Ampicillin (Amp) [Cat. No. K0292]	100 µg/ml in ddH_2O	Roth, Karlsruhe
Chloramphenicol (Cam) [Cat. No. 3886.3]	30 µg/ml in 96 % EtOH	Roth, Karlsruhe
Kanamycin sulphate (Kana) [Cat. No. T832.3]	50 µg/ml in ddH_2O	Roth, Karlsruhe
Hygromycin B Solution [Cat. No. 31282-04-9]	800 µg/ml in ddH_2O	Roth, Karlsruhe
Penicillin (P) [Cat. No. A1837]	100 U/ml in MEM	Applichem, Darmstadt
Puromycin (Puro) [Cat. No. A11138-03]	1 µg/ml in RPMI	Invitrogen, Carlsbad
Streptomycin (S) [Cat. No. A1852]	100 U/ml in MEM	Applichem, Darmstadt
Trimethoprim (TMP) [Cat. No. T7883-5G]	10 µM/ml in DMSO	Sigma-Aldrich, St Louis

7.1.6 Buffers and media

Buffers

1x Phosphate saline buffer (1x PBS)

2 mM KH_2PO_4
10 mM Na_2HPO_4
137 mM NaCl
2.7 mM KCl, pH 7.3

1x Tris-acetate-EDTA buffer (TAE)

40 mM Tris
1 mM $Na_2EDTAx2H_2O$
20 mM Acetic acid 99 %, pH 8.0

0.8 % Agarose Gel

80 mM Agarose
1x TAE buffer
4 µl Ethidium bromide 10 mg/ml

Media and supplements for cultivation of bacteria (*E.coli*)

LB medium (1l)

10 g Bacto™ Tryptone
5 g Bacto™ Yeast Extract

SOB medium (1l)

20 g Bacto™ Tryptone
5 g Bacto™ Yeast Extract

10 g NaCl
15 g Bacto™ Agar

0.584 g NaCl
0.186 g KCl
pH to 7.0

SOC medium

SOB medium
20 mM Glucose

Plasmid preparation solution

Buffer (P1)

50 mM Tris HCL pH 8.0
10 mM EDTA
100 µg/ml RNase

Neutralisation Buffer (P3)

3 M K-Acetate pH 5.5

Lysis Buffer (P2)

200 mM NaOH
1% SDS

Buffer TE

10 mM Tris HCL pH 7.4
1 mM Na_2EDTA

Media and supplements for cultivation of mammalian cells

Name	Cat. No.	Manufacturer
Biocoll Separating solution, density 1.077 g/ml	Cat.No. L6115	Biochrom AG, Berlin & Sigma-Aldrich, St Louis
Dulbecco's Modified Eagle Medium (DMEM) / Ham's F-12 (1:1)	Cat. No. F 4815	Biochrom AG, Berlin
Dulbecco's Modified Eagle Medium (DMEM)	Cat. No. P04-03500	PAN-Biotech, Aidenbach
Fetal bovine serum (FBS)	Cat.No. S 0415	Biochrom AG, Berlin & PAN-Biotech, Aidenbach
Fetal calf serum (FCS)	Cat.No. P30-1506	PAN-Biotech, Aidenbach
Minimum essential Medium Eagle (MEM)	Cat.No. F 0315	Biochrom AG, Berlin & PAN-Biotech, Aidenbach
Non-Essential Amino acids (NEAS)	Cat. No. K0293	Biochrom AG, Berlin
RPMI 1640 (w/o Glutamine)	Cat.No. F 1215	PAN-Biotech, Aidenbach
Trypsin	Cat.No. L 2103-20G	Biochrom AG, Berlin

Fluorescence in situ hybridization (FISH) solutions

Hypotonic solution

0,075 M KCl

autoclaved

Fixative

Methanol:Acetic acid 3:1

Detergent wash solution

4x SSC

0.5 % Tween-20

Hybridization buffer

50 % Deoinized formamide (v/v)

10 % Dextran sufate (v/v)

2x SSC

1 x Phosphate buffer, pH 7.0

Probe

Salmon sperm

20x SSC

87.6 g NaCl

44.1 g TriNaCitrate dehydrate

to 500 ml in ddH_2O, pH to 7.4

Stringency wash solution

50 % 2x SSC

50 % Deionized formamide

Pepsin solution

0.01 % pepsin in 10 mM HCl

Western blot solutions

10x SDS PAGE running buffer

250 mM Tris

1.9 M Glycine

1 % SDS

Transfer buffer

25 mM Tris

192 mM Gycine

20 % (v/v) MeOH

2x Stripping buffer

50 mM Glycine

2 % SDS

pH 2

RIPA buffer

20 mM Tris-HCl

150 mM NaCl

1 % (v/v) Nonidet P-40

0.5 % (w/v) Sodium deoxycholate

0.1 % (w/v) SDS

Complete® Mini protease/phosphatase inhibitor cocktail

7.1.7 Bacteria, cells and viruses

Bacteria

Name	Features	Reference
GS1783	DH10B λcI857 Δ(cro-bioA)<>araC-$P_{BAD,}$ I-SceI	[118]
Stbl3	F$^-$mcrB mrrhsdS20(r_B^-, m_B^-) recA13 supE44 ara-14 galK2 lacY1 proA2 rpsL20(Str^R) xyl-5 λ$^-$leumtl-1	ThermoScientific, Waltham
Top10	F$^-$mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1	Invitrogen, Carlsbad

Cells

Name	Features	Reference
CEFs/ CECs	Chicken embryo fibroblasts/cells, primary cells, VALO SPF strain	Primary cells
HEK293T reporter cells: DR-GFP; SA-GFP; EJ2-GFP	Human epithelial kidney cell line, SV40 T-antigen with a GFP reporter for double strand break repair	[119]
MDCC-MSB1	Chicken hematopoietic lymphoblast cell line	[120]
RECC-CU91	Reticuloendotheliosis virus-transformed avian T-lymphoblastoid cell line	[121–123]
U2OS (EJ5-GFP)	Human epithelial osteosarcoma cell line	[119]
CRs	Continuous *Cairina* retina cell lines from the Muscovy duck	[124]

Viruses

Name	Features	Reference
pRB-1B (S+)	Bacterial artificial chromosome (BAC) codifying for a vvMDV strain RB-1 with repaired US2 deletion and gC and UL13 mutations	[125]
ΔmTMR	RB-1B that lacks the mTMR sequences and is defective in integration into host telomeres	[55]

7.1.8 Plasmids

Name	Features	Reference
pcDNA3.1+	Mammalian expression vector; T7prom, f1 ori, pBR322 ori, AmpR, pCMV, pSV40, NeoR	Invitrogen, Carlsbad
pLKO-shDPS	Mammalian expression vector for shRNAs, 7SK, mU6, U6, shPML, shDaxx, shSp100, CPPT, hPGK, PuroR, sin 3'LTR, f1 ori, AmpR, pUC ori, 5'LTR, RRE	[126]
pCMV-dR8.91	Mammalian expression vector for envelope, AmpR, CMV promoter, VSV-G, SV40 polyA	Addgene
pHA-N-Dest	Low copy pHA vector	Addgene
pUC19	E.coli cloning vector; AmpR, ColE1 ori	New England Biolabs

7.1.9 Primers

Table 2. Primers and probes for qPCR, construction and sequencing of recombinant viruses and plasmids for the UL12 and UL29 project.

Construct name		Sequence (5' → 3')
vUL12 mut1	**For**	CTCTTTCGTTTTCGGGACTCATCTTTGCAAGATTTGGAGAGTGGTTACTAGGTTCCTAGTTCCATATAGGGATAACAGGGTAATCGATTT
	Rev	CAGTAGCAAGAACGGCTGATTTGTTGTTATGGAACTAGGAACCTAGTAACCACTCTCCAAATCTTGCGCCAGTGTTACAACCAATTAACC
vUL12 rev1	**For**	TTCGTTTTCGGGACTCATCTTTGCAAGATTTGGAGAGTGGAAACTTGGTTCCTAGTTCCATAACAAGCCAGTGTTACAACCAATTA
	Rev	TAGCAAGAACGGCTGATTTGTTGTTATGGAACTAGGAACCAAGTTTCCACTCTCCAAATCTTGCAATAGGGATAACAGGGTAATCG
vUL12 mut2	**For**	GTGCCACAATTCACTGTTCGCTTGTAGTCTAGTTAACGATTGAGCTCATTTGCACCAGCGAGAAGTAGGGATAACAGGGTAATCGATTT
	Rev	GGTTAATTGGTTGTAACACTGGCTAGTCTAGTTAACGATTGAGCTCATTTGCACCAGCGAGAAGCATGAATTGATGGTGACAAATTAT
vUL12 rev2	**For**	TGCCACAATTCACTGTTCGCTTGTAGTCTAGTTAACGATTCCACCATTTTGCACCAGCGAGATAGGGATAACAGGGTAATCGATTT
	Rev	TAATTTGTCACCATCAATTCATGCTTCTCGCTGGTGCAAAATGGTGGAATCGTTAACTAGACGCCAGTGTTACAACCAATTAACC
vUL29 mut1	**For**	GCCTATCGGTCCGCCGCAAAGTTTCACGCTTTTTCCTACAAGCTTCTAAATGTTGTTGTTAGGGATAACAGGGTAATCGATTT
	Rev	GGTTAATTGGTTGTAACACTGGCCTTTTTCCTACAAGCTTCTAAATGTTGTTGTTGGATAGTTGACAGAATCTAGTGCGAGAG
vUL29 rev1	**For**	AGCCTATCGGTCCGCCGCAAAGTTTCACGCTTTTTCCTACACCATCCATAATGTTGTTGTTGGATAGGGATAACAGGGTAATCGATTT
	Rev	CTCTCGCACTAGATTCTGTCAACTATCCAACAACAACATTATGGATGGTGTAGGAAAAAGCGTGGCCAGTGTTACAACCAATTAACC

Table 2. Continuation

Construct name		Sequence (5' → 3')
vUL12 mut1/rev1 (Seq)	**For**	TCGGACGGAAGATTGGCT
	Rev	CCACAGAGCCGAGTTTGATAA
vUL12 mut2/rev2 (Seq)	**For**	CACTGGGATAGTTCACTTCTCATTTC
	Rev	TAAACATTTTTCCCTATTTAAACAACCTGT
vUL29 mut1/rev1 (Seq)	**For**	ATAAATATATGACCTCTTTAAATAATTCGG
	Rev	TATGAGTTCCCTTTTGTTTTACATCTTT
ICP4 (qPCR)	**For**	CGTGTTTTCCGGCATGTG
	Rev	TCCCATACCAATCCTCATCCA
	Probe	FAM-CCCCCACCAGGTGCAGGCA-TAM
iNOS (qPCR)	**For**	GAGTGGTTTAAGGAGTTGGATCTGA
	Rev	TTCCAGACCTCCCACCTCAA
	Probe	FAM-CTCTGCCTGCTGTTGCCAACATGC-TAM
ICP8 low copy plasmid – CMV insertion	**For**	CAAAGTTTCACGCTTTTTCCTACACCATCCATAATGTTGT TGCACCGTACACGCCTACCG
	Rev	GTTATCTAGGAAGCTGATGCGGCCGGCCCTTAATTAACC ATGTACGGGCCAGATATACGC
ICP8 low copy plasmid – CMV (Seq)	**For**	ACCCCCACTGCCCAAAC
	Rev	GCGAGTTTACGTGCATGGAT
ICP8 low copy plasmid – BGH insertion	**For**	TCGTATAGCATACATTATACGAAGTTATCTAGCAGATCTG TTTGTCTTCCCAATCCTCCC
	Rev	CCGAGAAGCGCGTACCAGTGTTATCTGTAGATATGTTGT AAAGCATGCATCTAGAGGGCC
ICP8 low copy plasmid – BGH (Seq)	**For**	AATGCCTGTCAAGGGCAAGT
	Rev	GAGTCCGAATGGTGCTATCC
UL12 amplification for pUL12 plasmid (and Seq)	**For**	AGCGTTTAAACTTAAGGCCACCATGGAACTAGGAACCAA GTTTCC
	Rev	CCGAGCTCGGTACCTTAAATACGACACTGCTTGG

Table 3. Primers for construction and verification of shRNAs for the UL12 and UL29 project.

Construct name and primers		Sequence (5' → 3')
shRNA UL12 #1	**For**	CACCGGGGCATTACGATCGACTGTGATCAAGAGTCACAGTCGATCGTAATGCCCTTTTTT
	Rev	CAGCAAAAAAGGGCATTACGATCGACTGTGACTCTTGATCACAGTCGATCGTAATGCCCC
shRNA UL12 #2	**For**	ATCAAAAAAGTGGAGCCCCAATGGAACAATCTCTTGAATTGTTCCATTGGGGCTCCACGAGG
	Rev	GGTACCTCGTGGAGCCCCAATGGAACAATTCAAGAGATTGTTCCATTGGGGCTCCACTTTTT
shRNA UL12 (pLKO seq)	**For**	ATTTCTTGGGTAGTTTGCAG
shRNA UL29 #1	**For**	CACCGGGTAGTCACCGAATTATTTAATCAAGAGTTAAATAATTCGGTGACTACCTTTTTT
	Rev	CAGCAAAAAAGGTAGTCACCGAATTATTTAACTCTTGATTAAATAATTCGGTGACTACCC
shRNA UL29 #2	**For**	ATCAAAAAAGTTGGATATGCCGATAGGATACTCTTGATATCCTATCGGCATATCCAACGAGG
	Rev	GGTACCTCGTTGGATATGCCGATAGGATATCAAGAGTATCCTATCGGCATATCCAACTTTTT
shRNA UL29 (pLKO seq)	**For**	ATTTCTTGGGTAGTTTGCAG

Table 4. Primers and probes for qPCR and construction of recombinant viruses, plasmids and shRNAs for UL30cDHFR project.

Construct name and primers		Sequence (5' → 3')
vUL30 cDHFR Mutagenesis	**For**	ATAGCTTTCCGTATTCTAACAGCAACTCCCCATCGATAT GGTGGTTCTGGTGGTTCTTAC
	Rev	GGTTAATGCTGAAGATGTTTATTGTAAATTATGCGATCT TAATATCGATGGGGAGTTGCTGTTAGAATACGGAAAGC TATATTCAGCTTTCGACGAGTTTCTTCCTCCTCGTTGAT TATCGCCGCTCCAGAATCTCAA
vUL30 cDHFR Seq	**For**	CTGTTGGGGACACTATGCGTAACTTTTAAG
	Rev	CCGGAAAGGCTCTCCACCTCCACTA
Mini F GFP Mutagenesis	**For**	CTGTTGGGGACACTATGCGTAACTTTTAAG
	Rev	CCGGAAAGGCTCTCCACCTCCACTA
ICP4 (qPCR)	**For**	CGTGTTTTCCGGCATGTG
	Rev	TCCCATACCAATCCTCATCCA
	Probe	FAM-CCCCCACCAGGTGCAGGCA-TAM
iNOS (qPCR)	**For**	GAGTGGTTTAAGGAGTTGGATCTGA
	Rev	TTCCAGACCTCCCACCTCAA
	Probe	FAM-CTCTGCCTGCTGTTGCCAACATGC-TAM
shRNA UL30#1	**For**	CACCGGCTTCTCTAGGTTTCATGTATTCAAGAGATACAT GAAACCTAGAGAAGCTTTTTT
	Rev	CAGCAAAAAAGCTTCTCTAGGTTTCATGTATCTCTTGAA TACATGAAACCTAGAGAAGCC
shRNA UL30#2	**For**	ATCAAAAAAGCAAAGTCGATACTGATACCACTCTTGATG GTATCAGTATCGACTTTGCGAGG
	Rev	GGTACCTCGCAAAGTCGATACTGATACCATCAAGAGTG GTATCAGTATCGACTTTGCTTTTT
shRNA UL30 (pLKO Seq)	**For**	ATTTCTTGGGTAGTTTGCAG

7.2 Methods

7.2.1 Molecular biology techniques

7.2.1.1 The Red recombination system and *en passant* mutagenesis

In the early 1990s, Bacterial artificial chromossomes (BACs), which were based on the single-copy F-plamid of *E.coli*, were used for cloning extensive DNA fragments, which were larger than 300 kb. Considering that herpesviruses possess large genomes compared to other viruses and the stable maintenance and uncomplicated manipulation of a cloned DNA make BACs a very useful biological tool for manipulating virus DNA. Evidences show that the first herpesvirus genome cloned into a BAC with efficient reconstitution of virions in eukaryotic cells was of MCMV (murine cytomegalovirus) in 1997. In order to successfully control and manipulate BACs scientists have used the Red recombination system, which is a technique developed upon the λ-phage recombination system, and currently is widely used to introduce any type of alterations to the genome, including insertions, deletions, tags or point mutations.

The components of the recombination system are the *Exo, Beta* and *Gam* proteins, which are all regulated by a temperature-inducible promoter. The *Exo* protein is a 5'-3' exonuclease that produces 3' single strand overhangs, which are protected by the Beta protein. Here, *Beta* is a protein that initiates the annealing of complementary single strands of DNA, whilst *Gam* is a protein that inhibits the degradation of double strand breaks controlled by *E. coli RecB/C/D* enzymes. What is interesting about the Red recombination, is the fact that in order to initiate, the system only requires homologous sequences of only 30 to 50 bp long; thus, fragments such as those PCR-amplified can be used as targeting cassettes, which are then introduced through electroporation.

In this dissertation, a two-step markerless Red-mediated recombination system was used. Here, we used an *E.coli* strain named GS1783, which harbors the temperature-inducible λ-phage Recombination system together with an *I-SceI* endonuclease containing an 18bp recognition site, under an arabinose sensitive promoter, which together was able to promote the DNA cleavage upon temperature change. The two-step Red-mediated mutagenesis, or also known as *en passant* mutagenesis has as first step the PCR product of a linear DNA product (Figure 9). This first linear DNA product is the one that contains the specific desired mutation in frame with a resistance marker cassette, which in this dissertation were the *aphAI* kanamycin resistance gene and the *I-SceI* restriction site. The overhangs of this linear DNA product contain primers that harbor homologous sequences for the first step of the Red recombination system (A/B & C/D), the specific mutation (represented with a X) and the

nucleotide sequence duplications for successful removal of the resistance marker in the second step of the Red recombination (B/C). Additionally, these primers also need to contain a homologous sequence (black lines) in order to anneal to the pEP Kan-S plasmid, allowing proper amplification of the whole block.

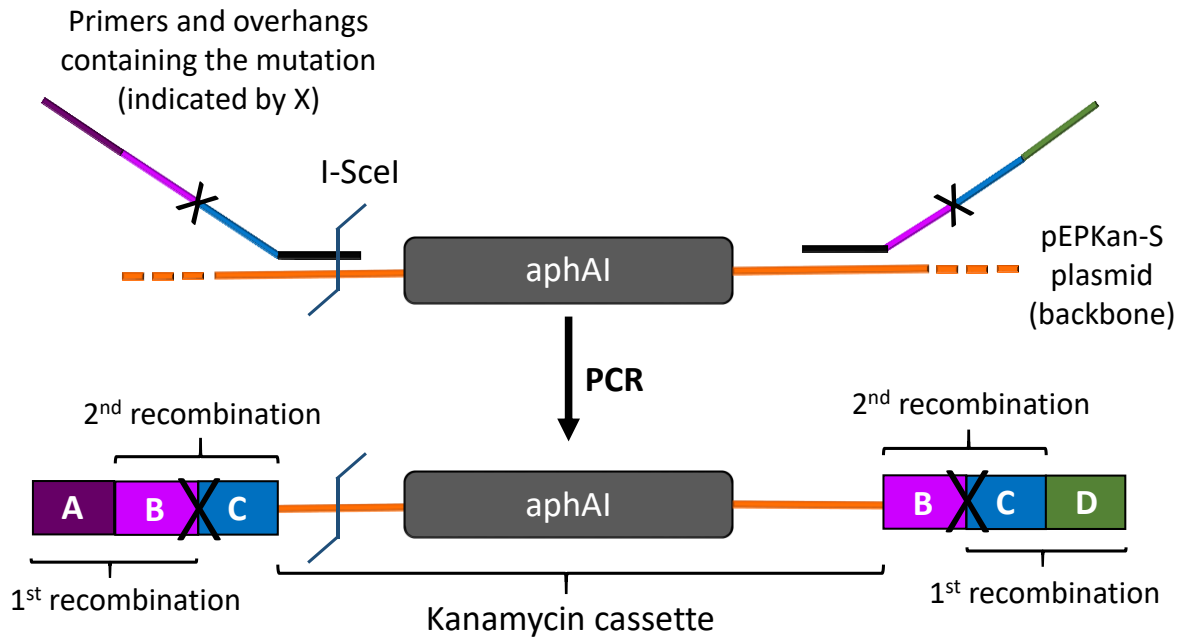

Figure 9. PCR result amplified from the plasmid pEP Kan-S (containing selection marker) in order to insert or delete point mutations into the desired genome. "X" represents the location containing the mutations. Both regions A/B and C/D located at the ends of the PCR product allow the recombination of the electroporated PCR product into the specific locus in the genome – this first step is named as 1st Red recombination. The repeated squences B/C permits the scarless removal of the kanamycin cassette containing the selection marker upon *I-SceI* cleavage during the 2nd Red recombination step, resolution.

Once the PCR product is generated, it will be electroporated inside a BAC-containing GS1783 bacteria, where the 1st Red recombination process happens through the homology between A/B and C/D regions and the genome. This event leads to the insertion of the entire cassette, which now contains the desired mutation, into the specific locus of the genome. This new DNA generated after the insertion of the cassette is designated as intermediate clone, which now can be positively selected by the kanamycin resistance gene that it contains. Following this, the induction of *I-Sce*I expression creates a DSB, event which here is named as resolution, permitting the 2nd Red recombination event to happen. The proper removal of *I-Sce*I leads to the smooth removal of the selection marker, yet remaining the mutation intact, which gives rise to the final clone. This technique has been used to generate all mutant viruses used in this research.

7.2.1.2 Generation of lentivirus plasmids

Recombinant plasmids here are referred to the ones used in the generation of expression vectors for lentivirus assembly. First, oligos for both UL12 and Ul29 shRNAs were designed using VectorNTi®, where both hairpins were targeting the n and c termini of proteins UL12 and ICP8, separately. In order to initially phosphorylate the oligos, those were on a concentration of 100µM in dH_2O and mixed with a solution containing 1µl of 10X T4 ligase buffer, 7,5 µl dH_2O and 0,5 µl T4 PNK kinase. Next, the mix was incubated at 37ºC for 1 h and had the ONK kinase heat-inactivated by heating up to 65ºC for 20 min. As a result, it was obtained a solution of oligos in the final concentration of 10 µM, which was stored in -20ºC. Following oligos phosphorylation, 10 µM of the oligos were added to 60 mM HEPES pH 7.4 mM potassium acetate buffer to initiate the annealing step of phosphorylated oligos. This solution was then put in the thermocycler and ran on a program set for 95ºC for 5 min, followed by a gradual cooling step that reduced the temperature by 0.6ºC/min until it reached 30ºC. After heating and slow cooling, samples were put on ice and directly ligated on a ratio of 3:1 into 50µL of digested vector. After the oligos have been phosphorylated and annealed, both shRNA oligos were cloned into the pLKO5.2XshRNA plasmid in order to be properly assembled *in vitro*. To ligate two annealed shRNA oligos into the pLKO5.2XshRNA plasmid, two sequential cloning steps were performed. First, the first shRNA, 3' was inserted to the huU6 promoter using BsmBI sites whilst the second shRNA was inserted in the anti-sense orientation controlled by the 7SK promoter, using BfuAI sites. For the shRNA#1. The pLKO5.2xshRNA plasmid was first digested with BsmBI and purified for ligation. Ligation then was performed with 100 ng of the vector DNA and 3X volume of the annealed oligos and 4 µL of T4 ligase transformed into 100 µl of Stbl3 chemically competent cells. Positive clones were tested via enzymatic digestion and restriction fragment length polymorphism (RFLP) band patterns. The insertion of the shRNA#2, for both UL12 and IPC8 was performed using the same protocol as for the shRNA#1, except that the first digestion step required an additional 0,5 µl of an annealed oligo containing the *BfuA*I site to ensure the vector was fully cut.

7.2.1.3 DNA mini and midi-preparation

In order to isolate exogenous DNA from bacteria, a standard alkaline lysis protocol was used. In this case, bacterial culture was cultivated overnight (o/n) at 32ºC in 4 ml of LB medium, which contained the respective antibiotics. On the following day, the 4 ml of bacterial culture was transferred into a falcon tube and pelleted for 5 min at 5,000 rpm, with the remaining supernatant being discarded afterwards. The remaining pellet was then resuspended in approximately 300 µl of P1 buffer, followed by the addition of 300 µl of P2 buffer and kept for

5 min at RT, which led to cell lysis. In order to neutralize the reaction and to promote the precipitation of proteins, a final step consisting on the addition of 300 µl of buffer P3 to the solution was performed. Next, the mixture was kept for 10 min on ice and then centrifuged for 10 min at 10,000 rpm to separate the proteins and cell debris for later removal. Following centrifugation, the supernatant was removed and transferred into a new eppendorf tube, where 900 µl of phenol:chloroform solution were added in order to eliminate any undesired protein debri. This solution was then vortexed briefly and centrifuged for 10 min at 10,000 rpm. As a centrifugation result, there were two phases in the tube, an aqueous phase containing the DNA and the phenol:chloroform solution, separated. The aqueous phase was transferred into a new eppendorf tube, mixed manually with 450 µl of isopropanol and kept for 10 min at -20ºC. Next, the samples were centrifuged in a refrigerated (4ºC) centrifuge for 15 min at 10,000 rpm in order to perform DNA precipitation. Once the DNA was precipitated, it was washed in two steps with 70% ethanol. The remaining ethanol in the tube was completely removed by heating the samples in a heat block for 5 min at 37ºC. Finally, the clean DNA was mixed at 37ºC for 30 min with TE-buffer containing RNase A (100 µg/ml) and stored at either 4ºC or -20ºC for later use.

In the case of MIDI DNA preparations, a similar protocol as for MINI preparation was performed, except that the reagents used were from the BAC100 Kit (Macherey-Nagel, Düren) and specific instructions for BAC DNA extraction from the manufacturer were followed.

7.2.1.4 Electrocompetent bacteria

In this study, the *E.coli* stain GS1783 containing the BAC clone of the MDV strain RB-1B was used for viral DNA manipulation. BAC MDV RB-1B clones or mutants containing either point mutations, insertions or deletions were cultivated overnight at 32ºC in 4 ml LB medium with the proper antibiotics added to it, which in this case was chloramphenicol (Cam). On the following day, 1 ml of the overnight culture was mixed with 200 ml of LB medium and incubated at 32ºC under vigorous shaking at 220 rpm in order to stimulate bacterial growth. Once optimal bacterial growth was achieved after 2-4 hours (OD_{600} 0.5-0.7), the bacterial solution was submitted to a heat shock step in order to activate the Red-recombination system. This step consisted of initial heating at 42ºC for 15 min at 220pm in a water bath, followed by a rapid cooling on ice for 20 min under continuous shaking. After the heat shock, the bacterial culture was centrifuged for 5 min at 4ºC at 5,000 rpm, generating a pellet. The bacterial pellet was washed during three washing steps with an ice-cold 10% glycerol solution, whilst the final wash was followed by centrifugation at 5,000 rpm, for 5 min at 4ºC. Next, the bacteria were resuspended in 10% glycerol, aloquoted in 1.5 ml eppendorf tubes and stored at -80ºC.

Following this protocol, bacteria were electrocompetent and ready to use for viral DNA manipulation *in vitro*.

7.2.1.5 Chemically competent bacteria

Chemically competent bacteria were used here to promote the ligation of the shRNAs into the pLKO5.2XshRNA plasmid. Initially, Stbl3 bacteria sample were taken from a glycerol stock and inoculated into a flask containing 50 mL of LB medium, where it was kept growing overnight at 32ºC whilst shaking vigorously. The next day, 500 µl of the overnight broth was taken and incubated in a new flask containing 50 mL of LB medium. After the right OD_{600} was obtained (usually after 2-4 h), the culture was centrifuged at 8,000 rpm for 5 min in sterile tubes. After this step, the supernatant was removed and the pellet was kept on ice, where it was resuspended in 5 mL of ice-cold $CaCl_2$. After resuspension, the solution was distributed into pre-chilled 1,5 mL ultra-centrifuge tubes, where it was again centrifuged and pelleted. Next, the pellet was resuspended in 500 µl ice-cold $CaCl_2$ and aliquoted in 50 µl into eppendorf tubes. Electrocompetent bacteria were stored at -80ºC until further use.

7.2.1.6 Generation of MDV mutants

In order to generate MDV mutants, 100 ng of DpnI-digested PCR product containing the mutations were added to thawed electrocompetent GS1783 bacteria containing the respective MDV BAC clone. For the first step of the Red recombination to occur, the bacteria were electroporated at 1.25 kV, 25 µF and 200 Ω. Next, 900 µl of pre-warmed LB medium were added to the bacteria in order to aid bacterial growth post-electroporation. Following 1h incubation at 32ºC at 220 rpm, bacteria cells were centrifuged for 2 min at 5,000 g. Here, most of the remaining supernatant was discarded and the pellet was resuspended in approximately 100 µl of residual medium. The resuspended bacteria were streaked out on LB agar plates, each containing the respective antibiotics used for positive selection (usually chloramphenicol-Cam, and kanamycin-Kana). As the PCR product that was electroporated in the bacteria contained a kanamycin cassette, after 48h incubation at 32ºC, clones that grew were positive and selected for RFLP screening in order to confirm the correct banding pattern on the gel.

Following the RFLP screening and the confirmation of the positive clones, these were cultures overnight at 32ºC in 2 ml solution of LB medium containing Cam+Kana. This culture would then be used for the resolution step, also called the second step of the Red recombination process, which is performed to facilitate the excision of the kanamycin cassette. On the next

day, 2 ml of LB+Cam were inoculated with 100 µl of the overnight culture. Next, the mixed solution was kept shaking for 3-4 h at 32°C, when 2 ml of LB+Cam containing 2 % arabinose were added and incubated for 60 min at 32°C and 220rpm to induce the *I-Sce*I expression. In order to activate the Red recombination system, the same bacteria were then incubated for 30 min at 42°C and 220rpm followed by a 4 h incubation at 32°C to allow recombination to happen. Agar plates containing Cam+1% arabinose were used to host the bacterial culture, which were distributed on 10^{-4} and 10^{-6} dilutions in LB.

Cam+1% arabinose plates containing the bacteria culture were kept at 32°C for 48 h. After this period, individual colonies were picked and plated on agar plates containing LB+Cam and LB+Cam+Kana for another round of selection. Clones that were Kana-sensitive were named ad final clones and taken for RFLP screening.

7.2.1.7 PCR & Sequencing

For the generation of MDV mutants used in this study, a two-step PCR protocol was followed, where the first annealing temperature and the second denature and annealing times varied. All PCR products generated for mutagenesis were created using Taq DNA polymerase.

Table 5. General two-step PCR settings for the generation of MDV mutants

Temperature (°C)	Time (Sec)	PCR Step		Cycles
94	300	Initial denaturation		
94	30	Denaturation		
Differs according to primer sets	30	Annealing	1st Step	10 cycles
72	Varied	Elongation		
94	30	Denaturation		
68	30	Annealing	2nd Step	30 cycles
72	Varied	Elongation		
72	600	Extension		

The PCR products generated for sequencing purposes to confirm if recombination occurred properly were generated following the protocol seen in table X below. All PCR products generated for sequencing were created using Taq DNA polymerase.

Table 6. Sequencing PCR protocol

Temperature (ºC)	Time (Sec)	PCR Step	Cycles
95	300	Initial denaturation	
95	30	Denaturation	
Differs according to primer sets	30	Annealing	30 cycles
72	Varied (approx. 1 min)	Elongation	
72	600	Extension	

7.2.2 Cell cultures

7.2.2.1 Culture of eukaryotic cells

In this research project, different eukaryotic cell lines were used for different purposes. Here it will be described the culture of the following cell lines *in vitro*: chicken embryo cells (CECs), reticuloendotheliosis virus-transformed avian T-lymphoblastoid cells (CU91s), *Cairina* retina cells (CRs), human embryonic kidney 293 cells (HEK293s) and human osteosarcoma cells (U2OS).

CECs were firstly used here to replicate efficiently MDV *in vitro*. These cells originated from Valo-SPF embryos as described previously [127]. Initially, eggs were kept in the incubator for 11 days at 37.8ºC with a humidity level of 50-60%. Before the eggs were hatched, embryos were carefully extracted from the eggs, and had their head, internal organs and extremities properly removed. The remaining part of the embryo was then immersed in PBS to have the excessive blood removed. Next, the embryo was reduced into small pieces by cutting it and the parts were washed in PBS on a magnetic stirrer for approximately 10 min. Following the stirring, 3 digestions with 100 ml of a 0.05% Trypsin solution were done, and after each step, the cell suspension was collected and filtered on a sterile gauze membrane, which later was placed in a 10% FBS MEM solution. The cells in the solution were aliquot in 50 ml Falcon tubes and separated by centrifugation for 10 min at 12000 rpm. The pellets generated were pooled and washed with 1xPBS before resuspension in 100 ml of 10% FBS MEM. The quality and the final concentration of cells were acquired using an inverted microscope and analysing the number of cells plated into tissue culture material. After the first plating of CECs, the following passages made to amplify the number of cells was done by removing the medium, washing the cells with PBS and incubating them with 0.05% Trypsin at 37ºC for an approximate time of 1-2 min, or until the cells were completely detached from the dish. Once cells were properly detached, MEM containing 10% FBS was added in order to inactivate the Trypsin. Next, cells were resuspended and then split at a 1:2 or 1:3 ration into new dishes.

CU91 cells present in suspension were maintained at 41ºC in a 5% CO2 atmosphere and propagated in RPMI 1640 medium supplemented with 1% sodium pyruvate, 1% nonessential amino acids, 10% FBS, and penicillin–streptomycin. Every second day the cells were amplified in the ratio of 1:2 or 1:4, depending on the experiment being carried on and the day it would happen. Usually, one day before any integration assay or transduction/ infection experiment, cells were amplified in the ratio of 1:2 to ensure good cell viability.

CR cells were maintained in Dulbecco's MEM/Ham's F-12 supplemented with 5% FBS, and penicillin–streptomycin. CR cells were amplified in a similar manner to CECs, as described previously.

Cells used for the double strand break repair, HEK293 and U2OS, were cultured in DMEM supplemented with 10% FBS and penicillin-streptomycin. Both cell lines were kept at 37ºC. HEK293s and U2OS were amplified in a similar manner to CECs, as described previously.

7.2.2.2 Lentivirus transduction

CU91 cells containing shRNAs to knockdown UL12 and UL29 for the integration assay were generated through lentiviral transduction. Here, lentiviruses were prepared using the lentiviral packaging vectors: pCMVdR8.91 and pVSV-G plus the respective pLKO5.2xshRNA, which is the transfer vector. For successful lentiviral transduction, the following materials were used in addition to the lentiviral packaging vectors: PEI 1mg/ml, Opti-MEM medium with no serum or antibiotics added, 293T cells and 0.45µm filters. On the first day, prior to transfection, 293T cells were counted and 4-4x10^6 cells were plated per 10cm dish, which led to approximately 80% cellular confluence. Next day, the packaging vectors were transfected into 293Ts using PEI with an incubation period of 20 min at room temperature. On the third day, after approximately 16h post-transfection, medium was removed and replaced with 5 ml of new medium containing 10mM sodium butyrate. After 6 h, the medium containing sodium butyrate was removed and cells were washed with fresh medium prior to virus harvest on the next day. The last step on lentivirus transfection is the harvest. Using a sterile disposable 10 ml syringe, cellular supernatant containing the lentiviruses was harvested and passed through a disposable 0.45µm filter into a sterile 50ml tube. Finally, lentiviruses were aliquot into 1.5ml Eppendorf tubes and stored at -80ºC. CU91 cells were transduced with 5µl, 50µl and 500µl of lentiviruses in order to guarantee optimal transduction. After 2 days, CU91 cells were positively selected for puromycin (UL29 shRNA) at a concentration of 1µg/ml and after 5 days for hygromycin (UL12 shRNA) at a concentration of 400µg/ml. In order to monitor and validate UL12 and UL29 shRNA constructs knockdown efficacy, CR cells were transduced with

shRNAs against UL12 and UL29 and later infected with CECs containing MDV. Here, CR cells transduced with shRNA against UL12 were selected for hygromycin after 6 days at a concentration of 200µg/ml, whilst CR cells transduced with shRNAs against UL29 were selected for puromycin after 4 days at a concentration of 1µg/ml. Plaque sizes were measured six days post infection to analyse shRNA knockdown indirectly represented by plaque sizes.

7.2.2.3 Immunofluorescence

Considering that the majority of the viruses used in this research did not contain a GFP gene in their backbones, immunofluorescence staining was used to aid the visualisation of CECS and plaques in different applications, such as plaque size assays, growth kinetics and virus titrations. Initially, medium was removed from cells, and these were washed with PBS once. Cells were carefully fixed with ice-cold 90% acetone and kept for 10min at -20^{o}C. Next, cells were air dried and then blocked for 30min with PBS containing 3% FBS whilst gently shaking. After blocking, cells were stained with the first antibody: anti-MDV chicken serum (1:2500) diluted in PBS with 3% FBS for 1 h [117]. Next, cells were washed 3 times with PBS and then incubated for 1h with the second antibody: Alexa Fluor-568 (1:1000) diluted in PBS 3% FBS. After the last incubation, cells were carefully washed three times with PBS and kept at 4^{o}C. Good levels of immunofluorescence were detected early to the staining, where viral plaques were counted and pictures taken using the Zeiss AxioVet S100 fluorescence inverted microscope.

7.2.2.4 Virus reconstitution and amplification

In order to reconstitute MDV recombinants *in vitro*, CECs and calcium phosphate were used. Initially, CECs were plated on a 6-well dish on the day prior to the transfection. Next morning, pCSGGS-NLS/Cre plasmid was transfected with 1µg of BAC DNA. The cre plasmid has to be transfected concomitantly to the BAC DNA, as it contains an enzyme, cre recombinase, which allows the proper removal of the mini-F sequence present in the MDV BAC backbone. For an efficient calcium phosphate transfection, viral DNA was initially dissolved in a final volume of 50µl containing 10mM Tris-HCl before it was added to 348µl of tri-distilled water. This solution was kept for 30min at RT followed by the addition of 62µl of $CaCl_2$ in a drop-wise manner under soft agitation. Samples were kept at 4^{o}C overnight. Next day, 500µl of 2X HBS were added in a drop-wise manner to the sample and kept for 15 min at RT prior to transfection. Next, each 6-well containing CECs had the medium replaced by 500µl of fresh MEM containing 10% FBS and added 500µl of the DNA-mix, totalising 1ml. The 6-wells now

containing 1ml of the mixed solution were incubated for 3h at 37ºC. Following the incubation time, medium was removed from the 6-wells and a single PBS wash was performed. Next, cells were submitted to a glycerol shock using a 1XHBS solution containing 15% sterile glycerol for exact 2min and 15sec. After the glycerol shock cells were carefully washed with PBS and incubated with new medium at 37ºC. On the following days, transfected cells were checked daily to monitor cell viability post-transfection. If cells were viable and confluent, 10% FBS MEM was replaced by 0.5% FBS MEM.

Approximately after 5 days post-transfection, viral plaque formation could be observed. In order to amplify virus DNA *in vitro*, viruses were propagated on CECs for 2 to 7 passages, until a desired viral titer was obtained. Initially, infected and uninfected CECs were added together to the same plate until an appropriate density and confluence was achieved. When cells were dense enough, FBS concentration was gradually reduced from 10% to 0.5% to slow cellular multiplication. After a good number of plaques were observed on the plates, viral stocks were trypsinized and resuspended in aliquots of 10%FBS MEM containing 8% DMSO. Viral aliquots were kept at -80ºC for a day and later stored in liquid nitrogen.

7.2.2.5 Virus titration, plaque size assay and growth kinetics

Concentrated stocks of CECs infected with MDV were used for plaque size assays and growth kinetics. In order to determine the correct concentration of plaque forming unit per ml (PFU/ml) from each stock, virus titration was performed. Here, a sample was taken from liquid nitrogen and diluted in 10 fold dilutions (10^{-2}, 10^{-3} and 10^{-4}) in duplicates (2 wells each from a 6-well plate). Uninfected CECs were trypsinized and plated in a new 6-well dish with 100µl of the diluted viral solution containing the three dilutions. After 6 days post-infection, wells containing 10-100 plaques were used to determine the viral titer.

Considering that MDV is a cell-associated virus, cell-to-cell spread features were evaluated by plaque size assays. Initially, fresh $1x10^6$ CECs were co-infected with 100 PFU of the viral sample and kept with MEM containing 10% FBS. Next day, medium was replaced for MEM containing 5% FBS. At 5 dpi, CECs were fixed and stained and images of 20 to 50 randomly selected plaques were taken. Plaque size areas were determined using Image J software (NIH). In order to determine the replication properties of MDV mutants, the multi-step growth kinetics techniques has been used, as described previously [56]. Here, $1x10^6$ CECs were co-infected with 100 PFU of different recombinant virus, including the WT. After each day post-infection, during 6 days, cells were harvested, titrated and fixed 5 days post-titration using 90% ice-cold acetone. In order to evaluate viral growth kinetics, viral load (PFU/ml) was determined for each time point and plaque numbers were counted for each dpi.

7.2.2.6 *In vitro* integration assay

In order to determine the integration efficiency of MDV mutants, $1x10^6$ CECs were initially infected with 30.000 PFU/ml of the respective virus overnight and kept with MEM supplemented with 10% FBS. On the next day, CECs were briefly washed with PBS and the medium was replaced for MEM 1% FBS kept for 4 days. At 3 dpi, CU91 cells, which here are the target cells for detection of viral integration, were split in a 1:2 ratio for co-infection on the next day. At day 4 post-infection cell media was removed from CECs and approximately $1x10^6$ CU91 cells were added per well for overnight co-infection at 41ºC with RPMI medium (Figure 10). Next day, CU91s, infected with the recombinant MDV containing a GFP sequence in the mini-F region of the BAC, were centrifuged at 250g, at RT for 6 min and washed with PBS prior to FACS sorting of the infected cells.

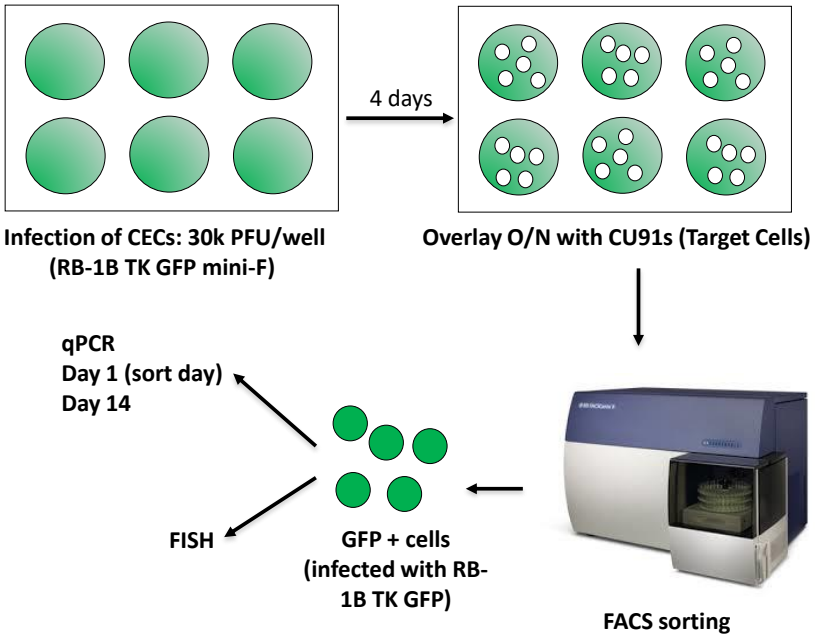

Figure 10. Schematic representation of the integration assay developed to evaluate viral maintenance in CU91 cells.

In order to evaluate the role of UL30 in MDV's genome maintenance, the integration assay was adapted. Here, infection of CECs with 30k PFU/well happened with the vUL30cDHFR containing a GFP gene in the mini-F region of the BAC (vUL30cDHFR-GFP). Initially, two 6-wells containing CECs were infected with vUL30cDHFR-GFP and supplemented with medium containing 10mM of TMP. This way, vUL30cDHFR-GFP was able to grow and form enough plaques to sustain a solid co-infection with CU91s after 4 days. On day 5 post-infection, medium was removed from both 6-wells infected with vUL30cDHFR-GFP and washed at least

5 times to ensure that all the remaining TMP has been removed. Next, $1x10^6$ CU91s were added to each well of both 6-well plates, but here, 10mM of TMP was added to only one of the 6-well dish in order to stop virus replication, whilst the other 6-well had TMP normally supplemented at 10mM per well, in the medium (Figure 11). After the overnight overlay with CU91s, cells proceeded for FACS analysis as previously described.

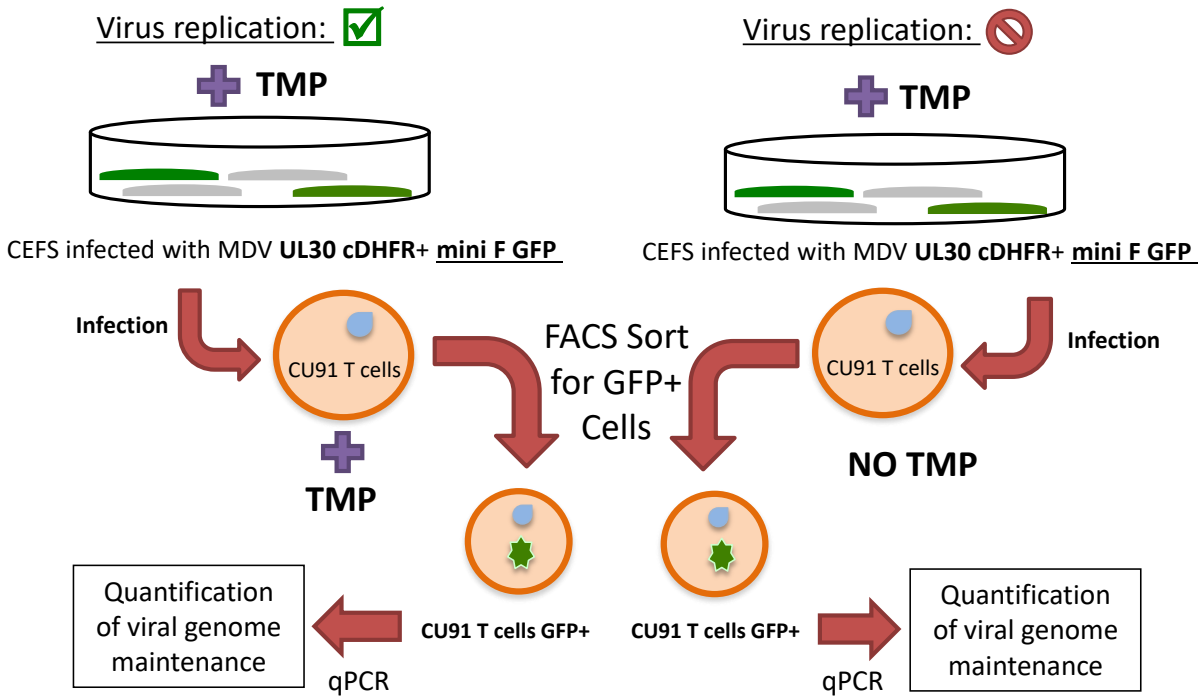

Figure 11. Schematic representation of the integration assay developed to evaluate viral maintenance in CU91 cells. CECs are initially infected with 30k PFU/ml of vUL30DHFR containing a GFP protein in the mini-F region of the BAC and kept for 4 days with TMP in the medium. On day 4 post-infection, CECs are co-seeded with CU91s in suspension, allowing the virus to transfer from CECs to CU91s. Here, TMP is kept in one sample and removed from the other. Next day, CU91s are FACS sorted for positive GFP signals and qPCR is performed to quantify viral and cellular genomes on day 0 (day of the sorting) and day 14 post-sort.

After FACS sorting for GFP positive cells, CU91s were recovered and kept at 20% FBS RPMI medium supplemented with 1% sodium pyruvate, 1% nonessential amino acids, 10% FBS, and penicillin–streptomycin, at 41°C in a 5% CO_2 atmosphere. Sorted cells were cultured for 14 days, and qPCR samples were taken at day 1 and day 14 post sort. FISH samples were taken and prepared on days 6, 8, 10 and 14 post sort.

7.2.2.7 qPCR

In order to quantify the MDV genome, DNA was isolated using a RTP® DNA/RNA Virus Mini Kit according to instructions from the manufacturer. The qPCR reaction was set up by initially generating a master mix for ICP4 and one for iNOS, separately. Each master mix contained 10µl Master Mix (including polymerase), 0.12 µl of each primer (concentration of 100µM), 0.5µl probe (10µM) and 9.5µl of DNA sample. MDV genome copies were determined by qPCR on a 7500 Fast Real-Time PCR System (Invitrogen, Grand Island) using primers and a TaqMan probe specific for the MDV ICP4 late gene. Here, ICP4 gene copy numbers were normalized against the cellular genome copies of the inducible nitric oxide synthase (iNOS) gene, as described [125]. Both primers and probes used for qPCR have their sequences presented in Table 7.

Table 7. qPCR Primers and Probes

Construct		Sequence (5' → 3')
ICP4	**For**	CGTGTTTTCCGGCATGTG
	Rev	TCCCATACCAATCCTCATCCA
	Probe	FAM-CCCCCACCAGGTGCAGGCA-TAM
iNOS	**For**	GAGTGGTTTAAGGAGTTGGATCTGA
	Rev	TTCCAGACCTCCCACCTCAA
	Probe	FAM-CTCTGCCTGCTGTTGCCAACATGC-TAM

7.2.2.8 Metaphase preparation and fluorescent *in situ* hybridization

In order to confirm virus integration *in vitro*, it is crucial to arrest the cells infected with the virus during metaphase, when chromosomes are better separated and can be easily identified. Here, metaphase chromosomes were prepared from the CU91 cells during different passages. In order to arrest cells in metaphase, cell cultures were treated with 0.1µg/ml colcemid for 3-4h. In this case, colcemid depolymerises microtubules and limits microtubule formation, and additionally it also inactivates spindle fiber formation, which is required to properly separate chromosomes during metaphase. Following incubation with colcemid, CU91s were kept at 42^{o}C during 10min in hypotonic solution. A single addition of 1ml of fresh ice-cold fixative was added to prevent cells from clumping. Next, CU91s were centifuged for 10min at 300g, and the supernatant was removed, whilst the pellet was washed twice with 5ml ice-cold fixative. Lastly, the cell pellet was carefully resuspended in 1ml of ice-cold fixative and stored at -20^{o}C.

After arresting the cells in methapase and fixing them in ice-cold fixative, the next step was to prepare the metaphase spreads. For this, a water bath was initially pre-warmed to 98ºC, and clean, unused microscope slides were briefly passed through the water vapour. Next, 10µl of chromosome suspension were dropped on the centre of the slide, which was carefully put on the side of a metallic place over the water bath to dry and evaporate the fixative. After the fixative was fully evaporated, the slide was passed again through the water vapour to ensure a homogeneous hydration of the slide. After this step, slides containing the chromosomes were kept aging overnight at RT, protected by a styrofoam box. On the next day, slides were dehydrated with 100% ethanol for 5min and any cellular debri, such as remaining cellular proteins, were eliminated via incubation of the slides in a pepsin solution pre-warmed at 37ºC for 1min30sec. Following the pepsin treatment, undesired RNA was also eliminated by incubation of the slide for 5min in 2xSSC solution containing RNAse. Next, slides were washed twice with 2xSSC for 3min each wash and shortly rinsed in Millipore water, following dehydration by gradually increasing ethanol dilutions: twice for 2min in 70%, twice for 2min in 90% and once for 4min in 100% ethanol. After the dehydration step, aging of the slide was performed at 65ºC for 1h. In order to create the MDV probe, 1.5µg of RB-1B BAC DNA were initially digested at 37ºC for 3h with the restriction enzyme *Hae*III, and then purified with a HI-Yield Gel purification kit. After the DNA probe digestion, it needed to be labelled for detection. Here, digoxigenin labelling of the DNA probe was performed with DIG-High Prime. Initially, 300ng of digested DNA present in the final volume of 16µl was denatured at 98ºC for 10min. Next, the sample was colled on ice, where 4µl of DIG-High prime were added to the DNA solution. This reaction was kept overnight at 37ºC and interrupted on the next day by the heat inactivation step, which happened at 65ºC for 10min. After heat inactivation of the probe, it was purified again using a High-Yield PCR purification kit and its final concentration was determined using Nanodrop (Thermo scientific, USA). Once purified, the inactive probe was hybridized, where 1.2µl of the MDV probe were mixed with 30.3µl of Hybridization buffer. Additionally, 0.5µl of salmon sperm DNA was added per slide to serve as a competitor DNA to reduce background during image collection.

The probe mix yet created was initially denatured at 75ºC for 10min, cooled down slowly on ice and added to the aged slides. A coverslip was placed on the probe and sealed with rubber cement in order to avoid evaporation of the probe during next steps. Here, slides were placed into an 80ºC incubator for 2min and slowly cooled to 42ºC, when it was left overnight. On the next day, slides' coverslips were removed and slides were washed in the order as follows: 44ºC, twice for 5min in 2xSCC; twice for 5min in stringency wash; twice for 5min in 2xSSC and lastly, twice for 5min in detergent wash. In order to detect fluorescence under the microscope, slides were incubated for 30min at 37ºC with 100µl of the antibodies solution

containing anti-DIG antibodies diluted in the ratio of 1:250 in detergent wash. Finally, a final wash was performed three times, each with a 4-min incubation time in detergent wash followed by a quick rinse in water. Lastly, a single drop of mounting media (Vectashield DAPI) was added to the slide and sampled were sealed with a coverslip before detection with the microscope Axiovision M1 (Zeiss).

7.2.2.9 Cellular recombination assay

To determine the roles of UL12 and UL29 in different double strand break repair pathways, a well-established protocol was used containing four different reporter cell lines: HEK293 DR-GFP, SA-GFP, EJ2-GFP and U2OS EJ5-GFP. These cell lines containing chromosomally integrated reporter genes have been developed to study the activation of four major double-strand break repair (DSBR) pathways following DNA damage [119]: Homologous Recombination (HR), Single strand annealing (SA), total non-homologous end joining (NHEJ) or alternative NHEJ (ANHEJ), respectively. Each cell line contains a green fluorescent protein (GFP) reporter gene that is disrupted by a 18-bp recognition sequence for the I-*Sce*I endonuclease and can no longer express GFP. The cell lines here used, has been designed in such a way that repair of the DSB using a specific DSBR pathway will restore the GFP reporter gene. Thus, repair can be measured in individual cells by monitoring the number of GFP-expressing cells using flow cytometry. Each of the reporter cell lines were transfected with an I-*Sce*I expression vector or an empty vector control (pLKO 3.1).

7.2.2.9.1 DNA repair quantification

To assess the role of UL12 and UL29 in these repair assays, the UL12 gene of the RB-1B strain was cloned into pcDNA3.1+. Because UL29 was thought to be toxic in *E.coli*, a low copy pHA vector for the expression of UL29 was used instead. These expression vectors here generated were used for the DNA repair assay together with the DSB GFP reporter cell lines, as previously described. Briefly, HEK293 HRGFP, SA-GFP, EJ2-GFP, or U2OS EJ5-GFP SA GFP cells were plated in 12 well plates treated with 0.01% poly-L lysine (Sigma-Aldrich, country). The next day, cells were transfected with the control vectors, the UL12 or UL29 plasmid in combination with the I-SceI expression vector (pCBA-SceI, Addgene: #26477), and an E2-Crimson transfection control (pE2-Crimson, Clontech, USA) using Lipofectamine2000, according to the manufacturer's instructions (Invitrogen, country). Cells were analyzed by flow cytometry 72 hours post-transfection, as previously described [128].

7.2.2.10 Statistical analysis

All statistical analyses were generated using GraphPad Prism7 software in these research projects. Initially, data sets were tested for normal distribution. Data from plaque size testing were converted to plaque diameters and analyzed with a one-way variance analysis (ANOVA). The kinetic growth data of Kruskal–Wallis and Mann–Whitney U tests were analyzed. Through Mann Whitney U test, DNA repair test data were analysed.

8. Results

Most of the results and their description present in this section have been peer-reviewed and published by the author & colleagues in the article “The Role of Marek's Disease Virus UL12 and UL29 in DNA Recombination and the Virus Lifecycle”, as previously stated.

8.1 Characterization of UL12 and UL29 in MDV life cycle

In order to evaluate the role of UL12 and UL29 in MDV replication, recombinant viruses were generated based on wild type RB-1B MDV BAC using two-step Red-mediated mutagenesis. To understand the role of UL12 in MDV’s lifecycle, we firstly abrogated the expression of the full-length UL12 protein, by replacing amino acid (aa) 6 and 7 with two stop codons (UL12 mut1) (Figure 12). As previously mentioned, a second shorter C-terminal form of HSV-1 UL12 has been described previously [86], we also inserted a stop codon at aa 136 into UL12 mut1 or wild type, generating UL12 mut2, with two mutations in UL12 and UL12 mut3, with only a stop codon at aa 136, respectively. To investigate the role of UL29 in MDV’s lifecycle, we replaced the start codon by a stop codon to abrogate its expression (UL29 mut) (Figure 14). To confirm that the observed phenotypes are due to the introduced mutations, revertant viruses were generated for all recombinant viruses, in which the original sequence was restored. Primers used for the generation of these recombinant viruses are listed in Table 2. Final clones were confirmed by multiple RFLP analyses, PCR, and Sanger sequencing of the target region.

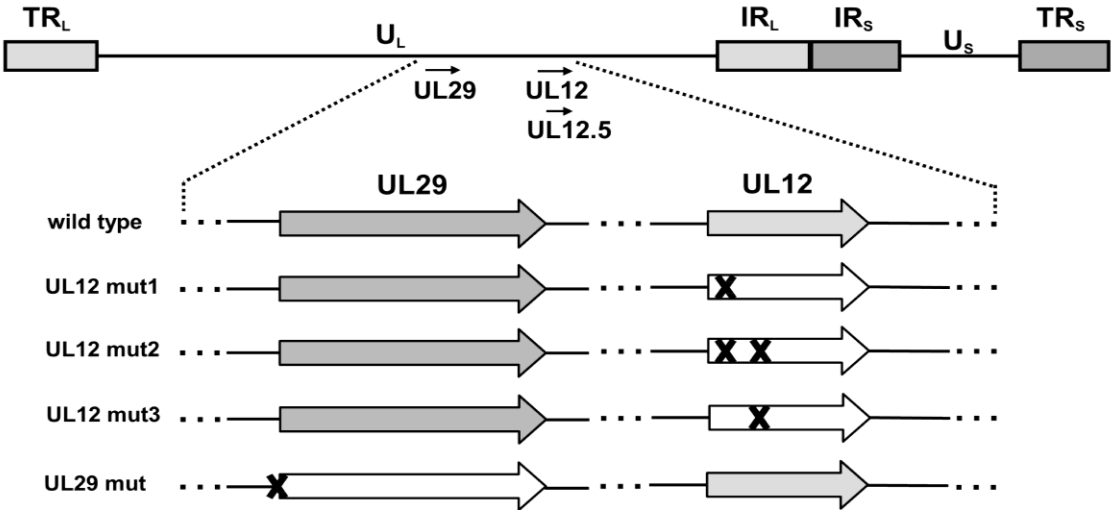

Figure 12. Generation and characterization of recombinant mutant viruses. Schematic representation of the MDV genome, with a focus on the UL region containing the UL12 and

UL29 genes. Mutations in the UL12 (stop codon insertion at aa 6/7 and/or aa136) and UL29 (stop codon at aa 1) are indicated with an 'X'.

Next, we investigated the replication properties of recombinant viruses. These were initially determined by plaque size assays, as here described. The introduced mutations in UL12 mut2 and UL29 mut1 completely abrogated MDV replication. Plaque sizes were measured post-transfection of the viral BAC DNA, followed by the analysis of 50 images of plaques randomly selected per well, and plaque areas determined using the ImageJ software (NIH), which were normalized to wild type virus. Replication properties were confirmed by multistep growth kinetics, as here previously described.

8.1.1 The n-terminus of UL12 is dispensable for virus replication

To determine the role of the n-terminus of UL12 in MDV replication, we replaced aa 6 and 7 with stop codons, generating UL12 mut1 (Figure 12), without affecting the overlapping coding sequence of UL13. Upon reconstitution of UL12 mut1, we performed plaque size assays and could demonstrate that abrogation of full-length UL12 significantly impaired virus replication by more than 30%, but yet did not abrogated it, when compared to wild type and the revertant viruses. We also confirmed this growth defect using multistep growth kinetics (Figure X). Because HSV-1 also produces a shorter isoform of UL12, termed UL12.5 that rather localizes to the mitochondria [86], we replaced the second start codon at aa 136, generating UL12 mut2 (Figure 1A) with a stop codon, to abrogate this putative protein. Interestingly, UL12 mut2 did not formed plaques upon virus reconstitution, indicating that replication was abrogated. However, only single infected cells were detected after transfection of the virus BAC DNA (Figure 13), while the revertant behaved equally to wild type virus. To determine if the insertion of the stop codon at aa 136 alone abrogates virus replication, we created a mutant virus that only harbors this mutation, named UL12 mut3. MDV replication was completely abrogated by the insertion of this stop codon (Figure 13), indicating that both, the c-terminus of UL12 and its putative isoform UL12.5, are essential for successful MDV replication.

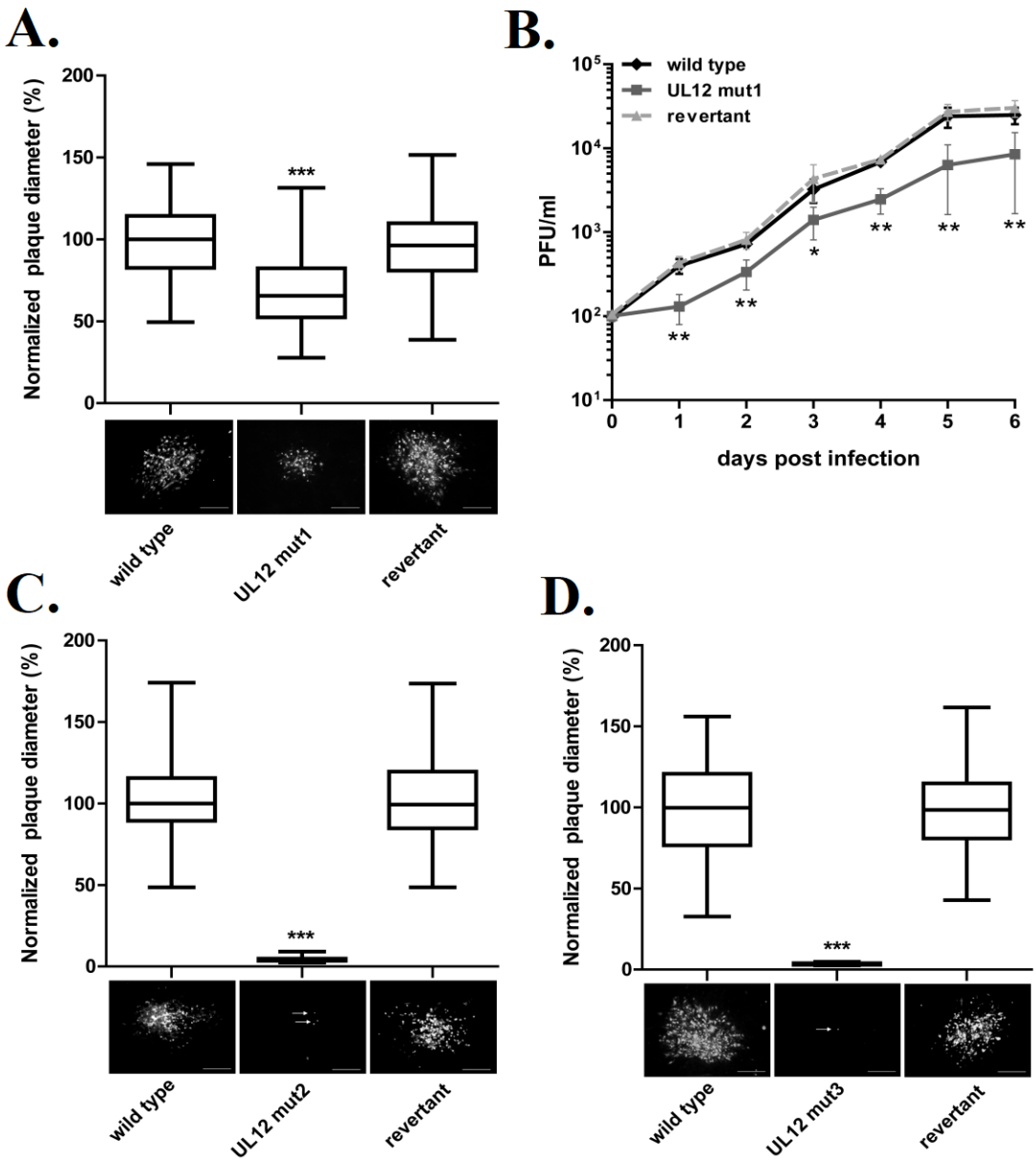

Figure 13: The effect of UL12 mutations on MDV replication. (A) Plaque size assay of UL12 mut1: CEFs were initally infected with 100 PFU of wild type MDV, UL12 mut1 or the revertant virus. The average plaque diameters from three independent experiments, normalized to wild type are shown in the box plots. Representative images of plaques are shown below, respectively (Scale bar, 100µm). (B) Growth kinetics assay: CEFs were infected with 100 PFU of wild type, UL12 mut1 and revertant virus and the titer determined from 0 to 6 dpi. In the plot are the mean titers of these viruses from three independent experiments ± SEM (**, P≤0.05; *, P= 0.01; Mann-Whitney U test). Wild type, (C) UL12 mut2, (D) UL12 mut3 and respective revertant MDV BACs were transfected into CEFs and images of plaques acquired six days post transfection. The average plaque diameters from three independent experiments are shown in box plots (normalized to wild type). Representative images of plaques are shown

below (Scale bar, 100µm). White arrows show single infected cells. Statistical differences in plaque diameters were determined using one-way ANOVA analyses (***, P<0.0001).

8.1.2 MDV UL29 is essential for virus replication

In order to investigate the role of UL29 in MDV replication, we created a mutant virus containing a stop mutation codon replacing the first start codon of the gene UL29. Upon virus reconstitution on CEFs, it could be observed that on day 6 post-infection there was plaque formation for the wild type RB-1B virus and the UL29 mut revertant virus. Shockingly, the wells transfected with UL29 mut contained no plaques, yet single infected cells were detected through antibody staining. These results, performed three times in an independent manner, showed that a MDV mutant lacking the protein ICP8 encoded by UL29 had its replication *in vitro* completely abrogated.

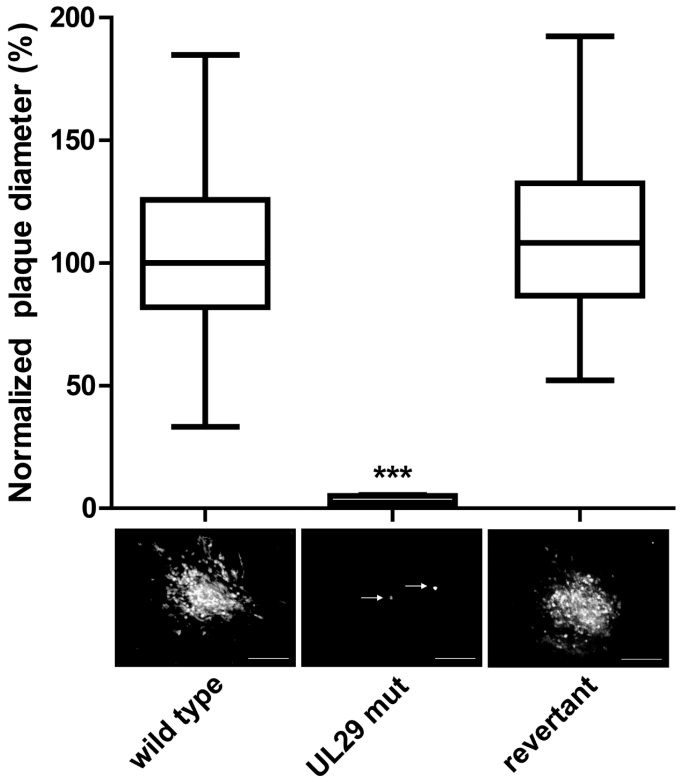

Figure 14. The role of UL29 in MDV replication. In this case, wild type, UL29 mut and reverse BACs were transfected into CEFs, and pictures of plaques were obtained six days later. The average plaque diameters of three independent experiments are shown in a box plot. Below are representative images of plaques (Scale bar, 100µm). White arrows show

single cells infected. A one-way ANOVA analysis (***, P<0.0001) determined statistical differences in plaque diameters.

8.2 MDV UL12 supports SSA during DNA recombination

It is known that in HSV-1, both proteins UL12 and UL29 when together form a complex that possess strand exchange activity [27,81]. Moreover, HSV-1's UL12 supports DNA repair through the single strand annealing repair pathway [80]. In the case of MDV, the roles of UL12 and UL29 were still unknown. To unveil this, we transfected four different well-established DNA repair reporter cell lines for HR, SSA, A-NHEJ and NHEJ, with UL12 and UL29 plasmids and I-*Sce*I expression plasmids [80,119,128] (Figure 15).

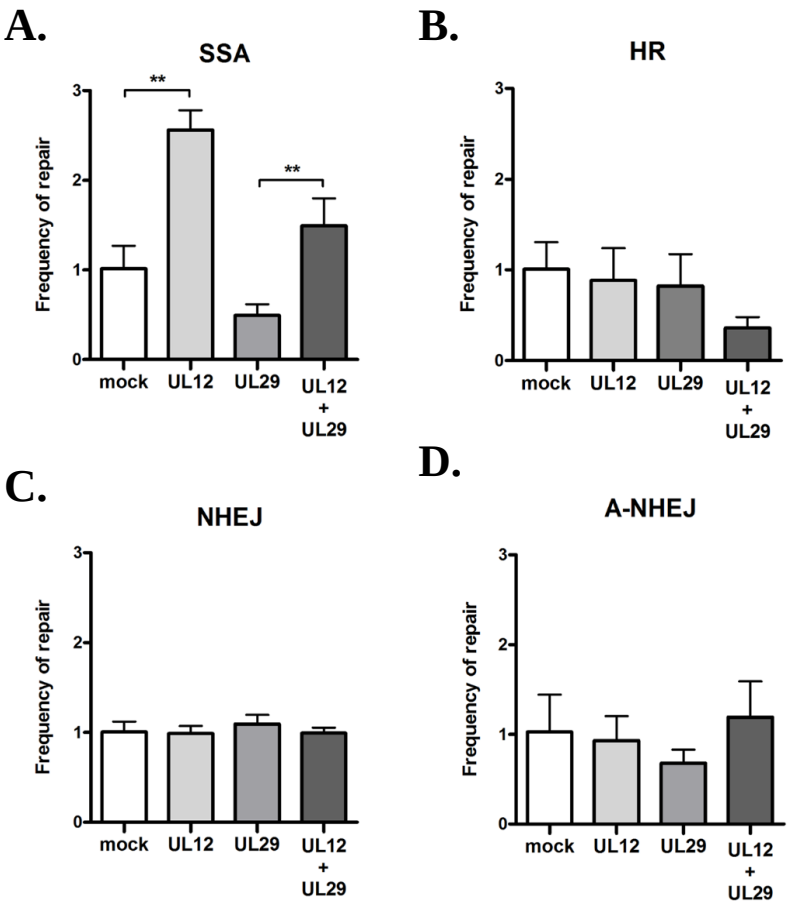

Figure 15. Role of UL12 and UL29 in DNA recombination. Four different DNA damage reporter cell lines were utilized containing an integrated DNA damage reporter that expresses GFP when an induced dsDNA break is repaired by (A) SSA, (B) HR, (C) NHEJ and (D) A-NHEJ. These cell lines were transfected with expression plasmids for UL12 and/or UL29 to assess whether these MDV proteins can aid in the specific DNA repair pathways. The mean

frequency of repair for the indicated expression constructs is shown from six independent experiments ±SEM. Statistics were performed using the Mann-Whitney U test (**, P<0.05).

Intriguingly, our results showed that MDV UL12 was able to increase SSA repair activity when compared to the control cells. No effect was observed for the other HR, A-NHEJ and NHEJ recombination pathways though. When transfecting the same cell lines with a UL29 plasmid, it was observed that UL29 did not enhanced the activity of UL12 or contributed to the activation of any of the other pathways.

8.3 A novel *in vitro* latency assay for MDV using CU91 cells

Integration studies targeting MDV's properties and the host chromosomes were so far majorly performed *in vivo.* In order to minimize the use of animals for experiments, optimize research costs and reduce workloads, we initiated the generation of a new *in vitro* latency model to study MDV integration. Prior to evaluate the roles of UL12, UL29 and UL30 in MDV integration, we tested the system with a wild type RB-1B and with the mTMR deletion mutant virus (ΔmTMR), this last one being well-known for having its integration capacity extinguished. When using both viruses, RB-1B for positive integration control and ΔmTMR for negative integration control, we were able to quantify the reliability of this integration system *in vitro* using CU91 cells [121–123] (Figure 16).

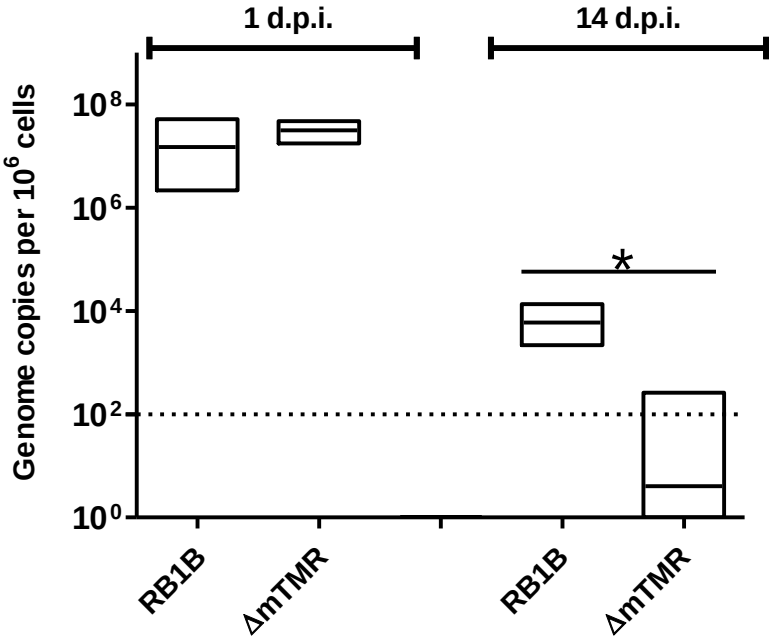

Figure 16: Establishment of an *in vitro* herpesvirus latency model in CU91s. CEFs infected with 30kPFU/ml of either RB-1B or ΔmTMR viruses expressing GFP were cultivated for 4 days. On day 4, CU91 T cells were overlaid overnight with CEFs infected. On day 5,

infected CU91s were taken for FACS sort. Next, samples were taken for qPCR analysis on day 1 (sorting day) and day 14 post-sort. Displayed are the box plots containing the mean levels of virus genome copies per million cells from three independent experiments. Statistics were performed using the Kruskal-Wallis test (*; P≤0.01).

On the same day cells were sorted, viral genome copies were statistically equal between RB-1B and ΔmTMR, indicating that CU91s were successfully infected with both viruses.

Considering that CU91 cells can uptake MDV latency *in vitro* [122], our results suggested that the integration assay developed using CU91 cells works reliably when assessing viral genome maintenance to indicate integration through the quantification of cellular and viral genome copies by qPCR extracted on different days.

8.4 Generation of shRNA for UL12 and UL29 knockdown using lentivirus transduction

To confirm if UL12 and UL29 was involved in MDV integration, we generated shRNAs targeting the 5'- and 3'-ends of UL12 and UL29 mRNA. The two shRNAs for each gene were cloned into the pLKO5.shRNA vectors. Lentiviruses generated were delivered into CR and CU91 cells as previously described [128]. CU91 cells were selected using hygromycin for UL12 shRNA or puromycin for UL29shRNA. Clonal lines were generated in these cell types, and shRNA-expressing clones were used for the *in vitro* latency assay.

Here, we used CR cells to validate the functionality of shRNAs in CR cells. In this case, CR cells were transduced with the respective shRNAs for UL12 and UL29, selected and later infected with MDV. Because UL12 and UL29 play major roles in MDV replication, as indicated previously here, CR cells containing shRNAs would limit virus replication, which would be phenotypically showed as reduced plaque sizes. (Figure 17).

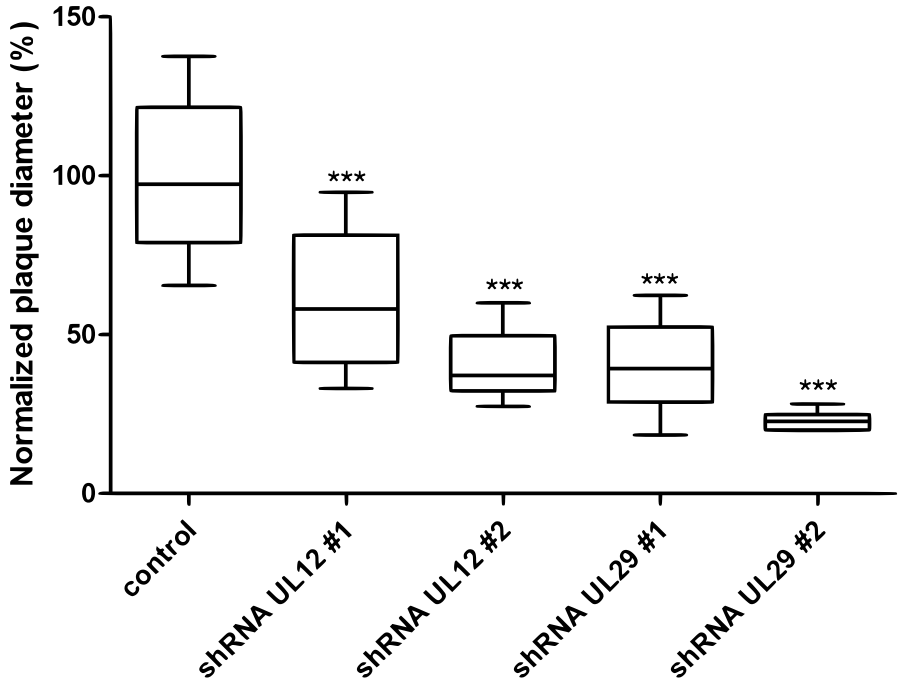

Figure 17. Validation of the UL12 and UL29 shRNA constructs. CR cells with or without UL12 or UL29 shRNAs were infected with MDV. Six days after infection, plaque sizes were measured. The average plaque diameters are displayed as a box plot (n=50). A one-way ANOVA (***, P<0.0001) has been used to determine statistical diameter differences in plaque.

These results indicated that there was a significant difference between the plaque sizes of the control (no shRNA) CR cells and CR cells containing the respective shRNAs. This shows that upon infection with MDV, transduced CR cells had fewer plaque formation. This indirectly indicates that MDV's UL12 and UL29 were being abrogated in the cells and that shRNAs generated were reliable tools to target expression of those genes.

8.5 UL12 and UL29 are dispensable for virus integration *in vitro*

To determine if UL12 plays a role in integration and genome maintenance, we used the UL12 mut1 virus that lacks the full-length UL12 protein. For HSV-1, only the full-length UL12 protein is located in the nucleus, where it helps to recombine DNA [86]. *In silico* predictions confirmed that MDV UL12's nuclear localization signal (NLS) is within the first 50 aa, as shown for HSV-1 previously [129]. With this, used the UL12 mut1 virus that lacks the full-length UL12 protein and the NLS needed for nuclear localization to determine if UL12 plays a role in integration and genome maintenance. CU91 cells were infected with wild type virus, UL12 mut1 and ΔmTMR). Upon infection, CU91s were cultured for 14 days to determine the level of virus genome maintenance within the infected culture over time. Equal virus genome copies were detected after one day post-infection (Figure 18). After passaging, the ΔmTMR genome was

almost completely lost due to its inability to integrate into host telomeres. Overall, no effect was observed for UL12 mut1 compared to the wild type virus, indicating that the full-length UL12 is dispensable for MDV integration and genome maintenance.

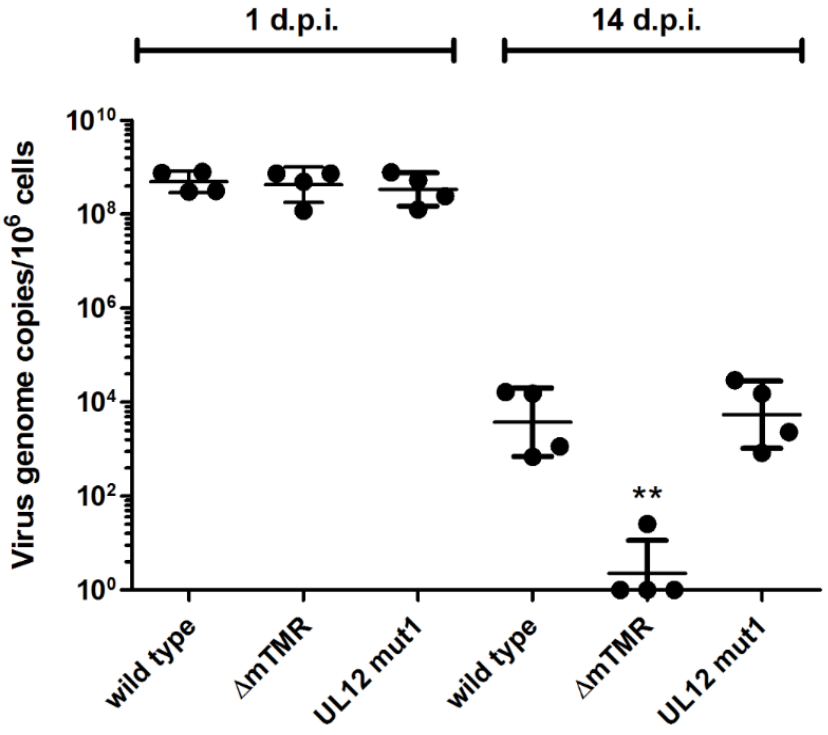

Figure 18. Viral genome maintenance in CU91 T cells infected with UL12 mut1. CU91 T cells have been infected with a variety of MDV mutants and cells that have been grown up for 14 days. Samples were taken on Day 1 and Day 14 for qPCR analysis. The figures show the mean level of copies per million cells of virus genomes from four independent experiments (a single dot shows each experiment). Statistics were made by Kruskal-Wallis (** P<0,01) test.

To confirm this observation, we used CU91s expressing shRNAs against UL12 or UL29 mRNAs that we validated in CR cells, as showed here previously (Figure 17). Here, two independent cell lines for each UL12 or UL29 genes were generated expressing two shRNAs each, targeting the 5'- end and the 3'- end of the UL12 or UL29 mRNA. These cells lines were used to verify if virus genome maintenance and, therefore, integration is compromised in the absence of these gene's products. On day 1 post-infection, comparable virus genome copy levels were observed. However, on day 14 post-infection, ΔmTMR genome was almost completely lost due to an impairment in integration, and, intriguingly, shRNAs against UL12 or UL29 had no effect on virus genome maintenance, which suggests that UL12 and UL29 are not essential for MDV integration (Figure 19).

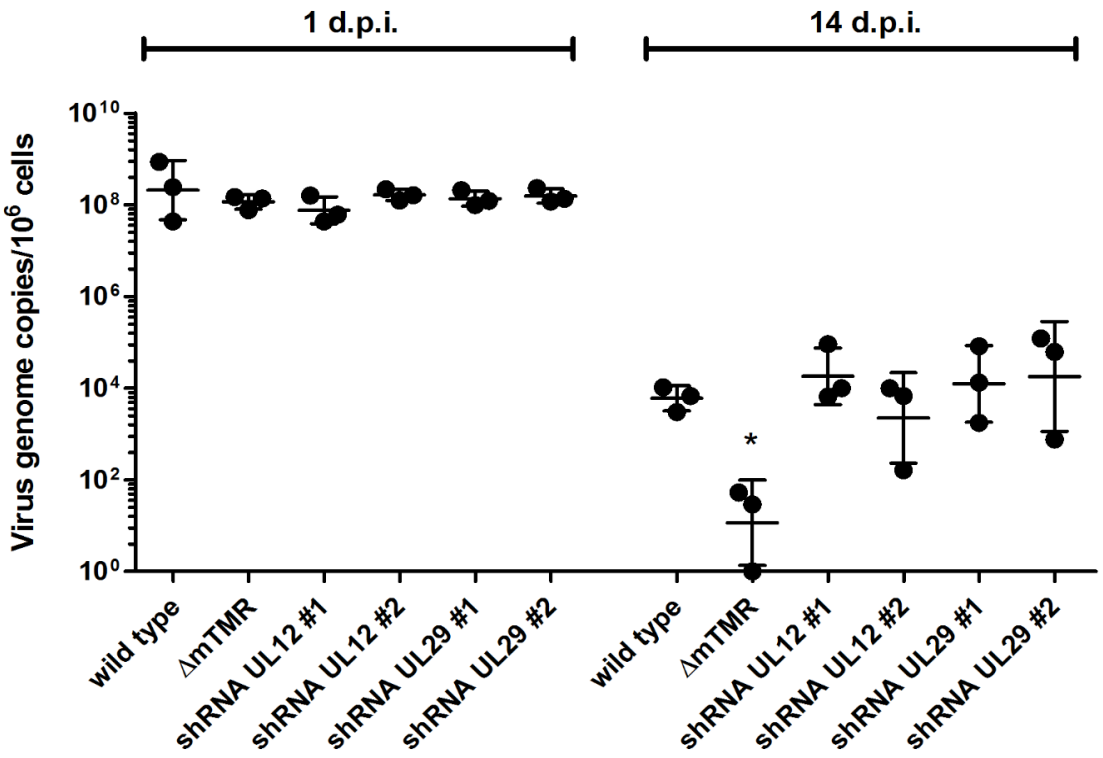

Figure 19. Role of UL12 and UL29 in MDV integration. MDV infected cells of CU91 T with or without UL12 or UL29 shRNAs. Samples were taken on Day 1 and Day 14 for qPCR analysis. The mean levels of virus copies are shown per million (bar) ±SD cells from three independent experiments (each experiment is shown with a single dot). A Kruskal-Wallis test (*; $p < 0,01$) was used to perform statistics.

8.6 Characterization of vUL30cDHFR

There are some evidences on temporal analysis of MDV infection suggesting that virus replication either happens concomitantly or prior to integration on B and T cells, and that this happens on in the initial days post-infection [109]. Additionally, despite being already described in the literature the role of UL30 in MDV as the main viral DNA polymerase [115], there were no evidences of the role of this gene and replication in MDV's integration process.

In order to address these questions, we generated a mutant virus containing a DD DHFR in frame with the UL30 gene. Here, the DD DHFR genomic sequence was added between the final stop codon and the last aa of UL30, being translated in frame with the main DNA polymerase, yet not interfering on its structure nor functionality. The new MDV mutant virus was generated containing the DD DHFR in frame with the UL30 gene (vUL30DHFR). As it

was hypothesized, vUL30DHFR would have its protein active or degraded, in an inducible manner when in the absence of presence of the ligand, TMP (Figure 20).

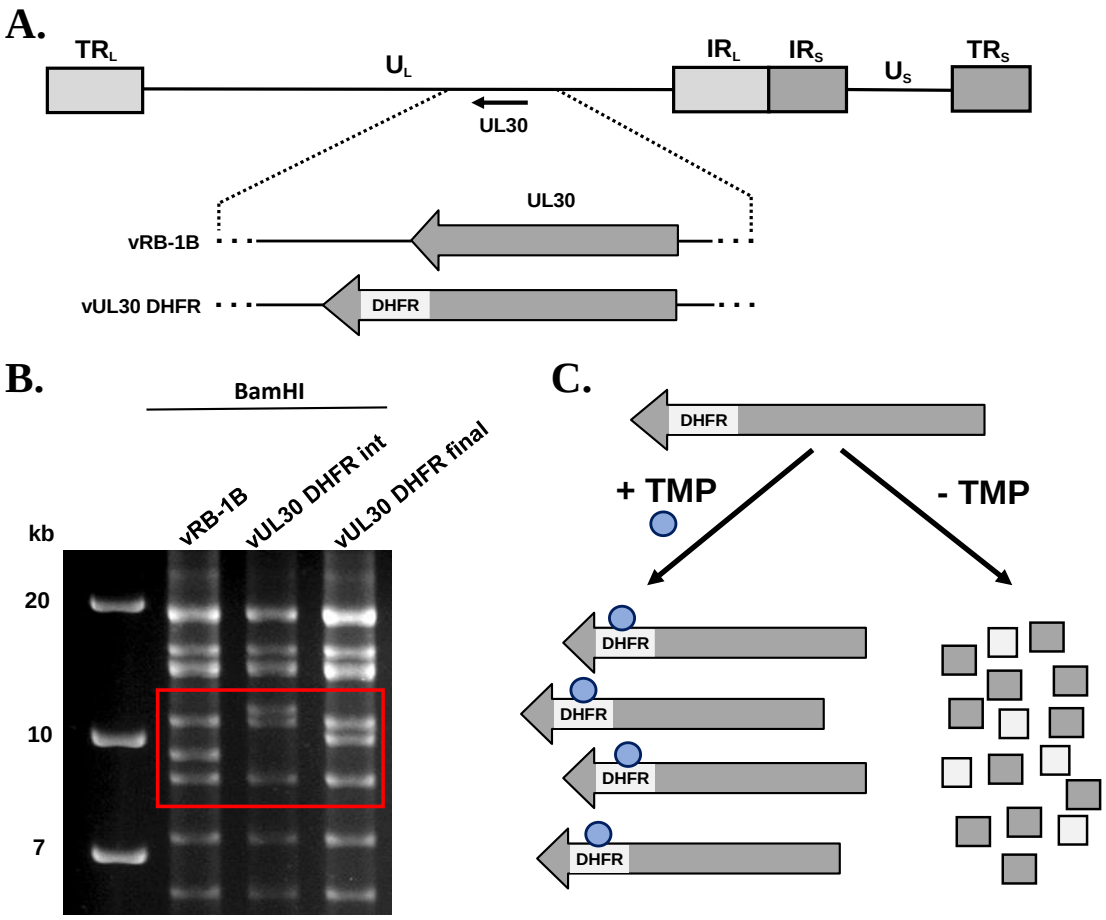

Figure 20. Characterisation of vUL30 DHFR mutant. (A) Schematic representation of the wild type MDV genome (RB-1B strain), with a focus on the UL region with the UL30 gene. The mutant virus generated contains a DD DHFR cassette in frame with the c-Terminus of UL30. (B) RFLP pattern of vRB-1B, vUL30 DHFR intermediate and vUL30 DHFR final mutants upon digestion with BamHI analyzed on a 0.8% agarose gel o/n at 65 V. Marker = 1 kb plus. Sizes of the marker fragments are indicated on the left. Red box highlights the fragment containing the target region, where the expected band shifts can be observed. (C) Schematic representation of functionality of the DHFR and TMP system. TMP present in the medium bind to the DHFR region and stabilizes it. In the absence of TMP, DHFR and the protein are rapidly degraded by proteassomes.

8.6.1 The DD DHFR system affects viral replication in a TMP dose-dependent manner

In order to evaluate if the DD DHFR system linked with the main viral DNA polymerase was affecting viral replication *in vitro*, we transfected CECs with vUL30DHFR and reconstituted the virus in different concentrations of TMP. Our results demonstrate that vUL30DHFR had an impact on virus replication in different concentrations of TMP added to the media. This was represented by plaque size assays performed at day 6 post-infection, which indicated that vUL30DHFR was indeed replicating in a dose-dependent manner of TMP (Figure 21 and Figure 22).

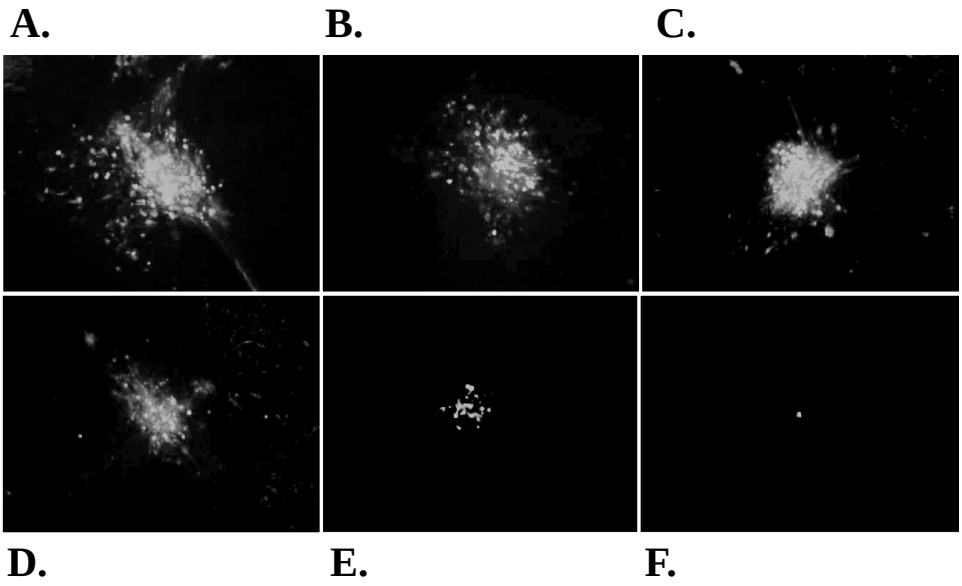

Figure 21. Representative images of vUL30DHFR plaque formation. vUL30DHFR was transfected into CECs and kept under different TMP concentrations; (A) 10µM; (B) 5µM; (C) 0.5µM; (D) 0.05µM; (E) 0.005µM and (F) NO TMP added on day 0 (transfection day).

Plaque size and growth kinetics assays were performed at least three times in an independent manner to confirm the phenotype of vUL30DHFR on different concentrations of TMP (Figure 22).

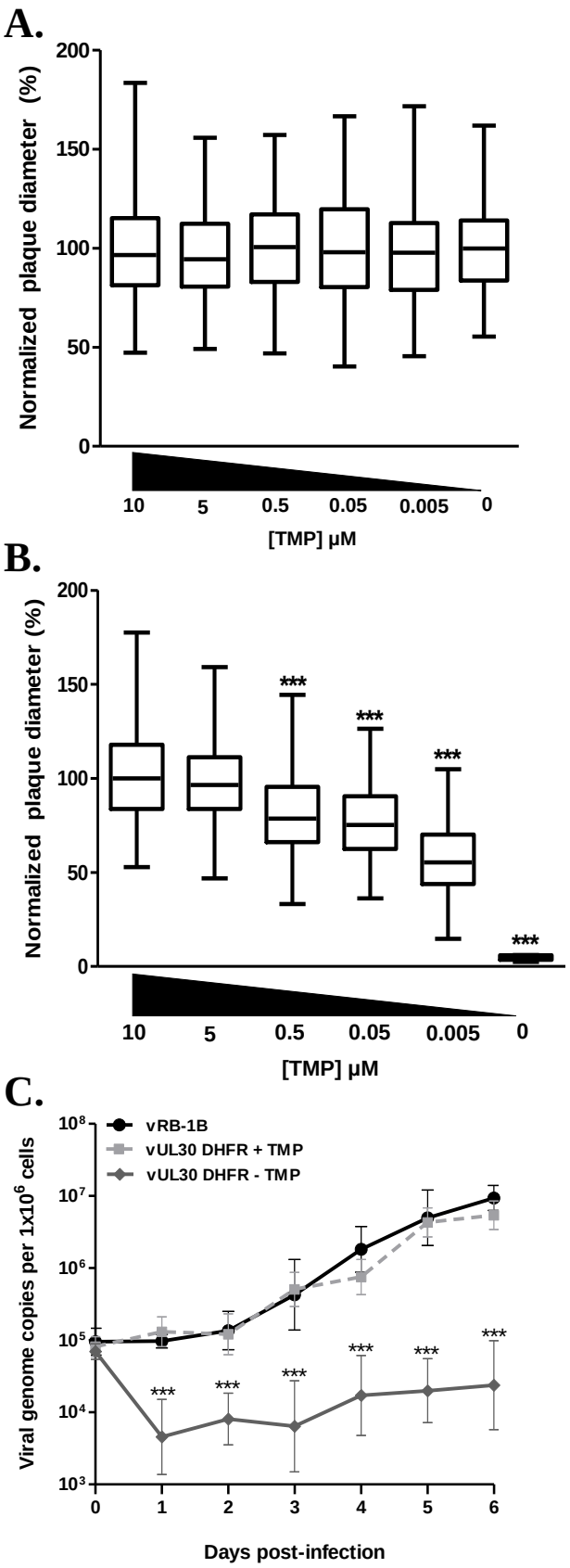

Figure 22. Characterisation of vUL30DHFR in different TMP concentrations. (A) Wild type MDV transfected into CECs in different TMP concentrations. The average plaque diameters

from three independent experiments are shown in a box plot (normalized to wild type without TMP). (B) vUL30DHFR transfected into CECs in an identical scenario as the wild type, with different TMP concentrations. The average plaque diameters from three independent experiments are shown in a box plot (normalized to wild type without TMP). Statistical differences in plaque diameters were determined using one-way ANOVA analyses (***, $p<0.0001$). (C) CEC cells were infected with 100 PFU of wild type and vUL30DHFR kept with 10µM TMP or without TMP. Titer determined at indicated times post-infection. Shown are the mean titers of these viruses from three independent experiments ± SEM (***, $p<0.0001$). Mann-Whitney U test).

8.6.2 UL30 and MDV integration in CU91 cells

In order to verify if UL30 was indeed necessary for viral integration in CU91 cells, we used the vUL30DHFR virus in the integration assay adapted, as previously described here. vUL30DHFR containing a GFP sequence inserted into the mini-F region of the BAC was used for FACS sorting during three independent experiments. The equal number of viral genome copies and cellular genome copies upon qPCR would represent viral integration or maintenance. When analyzing samples from days 1 and 14 post-infection, we were able to confirm that infection rate of on day 1 post-infection was statistically identical to all viruses in CU91 cells (Figure 23).

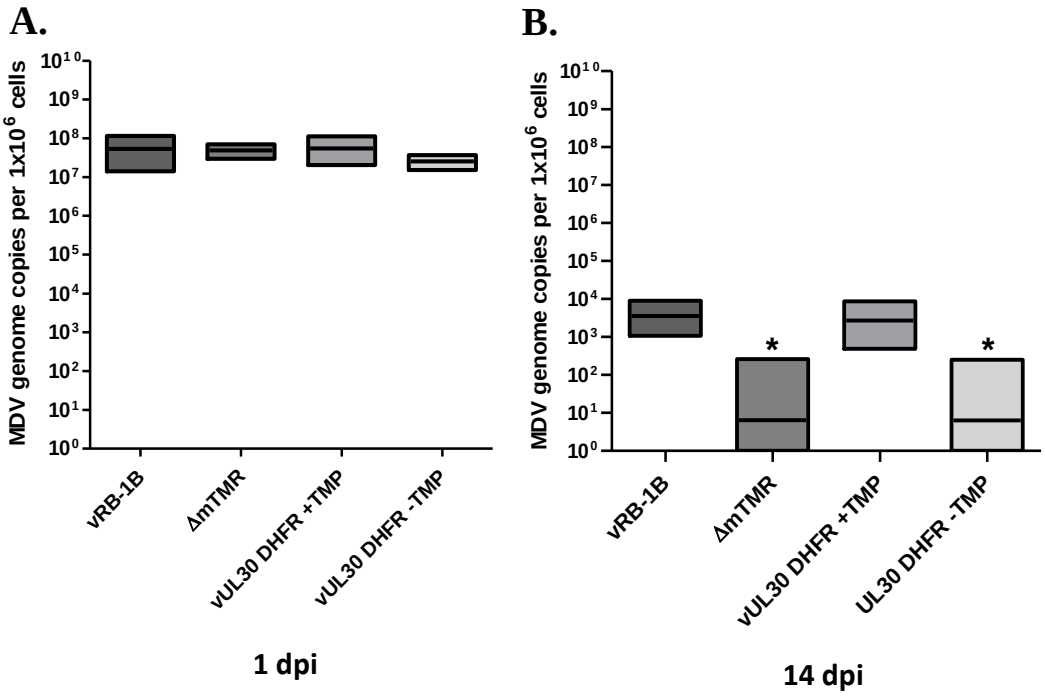

Figure 23. Role of UL30 in MDV integration. CU91 cells were infected with different viruses, vRB-1B; ΔmTMR, vUL30DHFR kept with TMP and vUL30DHFR kept without TMP. Samples

were taken for qPCR analysis on day 1 (A) and day 14 (B). Displayed are the mean levels of virus genome copies per million cells from three independent experiments. Statistics were performed using the Kruskal-Wallis test (*; $p<0.01$).

Interestingly, at day 14 post-infection, we were able to visualize a different pattern. Here, the viral-cellular genome copies ratio of wild type RB-1B and vUL30DHFR kept with TMP was very similar, confirming that integration was occurring. However, when comparing the viral-cellular genome copies ratio of vUL30DHFR kept without TMP, the ration between virus and cellular genome copies was reduced, which, intriguingly, was equal to the viral-cellular genome copies ratio of ΔmTMR, which has its integration potential impaired (Figure 23).

These results indicate that there was a significant difference between the viral-cellular genome copies ratio on day 14 post-infection of wild type RB-1B virus, vUL30DHFR kept without TMP and ΔmTMR, when using CU91 cells; while wild type RB-1B and vUL30DHFR kept with TMP presented no statistical difference.

9. Discussion

This thesis was developed with a focus on the viral factors involved in two aspects of the Marek's disease virus lifecycle: i) to elucidate the roles of genes UL12 and UL29 in MDV's replication and their involvement in the establishment of viral latency, and ii) to develop and test a recombinant MDV with an inducible expression of the DNA polymerase in order to verify the role of replication in MDV integration in CU91 cells.

9.1 The role of UL12 and UL29 in MDV replication

As it has been here previously presented, both UL12 and UL29 genes are highly conserved among members of the *Herpesviridae* family. In HSV-1, the alkaline exonuclease activity of UL12 has been described as performing an essential role in DNA replication and recombination [27,80]. Similar nuclease activities of UL12 has also been confirmed in other herpesviruses orthologues, including EBV [130–132], bovine herpesvirus type 1 [133], pseudorabies virus [134] and human cytomegalovirus (HCMV) [135]. Also in HSV-1, UL29 encodes the major ssDNA-binding-protein. This gene plays a vital role in viral DNA synthesis, viral gene expression control, and express high recombination levels during HSV infection, being indispensable for herpesvirus replication [27].

In HSV-1, UL12 plays an important role in replication as a UL12 null mutant shows a severe growth defect [136]. In 1983, Costa and colleagues suggested that the UL12 gene in HSV-1, which is encoded by a 2.3-kb mRNA, is embedded in a subgenic 1.9-kb mRNA encoding an n-terminally truncated version of UL12, which was named as UL12.5 [137]. This was later confimed by Martinez and colleagues, when they evidenced that, UL12.5 not just encoded a 1.9-kb mRNA but also ended in the production of a 60kDa size protein that does not possess exonuclease activity, is not enzymatically active, and that is expressed threefold less efficiently than the whole UL12, resulting in low abundance of this polypeptide [138]. These results found by Martinez *et al*. suggested that this polypeptide was still not able to replace the full-length UL12 in HSV-1, and therefore it could not compensate the growth defects of a UL12 null mutant virus. The UL12.5's inability to supplement a UL12 null mutant poses a dilemma and suggests that HSV-1 UL12's essential function lies within the first 126 amino acids. Later on, Reuven and colleagues showed the first evidences that UL12 and UL12.5 contained different intracellular locations, with UL12 present in the nucleus exhibiting recombination activity and UL12.5 in the cytoplasma. They also verified that UL12.5 could mediate ICP8 strand exchange with ICP8 as well [139]. Later, Corcoran and colleagues showed that the full-length UL12 amino-terminal region is required for nuclear localization and that show that UL12.5

specifically localizes in the mitochondria, with the mitochondrial location of UL12.5 being largely based on sequences between UL12 residues 185 and 245 (UL12.5 residues 59 to 119). Here we showed first evidences of the importance of genes UL12 and UL29 in MDV replication. In our initial experiments, we replaced aa 6 and 7 of UL12 for stop codons, as the first 5 aa of UL12 overlap with the c-terminus of the UL13 gene. The generated mutant virus, UL12 mut1 represented the null mutant and it was shown to still form viral plaques upon transfection of CECs, despite having a severe growth defect in terms of plaque sizes and numbers. This firstly indicated to us that perhaps UL12 was not essential for replication.

Following this result and what has been evidenced by UL12.5 in HSV-1, we decided to replace the second start codon of MDV UL12, located in aa 136, generating UL12 mut2. Upon transfection, we confirmed that virus replication was completely abrogated, as UL12mut2 displayed no plaque formation. To confirm the hypothesis that the c-terminus of UL12, rather than the full UL12, was essential for virus replication, we generated UL12 mut3, which contained only the stop mutation replacing aa 136. UL12 mut3 presented the same phenotype as UL12 mut2, confirming that the c-terminus of MDV UL12 is actively involved in viral replication, while the first 135 amino acids are dispensable for this process.

Because MDV's genomic structure is similar to that of HSV-1 and HSV-2, we initially hypothesized that the n-terminus of MDV UL12 would be mostly involved in replication, which was not the case. Differently from HSV-1, the c-terminus of MDV UL12 played a bigger role in replication, and suggested expressing exonuclease activity. Nevertheless, if the putative isoform of MDV UL12.5 follows the genomic trend seen HSV-1, our results indicate that MDV UL12.5 protein version is probably encoded after the first 135 aa of UL12, which could raise hypotheses that MDV UL12.5 is essential for virus replication and possesses exonuclease activity. Additional research would be able to confirm the exact location, function and size of MDV UL12.5 and shed light on the properties and functions of UL12 in MDV.

In order to evaluate the role of MDV UL29 gene in replication, we replaced the first start codon with a stop mutation. Our results showed an identical phenotype in virus replication between MDV and HSV, with both viruses depending on UL29 for successful replication. These evidences suggest that ICP8 and UL29 and their role in virus replication are strongly conserved between members of the *Alphaherpesviridae* subfamily.

9.2 The role of MDV UL12 and UL29 in DNA recombination

As it is known, the processes of DNA replication and recombination are closely tied in different types of organisms. In DNA viruses, the linkage between recombination and replication was first described for DNA bacteriophages T4 [140,141]. Similarly to T4 bacteriophages, λ can use different pathways to recombine its DNA and integrate into the bacteria's genome, a process that happens in high frequency. Some of those pathways involve the catalysis of virus proteins, while others depend on the host's recombination machinery [142].

In addition to HSV-1 UL12 and UL29 genes being involved in virus replication, studies have showed that both proteins are capable of performing *in vitro* strand exchange [79,80]. Moreover, it was suggested that HSV-1 UL12 and UL29 are reminiscent of the complexes encoded by λ phage in *Escherichia coli* that may stimulate recombination-mediated genetic engineering [80,143–147].

After unveiling the role of UL12 and UL29 in MDV replication, we decided to test whether MDV UL12&UL29 complex would also stimulate recombination, a biological process required for DNA repair and, as showed for λ phage, viral integration-mediated through genomic recombination.

Eukaryotic cells have developed at least four separate recombination pathways to repair double-strand breaks: HR, SSA, NHEJ and ANHEJ [80,119]. We used here different cell lines expressing a GFP reporter gene that would be expressed upon activation of these pathways, respectively [119].

Upon transfection of these cells with plasmids expressing UL12 and (ICP8) UL29, we could verify that SSA was significantly enhanced by UL12 upon DNA repair, while UL29 did not participated in any of those recombination pathways, nor supported UL12 in DNA repair. Our results here presented are consistent with previous data on the orthologues of HSV-1 that showed UL12 participating in DNA repair via the SSA pathway [80].

Differently from MDV, HSV does not integrate during the quiescent phase of infection, but rather remains in a circular episomal form [27,30].Thus, investigating the role of UL12 and UL29 in virus integration has never been possible.

HHV-6, another herpesvirus that belongs to the *Betaherpesviridae* subfamily is also capable of integrating its DNA in the host telomeres during latency [77,111]. Similarly to MDV, HHV-6 contains orthologue genes that express an exonuclease, U70, and a single-strand annealing protein, U41, respectively. A study conducted by Wight and colleagues showed that U70, similarly to MDV and HSV UL12, was able to enhance SSA as a pathway to repair DSBs, but surprisingly, U70 and U41 were not involved in the process of virus integration in human's

cells [128]. This was the first evidence that a viral exonuclease and a single-stranded annealing protein were not involved in virus integration. On this matter, nothing had been yet published on UL12 and UL29 in alphaherpesvirus integration.

9.3 The integration assay as a tool to assess virus integration *in vitro*

Once MDV has established an infection in the natural host it can establish latency in CD4+ T cells on resistant hosts. These T cells infected have showed to undergo transformation driven by the virus and develop deadly lymphomas [36,42,61]. Both latently infected and MDV-induced tumour cells harbour the virus genome integrated in the telomeres of multiple host chromosomes and genome integration is the mechanism that allows MDV to persist for life [42,55,148]. Because MDV and other herpesviruses of humans share this integration mechanism [111], MDV has been often see as a model for virus-induced oncogenesis, being extensively researched and studied [111]. For MDV, performing virus integration studies has only been possible *in vivo* due to the lack of suitable *in vitro* techniques.

Studies have demonstrated that reticuloendotheliosis virus-transformed chicken T cell line CU91 can be infected with MDV and supports a latent infection [121,122]. Making use of this cell line and aiming the reduction on the number of living animals used for research purposes, we initiated the development of a method to evaluate MDV latency properties *in vitro*.

As demonstrated in this piece of work, a quantitative *in vitro* assay to assess Marek's disease virus genome maintenance and integration based on qPCR proved itself to be a reliable tool to verify virus integration indirectly. During MDV lytic replication, virus genome is extensively present in the cell, while during latency, MDV integrates its DNA directly into the host telomeres, equalizing the ratio of cellular and viral genome copies. With this, copy numbers of the viral infected-cell protein 4 (ICP4) gene were normalized against the cellular genome copies of the inducible nitric oxide synthase (iNOS) gene, after qPCR analysis.

Despite such progress, it is undoubtedly agreed that the gold-standard technique to visualize virus integration in the cells is still FISH [149]. Because of many technical limitations with CU91, such as low viability and fragile stability of the cells *in vitro*, we had to adapt growth conditions, such as media, temperature and even the sorting process to ensure good cell viability. Additionally, we ran pilot studies to visualize and standardize detection of viral latency in the telomeres. Here, we progressed to the stage where we could identify and distinguish through FISH viral lytic replication from latent infection in CU91s (Figure 24).

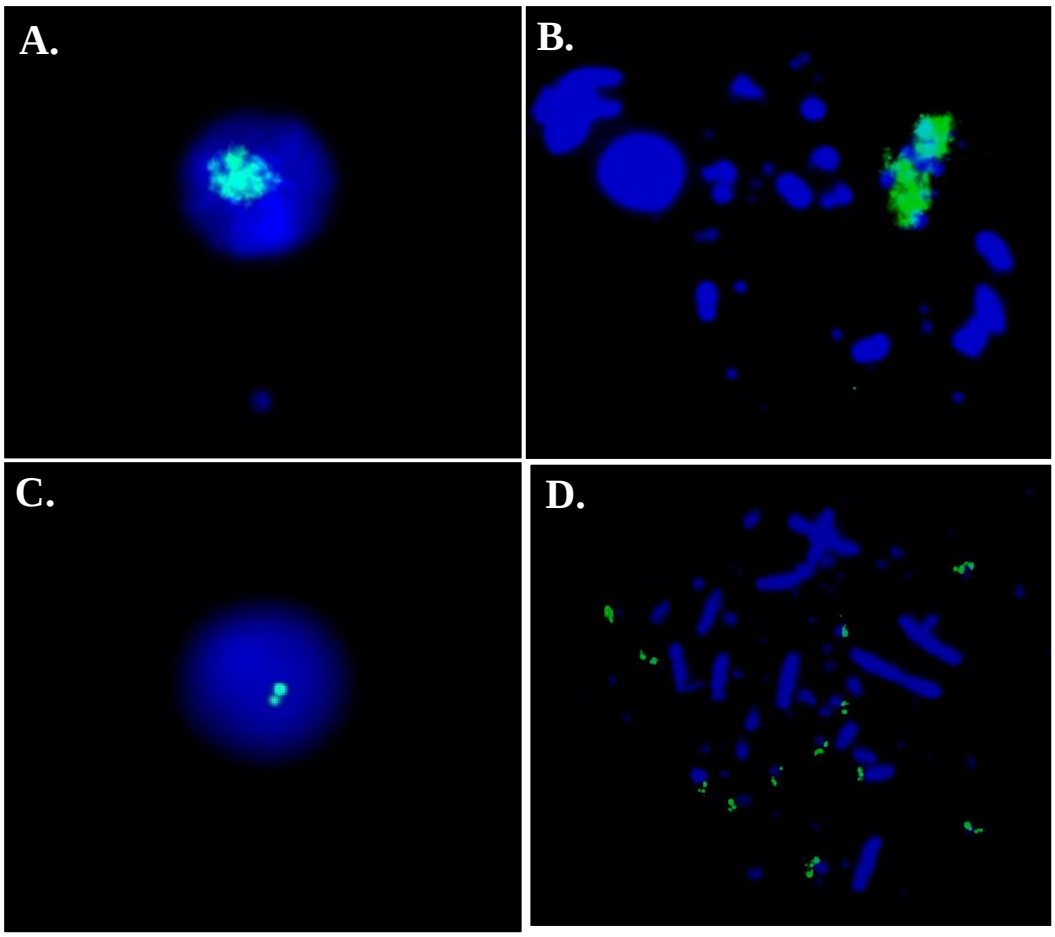

Figure 24: Characterization of lytic and latent infection through FISH in CU91s. Blue: Vectashield DAPI staining of the host cells and chromossomes; green: specific staining for MDV. (A) A single CU91 cell present in interphase, with multiple signals of MDV genome detection, suggesting lytic replication. (B) Metaphase spread preparation containing CU91s and its chromosomes, with lytic MDV replication occurring. (C) A single CU91 cell in interphase with a two green signals expressed, suggesting MDV integration. (D) Metaphase spread preparation of MSB-1 cells with reliable emission of green signals in the end of chromosomes, suggesting MDV integration.

The confirmation of herpesvirus integration events through FISH has been extensively used for human and animal viruses [55,77,110,149]. Although quantification of viral and cellular genome through qPCR is a reliable tool to indirectly address virus integration, the direct visualization of this event leaves no doubt behind. Currently, experiments are being performed to optimize this innovative MDV integration assay *in vitro*, augmentate cellular viability and reduce signal background from FISH. Our incessant progress to work around these limitations in order to establish FISH and properly visualize integration in the telomeres will pave the way for future integration studies capable of being delivered *in vitro*.

9.4 The role of UL12 and UL29 in MDV integration

One main aspect of MDV that distinguishes it from other herpesviruses is the fact that, together with HHV-6, MDV integrates its DNA directly into the host telomeres during the quiescent phase of infection [55,56,77]. Previous studies on herpesvirus integration showed that the homology between telomeric repeats of the host and the herpesviruses are essential for this integration process to occur, suggesting that this process is mediated by homologous recombination.

After assessing the role of UL12 and UL29 in MDV replication and DNA recombination and showing that UL12 is involved in SSA, we progressed towards the answer if UL12 and UL29 could be involved in MDV integration.

To address the role of UL12 and UL29 in MDV integration, we considered a variety of experiments that could answer this. One of them would be using UL12 mut1, UL12 mut2 and UL29 mut1 in the integration assay, considering that these viruses contain those genes abrogated by stop codon mutations. This option was disconsidered for UL12 mut2 and UL29 mut1 due to the fact that these viruses could not grow *in vitro*, and thus, infection of CU91s in our new integration assay would be compromised. Another option on the table was to use the DD DHFR in frame with the UL12 and UL29 proteins and use these mutant viruses in an identical scenario as the one established for vUL30DHFR. This idea seemed promising and we generated those respective mutants containing the DD DHFR cassette on the n- and c-terminus of the genes, separately. However, upon transfection we were not able to reconstitute none of the UL12 or UL29 mutant viruses in the presence of TMP, which suggests that the addition of the DD DHFR in either termini of the genes was disrupting one or multiple steps of protein synthesis, leading to the abrogation of virus replication. Although the results from the DD DHFR UL12 and UL29 mutants did not helped to address virus integration, they confirmed the role of those genes in replication.

After this, we went back to the initial stop codon mutants generated. Because UL12 mut1 had a growth defect, yet was able to grow *in vitro*, we proceeded with it in the integration assay. Similarly to HSV-1, only the full-length UL12 protein is located in the nucle*us,* where it helps to recombine DNA [86]. Considering that MDV UL12's nuclear localization signal (NLS) is within the first 50 amino acids, as shown for HSV-1 previously [129]*,* we firstly used the UL12 mut1 virus that lacks the full-length UL12 protein and the NLS needed for nuclear localization to determine if UL12 plays a role in integration and genome maintenance. Our results showed that ΔmTMR, at day 14 post-infection had its genome completely lost, indicating that integration was impaired, as expected. Differently, UL12 mut1 genome has been maintained

at levels comparable to wild type virus at day 14 post-infection, suggesting that it has not affected integration.

In order to confirm these results, we developed specific shRNAs to target UL12 or UL29 in CU91 T cells in our integration assay. Inhibition of UL12 or UL29 in chicken T cells did not affect the maintenance of the genome (Figure 19), suggesting that these proteins could be dispensed for integration in MDV. Interestingly, experiments with U41 and UL70 genes of HHV-6's orthologues for UL12 and UL29 showed that these proteins are also not essential for HHV-6A integration [128]. These evidences that the viral exonuclease and single-stranded binding protein do not play a role in integration could indicate that there are other cellular factors involved in it.

Our studies on MDV's UL12 and UL29 present the first line of evidence of the role of these two genes in the virus life cycle. To conclude, we assessed the high importance of these genes in MDV replication, the impact of UL12 in DNA recombination through SSA and the non-essential participation of UL12 and UL29 in integration.

9.5 The role of UL30 in the establishment of MDV latency in CU91 cells

It has been widely known and accepted that MDV's genome is found integrated in transformed tumour cells and B and T cells of susceptible animals infected. Additionally, this suggests that in order to transformation to happen, integration must be first established. [40,42,49,55,56,58,61,63,97,109,112,121,122,148]. Despite these facts, not much is known on the exact kinetics of the biological processes involved in MDV integration. One study conducted by Robinson and colleagues demonstrated that virus integration as an event, can happen on B and T cells during early stages of the infection, and evidenced that virus replication happened concomitantly to integration [109]. In spite of this, no studies so far have addressed the exact moment integration happens after MDV enters the animal body, or if replication is a precursory event to integration.

Here, we aimed at the UL30 gene in MDV, which encodes the main viral DNA polymerase, and tested in our integration assay if UL30, and consequently replication, influenced on viral integration. Thus, we generated a mutant virus containing a destabilization domain (DD) based on a genetic engineered DHFR gene of *E.coli*. We first inserted the DD DHFR sequence in frame with the UL30 gene, generating the MDV mutant vUL30DHFR. Here, the DD DHFR genomic sequence was added between the final stop codon and the last aa of UL30, being translated in frame with the main DNA polymerase, yet not interfering on its structure. As it was hypothesized, vUL30DHFR would have its DNA polymerase either active or degraded in

an inducible manner when in the absence of presence of the ligand, TMP. Upon characterization of vUL30DHFR *in vitro*, we have demonstrated that, indeed, vUL30DHFR was being controlled in a TMP dose-dependent manner that directly affected virus replication. In the maximum presence of the ligand, TMP, vUL30DHFR behaved identically as the wild type virus, whereas in the absence of TMP, replication was completely abrogated.

After confirming these properties of vUL30DHFR, we adapted our integration assay using CU91s to work in the presence and/or absence of TMP. We conducted the integration assay with vUL30DHFR on two separate conditions, with and without TMP. Here we evidenced that after 14 days post-infection, the level of viral copies of vUL30DHFR were reduced in the absence of TMP to an equal amount of viral copies of ΔmTMR's. Because ΔmTMR virus has integration compromised, we concluded that, in CU91 cells, vUL30DHFR lacking TMP interfered in virus replication and subsequently in virus integration.

Our results showed the first evidences of the functionality of a destabilization domain system to control MDV's protein expression *in vitro* and suggests that replication could possibly be involved in viral integration.

In order to confirm these facts and set in stone that replication is important for MDV integration, further experiments should be conducted, ideally *in vitro* and *in vivo*. *In vitro* experiments using a different T cell line, such as 855-19 T cells (MT3), (informal source of information from Tierärztliche Fakultät - LMU München), would confirm if these observations were only limited to CU91 cells of not. In addition to that, *in vivo* experiments using vUL30DHFR and the removal of TMP from the infected animals' water supply could help address the question of when and if replication happens prior to integration into the hosts' telomeres.

Nevertheless, it is important to consider that viral factors alone might not solely support integration, as there might also be other cellular factors involved in this process.

9.6 Other cellular and viral factors involved in MDV integration

Nowadays, it is known that MDV integration happens due to the contribution of viral structures that aid this process, such as the sTMR and mTMR [55,56]. For telomere integration, TMR sequences in these herpesvirus genomes are required, although the exact mechanism and factors needed for integration still need to be fully defined.

The putative viral recombinase U94 is another viral protein that could eventually facilitate the integration, in case of HHV-6. U94 is an adeno-associated virus integrase orthologist (Rep68) that is highly preserved in all HHV-6 strains. Additionally, it has recently been shown that the

U94 protein possesses all the functions necessary for homologous recombination to occur. A study conducted by Wallaschek and colleagues has shown that HHV-6A without U94 can still be found efficiently integrated into host telomeres, indicating that other viral or cellular factors can complement the loss of U94 during integration with HHV-6A [110]. MDV and other telomere herpesviruses do not encode an orthologue gene for U94, which suggests that this gene was somehow acquired in herpesvirus evolution during later stages.

There are two central recombinases that are involved in homologous DNA recombination, and which are also expressed by avian and mammalian cells, Rad51 and DMC1. Although DMC1 is unique to meiotic cells [92], Rad51 is active in somatic cells and is essential for DNA repair driven by HR [94,95,150]. In addition to that, Rad52, protein involved in SSA homologous sequence repair of DNA, is also present in these cells and could potentially be involved in aiding virus integration to occur [96]. Because of these proteins' cellular functions, they may be able to influence herpesvirus integration, although it remains open to questions whether or not they actually do.

In conclusion, this disparity on integration hypotheses involving different viral and cellular factors shows that telomere-integrating herpesviruses may have kept themselves promiscuous in terms of the route they have taken to telomeres throughout virus-host evolution. Moreover, it is also possible that herpesviruses may have been able to redundantly use different cellular and viral components involved in different recombination pathways in order to guarantee genome preservation and viral maintenance during different phases of the infection.

10. Zusammenfassung

“Neue Erkenntnisse über virale Faktoren, die am Lebenszyklus der Marek-Krankheit beteiligt sind”.

Das Marek´s Disease Virus (MDV) ist ein Alphaherpesvirus welches Hühner infiziert und verschiedenste klinische Symptome hervorruft, wie die Folgenden: Immunsuppression, Paralyse, neurologische Probleme und tödliche Lymphome. Interessanterweise, und im Kontrast zu den meisten anderen Herpesvieren, integriert MDV sein Genom in die Telomere von latent infizierten Wirtszellen. Es ist bekannt, dass Telomersequenzen im viralen Genom essentiell sind für diesen Prozess, der Mechanismus aber und die Faktoren, die die Integration beeinflussen sind nur unzureichend beschrieben.

MDV ist relativ nah mit dem humanen Virus Herpes Simplex Virus-1 (HSV-1) verwand und beide besitzen Gene, welche funktionell konserviert sind. Beide Virusgenome enthalten zwei Gene, welche eine 5´-3´ Exonuklease (UL12) und eine DNA-Einzelstrang bindendes Protein (UL29 oder auch ICP8) kodieren. Im Falle von HSV-1 interagieren die Proteine pUL12 und ICP8 miteinander und vermitteln die DNA-Rekombination, ein Prozess welcher höchstwahrscheinlich in der Virusreplikation involviert ist. Studien konnten zeigen, dass UL12 des HSV-1 einen schweren Replikationsdefekt von 100-1000 fach hervorruft, was es sehr wichtig macht für die virale Replikation. Auf der anderen Seite konnte gezeigt werden, dass UL29 ein essentielles Gen für die Replikation von HSV-1 darstellt. Weiterhin wurde gezeigt, dass die 5´-3´ Exonukleaseaktivität von pUL12 das Einzelstrangbinden während der HSV-1 Replikation versärkt, wohingegen ICP8 entbehrlich ist.

Trotz der vielen Fortschritte im Verständnis über die HSV-1 Gene UL12 und UL29, ist bisher nur wenig über die Orthologe in MDC bekannt. Die ersten Ziele dieses Projekt waren es die Rolle von UL12 und UL29 in der Replikation von MDV zu verstehen, zu verifizieren ob diese beiden Gene in der DNA Rekombination involviert sind, und ob der Replikationskomplex geformt aus UL12 und UL29 helfen kann das MDV Genom in Telomere zu integrieren.

Um die Rolle von UL12 und UL29 in der lytischen MDV Replikation zu untersuchen, wurde aa 6 und 7 in UL12 und aa 1 in UL29 mit Stopkodons ausgetauscht um die Proteinexpression beider Proteine individuell zu unterbinden. Mittels Transfektion dieser UL12 mutierten Viren konnte bestätigt werden, dass Stopmutationen in UL12 nicht zu einer kompletten Reduktion der Virusreplikation führten, jedoch die Größe und Anzahl der Plaques reduziert wurde. Im Anschluss wurde das zweite Methionin in UL12, welches sich an Position aa 136 befindet, zu einem Stopkodon mutiert. Transfektion dieser mutierten Viren zeigte, dass das Virus nicht mehr replizieren konnte, was zeigt, dass der C-Terminus von UL12 eine wichtige Rolle in der

Replikation spielt. Die Mutation von UL29 blockierte die Virusreplikation, was in Übereinstimmung mit den Daten für HSV-1 ist.

Nachdem die Bedeutung von UL12 und UL29 in der Replikation von MDV untersucht wurde, wurde die Rekombinationsaktivität dieser Proteine in den vier hauptsächlichen DNA Rekombinationsreparaturmechanismen in eukariotischen Zellen untersucht: das Einzelstrangannealing (single strand annealing, SSA), die homologe Rekombination (HR), das nicht-homologe Endenverschmelzen (non-homologous end-joinning, NHEJ), und das alternative nicht-homologe Endenverschmelzen (alternative non-homologous end-joinning, A-NHEJ). Mit Hilfe eines zellbasierten Assays und viraler proteinexpressions Vektoren, konnte gezeigt werden dass UL12 behilflich sein kann bei der SSA DNA Reparatur, wohingegen UL29 nicht aktiv war in der SSA.

Da UL12 bei der Reparatur homologer DNA behilflich sein kann, wurde die Rolle von UL12 und UL29 bei der Integration von MDV mit Hilfe eine Integrationsassays mit immortalisierten Hühner T Zelllinien untersucht. shRNAs wurden generiert um UL12 und UL29 auszuschalten und der Effekt dessen auf die Virusintegration wurde untersucht. Die Analyse der Ergebnisse zeigte, dass das Fehlen dieser beiden Gene keinen Einfluss hatte auf die virale Integration in T Zellen.

Das zweite Ziel dieser Arbeit war es zu untersuche ob die virale Replikation von MDV wichtig ist für die Integration des Virus in T Zellen. Es wurde auf das UL30 Gen fokussiert, welches die MDV DNA Polymerase kodiert, und es wurden mutante Viren hergestellt, welche eine desabilisierende Domäne in Verbindung mit UL30 (vUL30DHFR) enthalten. Diese desabilisierende Domäne gewährleistete die induzierbare Kontrolle der viralen DNA Polymerase. Die Ergebnisse zeigten, dass bei Block der DNA Polymerase und somit der Abschaltung der viralen DNA Replikation keine viralen Genome in T Zellen 14 Tage nach Infektion gefunden werden konnten. Weiterhin war dieser Effekt in einer ähnlichen Ausprägung bei MDV Mutanten vorhanden, welchen die viralen Telomerwiederhohlungen, die essentiell sind für die Integration, fehlen. Dieser Beleg ist der erste seiner Art und weist darauf hin, dass die virale Replikation in der Etablierung des latenten MDV Genoms involviert ist.

Zusammengefasst lässt sich sagen, dass die in der vorgelegten Arbeit erlangten Erkenntnisse zeigen konnten, dass MDV essentielle virale Faktoren kodiert, welche in der SSA DNA Reparatur eine Rolle spielen, die aber nicht essentiell sind für die Integration von MDV in Telomere. Abschließend lässt sich sagen, dass die vorliegende Arbeit eine Grundlage für weitere Studien darstellt, die den Zusammenhang von viraler DNA Replikation und Integration von MDV in Telomere untersuchen.

11. Summary

"*Novel insights on viral factors involved in the Marek's Disease virus' life cycle".*

The Marek's disease virus (MDV) is an alphaherpesvirus of chickens, causing various clinical symptoms including: immunosuppression, paralysis, neurological disorders and terminal lymphomas. Interestingly, in contrast to most herpesviruses, MDV integrates its genome into the telomeres of latently infected host cells. While it's known that telomere sequences in the virus genome are essential for this process, the actual mechanism and factors influencing this process remain poorly defined.

MDV is relatively closely related to the human virus herpes simplex virus 1 (HSV-1) and they possess genes that are functionally conserved. Both virus genomes contain two genes called UL12 and UL29, which encode for a 5'-3' exonuclease and a single stranded DNA binding protein (ICP8), respectively. For HSV-1, pUL12 and ICP8 interact with each other and promote DNA recombination, a process likely involved in virus replication. Studies have showed that HSV-1's UL12 when abrogated leads to a severe virus replication defect of 100-1000 fold, which makes it very important for virus replication. On the other hand, UL29 has been shown to be an essential gene for HSV-1 replication. Moreover, the 5'-3' exonuclease activity of pUL12 has been shown to enhance single strand annealing during HSV-1 replication, whilst ICP8 is dispensable.

Despite so many advances in understanding the HSV-1 UL12 and UL29 genes, little is known about their orthologues in MDV. The first aims of this project were to address the role of UL12 and UL29 in MDV replication, verify if these two genes are involved in DNA recombination, and if the recombination complex formed by UL12 and UL29 could help MDV integration into telomeres.

To address the roles of UL12 and UL29 in lytic MDV replication, aa 6 and 7 in UL12 and aa 1 in UL29 were replaced for stop codons, abrogating both proteins' expression individually. After transfecting these UL12 mutant viruses, we could confirm that the stop mutations in UL12 did not completely abrogate virus replication, although reduced the size and number of plaques formed. Next, we mutated the second methionine in UL12 located at aa 136 to a stop codon. Transfection of this mutant virus showed that it could not replicate, indicating that the c-terminus of UL12 is important for replication. In accordance with the data on HSV-1, mutation of UL29 completely blocked virus replication.

After addressing the importance of UL12 and UL29 in MDV replication, we assayed their recombination activity in the four main DNA repair recombination pathways in eukaryotic cells: single strand annealing (SSA), homologous recombination (HR), non-homologous end-joining

(NHEJ) and alternative non-homologous end-joining (A-NHEJ). Using a cell-based assay and viral protein expression vectors, we could show that UL12 can aid in SSA DNA repair, whilst UL29 was not active for SSA.

As UL12 can aid repair of homologous DNA, we investigated the role of UL12 and UL29 in MDV integration using an integration assay with an immortalized chicken T-cell line. We generated shRNAs to knockdown UL12 and UL29 and tested the effect on virus integration. This analysis showed that the absence of these two genes did not interrupt virus integration into T cells.

The second main aim of this work was to address whether MDV DNA replication is important for virus integration in T cells. We focused on the UL30 gene, which encodes the MDV DNA polymerase, and generated a mutant virus containing a destabilization domain-UL30 fusion (vUL30DHFR). This destabilization domain fusion enabled inducible control of the viral DNA polymerase. Our results showed that when the DNA polymerase is blocked and virus DNA replication is abrogated, there was no virus genome maintenance in T cells at 14 days post-infection. Moreover, this defect was of a similar magnitude to MDV mutants lacking the viral telomere repeats, which are essential for integration. This evidence is the first to suggest that virus replication is involved in the establishment of the latent MDV genome.

Taken together, the work presented in this thesis has shown that MDV encodes essential viral factors for virus replication and that are active in SSA DNA repair but are not essential for MDV telomere integration. Lastly this work has built a foundation for further studies on the links between virus DNA replication and MDV integration into telomeres.

12. References

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13. List of Publications

Here are all the publications completed by the author.

The outcome of this PhD led to two publications, which have been published prior to this thesis being submitted. Both articles were published in the journal *Viruses*, as below:

1) Previdelli RL, Bertzbach LD, Wight DJ, Vychodil T, You Y, Arndt S, Kaufer BB. **The Role of Marek's Disease Virus UL12 and UL29 in DNA Recombination and the Virus Lifecycle**

Viruses 2019, Jan 28; 11(2), 111; doi: 10.3390/v110201113

2) Kheimar A, Previdelli RL, Wight DJ, Kaufer BB.
Telomeres and Telomerase: Role in Marek's Disease Virus Pathogenesis, Integration and Tumorigenesis
Viruses 2017, Jul 4; 9(7); doi: 10.3390/v9070173

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16. Conflict of interests

There is no conflict of interest due to financial support of the work.

17. Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 17.10.2019

Renato Lopes Previdelli