Aus dem Institut für Virologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Marek's disease virus: from novel viral interleukin-8 (vIL-8) splice variants to inhibition with CRISPR/Cas9

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zur Erlangung des Grades eines
PhD of Biomedical Sciences
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vorgelegt von
Ibrahim T. Hagag
Tierarzt aus Sharkia, Ägypten

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“Excellence is never an accident. It is always the result of high intention, sincere effort, and intelligent execution; it represents the wise choice of many alternatives – choice, not chance, and determines your destiny.”

Aristotle
(384-322 BC)

Dedicated to Nadine
- I want you to know what my treasures are.

Contents

Figures and tables

Abbreviations

APS	Ammonium persulfate
AS	Alternative splicing
B cells	Bursa of Fabricius cells
BAC	Bacterial artificial chromosome
bp	Base pairs
BSA	Bovine serum albumin
C-terminus	Carboxyl-terminus
Cam	Chloramphenicol
Cam/Knm	Chloramphenicol/Kanamycin
Cas9	CRISPR associated protein 9
CD	Clusters of differentiation
CECs	Chicken embryo cells
CR	Carina retina cell line
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CVI988	Attenuated vaccine strain of MDV
CXCL	Cystine (X-amino acid) cystine ligand
dCas9	Deactivated Cas9 protein
DEPC	Diethylpyrocarbonate-treated water
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dpi	Days post-infection
DSBs	Double-strand breaks
E. coli	Escherichia coli
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ES	Exon skipping
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
For	Forward

GAGs	Glycosaminoglycans
GaHV-2	Gallid herpesvirus 2
gB, gC, gD, gH, gL	Glycoprotein B, C, D, H, and L
GFP	Green fluorescent protein
h	Hour
HCMV	Human cytomegalovirus
HDR	Homology-dependent Repair
HHV	Human herpesvirus
HR	Homologous recombination
HSV	Herpes simplex virus
HVEM	Herpes virus entry mediator
HVT	Herpesvirus of Turkey
ICP4	Infect cell protein 4
IF	Immunofluorescence
ILTV	Infectious laryngotracheitis virus
iNOS	Inducible nitric oxide synthase
IR	Internal repeat
kDa	Kilodalton
LAT	Latency-associated transcripts
LB	Luria-Bertani
MD	Marek’s disease
Md5	vvMDV strain (Prototype)
MDV	Marek’s disease virus
MDV-1	Marek’s disease virus serotype 1
MDV-3	Marek’s disease virus serotype 3
MEM	Minimal essential media
Meq	MDV EcoRI Q
Meq-sp	Meq splice variants
MHC	Major histocompatibility complex
MIIP	Migration invasion inhibitory protein
min	Minutes
ml	Millilitre
MM	Millimetre
mMDV	Mild strain of MDV

mRNA	Messenger RNA
mut	Mutant
NGS	Next-generation sequencing
NHEJ	Non-homologous end joining
O/N	Overnight
OD600	Optical density, 600 nm wavelength
ORF	Open reading frame
p.i.	Post-infection
PAM	Protospacer adjacent motif
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline tween
PCR	Polymerase chain reaction
pfu	Plaque-forming unit
Pol	Polymerase
pp38	Phosphoprotein 38
PSM	Positive-selection marker
PVDF	Polyvinylidene difluoride
qPCR	Quantitative PCR
RB-1B	Very virulent strain of MDV
Rev	Reverse
RFLP	Restriction fragment length polymorphism
RIPA	Radioimmunoprecipitation assay
RLORF	Repeat long open reading frame
rpm	Rotations per minute
RT	Reverse transcriptase
s	Seconds
SB-1	Strain of GaHV-3
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SPF	Specific pathogen free
T cells	Thymus cells
TAE	Tris-acetate-EDTA buffer
TALES	Transcription activator-like effectors
TCR	T cell receptor

TEMED	Tetramethylethylenediamine
TGN	Trans-Golgi Network
Th	Thymus helper cells
TPP	Transfection polypropylene tubes
TracrRNAs	Trans-activating CRISPR RNAs
TR_L	Terminal repeat long
TR_S	Terminal repeat short
UL27	Unique long 27 (glycoprotein B)
UL30	Unique long 30 (DNA Pol)
UL36	Unique long 36, (tegument protein)
UL49	Unique long 49 (tegument protein)
V.	Volt
vIL-8	Viral interleukin-8
vMDV	Virulent Marek's disease virus
vTR	viral telomerase RNA
vv+MDV	Very virulent + Marek's disease virus
vvMDV	Very virulent Marek's disease virus
VZV	Varicella-zoster virus
WB	Western blot
WM	Whatman
WT	Wild type
ZF	Zinc finger

1. Introduction

1.1. Herpesviruses

1.1.1. Taxonomy of herpesviruses

Herpesviruses are double-stranded DNA viruses that infect a wide range of hosts including humans and animals [1]. They produce lytic and latent infections with intermittent reactivations and shedding of infectious viral particles. Herpesviruses belong to the order Herpesvirales that includes three main families: Alloherpesviridae, Malocoherpesviridae, and Herpesviridae. Herpesviridae is classified into three subfamilies, Alpha-, Beta-, and Gammaherpesvirinae [2, 3].

Alphaherpesviruses include Mardiviruses, Iltoviruses Simplexviruses, Varicelloviruses, and Scutaviruses. Marek's disease virus (MDV) belongs to Mardiviruses and causes characteristic lymphomas in chickens. Infectious laryngotracheitis virus (ILTV) belongs to Iltoviruses and causes infectious laryngotracheitis in chickens. Simplexviruses include herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) that cause facial and genital lesions in humans. Varicella-zoster virus (VZV) belongs to varicelloviruses and produces characteristic shingles in humans. Scutaviruses cause respiratory infections in turtles [2-4].

Betaherpesviruses are slow-replicating viruses that infect a narrower host range with an establishment of latency in monocytes. They include human herpesviruses 6 and 7 (HHV-6 and 7), and human cytomegalovirus (HCMV), which is considered the prototype for betaherpesviruses [5]. Epstein-Barr virus (EBV) and human herpesvirus 8 (HHV-8) belong to gammaherpesviruses that infect a narrow host range and cause latency in the host lymphocytes [2].

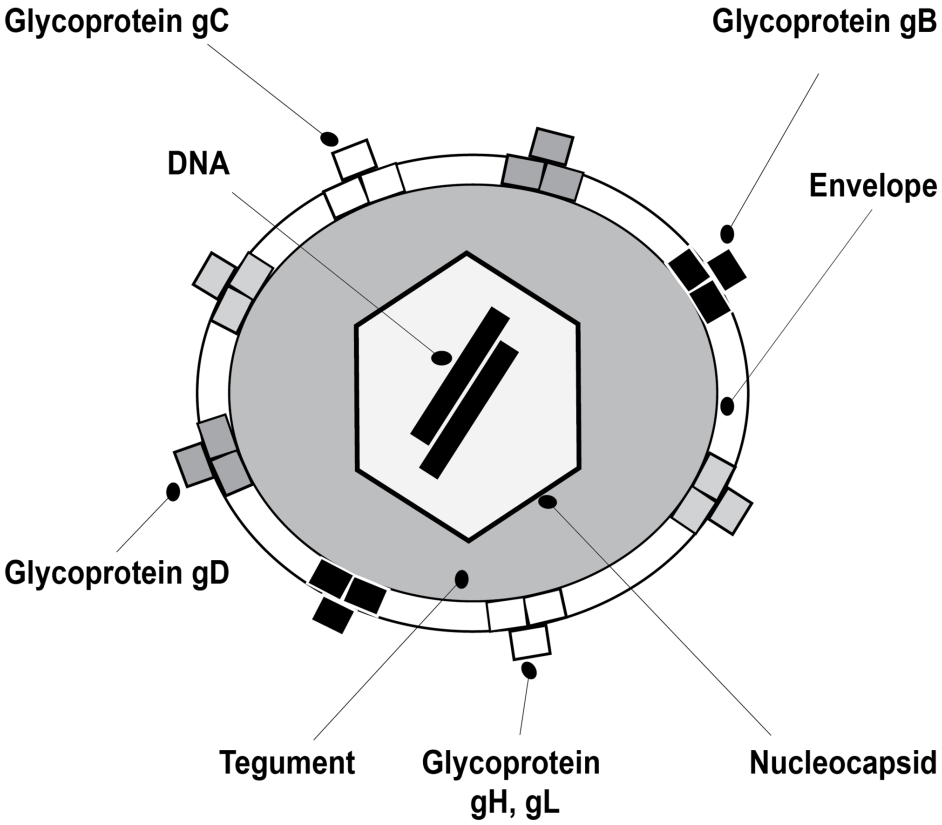


Figure 1: Virion of alphaherpesviruses. Herpesviruses are enveloped viruses with icosahedral symmetry, spherical to pleomorphic shape, and a diameter of 150-200 nm. The envelope composed of lipid bilayer impeded with several glycoproteins and surrounds an amorphous layer known as “tegument”. A linear double-stranded DNA of 120-240 kilobase pair is surrounded by a nucleocapsid that consists of 162 capsomers [6, 7].

1.1.2. The herpesvirus replication cycle

Understanding the replication cycle of herpesviruses is important for the assessment of their pathogenicity and developing antiviral therapeutics. Over the last years, HSV-1 was extensively studied and therefore is used as a prototype model for the replication of alphaherpesviruses [8]. Briefly, HSV-1 virions enter susceptible cells via a fusion process between the viral envelope and cell receptors. Next, viral nucleocapsid and tegument proteins enter cytosol where viral DNA is transported to the nucleus via nuclear pores. Viral replication and the synthesis of new DNA components start in the nucleus. Once a full-length viral DNA is formed, viral procapsids are packaged with DNA to form nucleocapsids that bud through the nucleus into the cytoplasm. Mature viral particles are formed when nucleocapsids get both tegument and envelope from trans-Golgi networks. Finally, mature viral particles are released from the cell via exocytosis [8].

Herpes simplex viruses own surface glycoproteins that interact with specific cell receptors to mediate the fusion process. Initially, gC and gB glycoproteins attach to glycosaminoglycan (GAGs) cellular receptors, which is followed by further binding of gD glycoprotein to other cell surface receptors known as integrins, herpes virus entry mediator (HVEM), and nectins according to viral species [9]. Once the gD glycoprotein is rigidly attached, a cascade of conformational changes of gD is started, allowing the interaction with other glycoproteins gH and gL. This multi-protein fusion complex allows the release of nucleocapsids into the cytosol [10]. In the cytosol, tegument proteins allow viral DNA to enter the nucleus where it becomes circular [11-13].

In the nucleus, the UL48-encoded tegument protein VP16 recruits cellular transcription factors to start the transcription of immediate early α genes as ICP4 (infected cell protein 4). ICP4 is essential for the inhibition of innate cellular defence mechanisms and the expression of β genes [14, 15]. The β gene-encoded DNA polymerase UL30 is required for DNA replication as it builds newly synthesized viral DNA strands [15]. γ genes encode for structural viral proteins and their expression is activated by the DNA replication process [16, 17]. DNA replication initiates as a long head to tail concatemers and mature DNA concatemers are cleaved and packaged into viral procapsids. This process is known as rolling circle replication, which is the most acceptable model for the replication of alphaherpesviruses [16, 17].

Assembled viral particles are shuffled to the nuclear membrane where they acquire a primary tegument from the inner lamina of the nuclear membrane that facilitates the budding of viral particles from the nucleus [18]. Budding of the new viral particles from the nucleus leads to the formation of enveloped viral particles that fuse with the nuclear membrane releasing viral capsids into the cytoplasm [19]. Newly synthesized viral particles further interact with tegument UL36 and UL49 proteins and acquire their envelope by budding through Trans-Golgi Network (TGN) [18-20].

Although MDV replication is thought to be analogous to other alphaherpesviruses, knowledge about MDV replication is limited due to its highly cell-associated behaviour. MDV virion lacks three morphogenesis steps that include nuclear egress, acquiring of the secondary tegument, and exocytosis [21]. Moreover, the mechanism of cell-to-cell transfer of MDV virions is poorly understood. However, there is a line of evidence that gB and gD glycoproteins contribute to MDV spread from infected to uninfected cells by forming intracellular bridges [22].

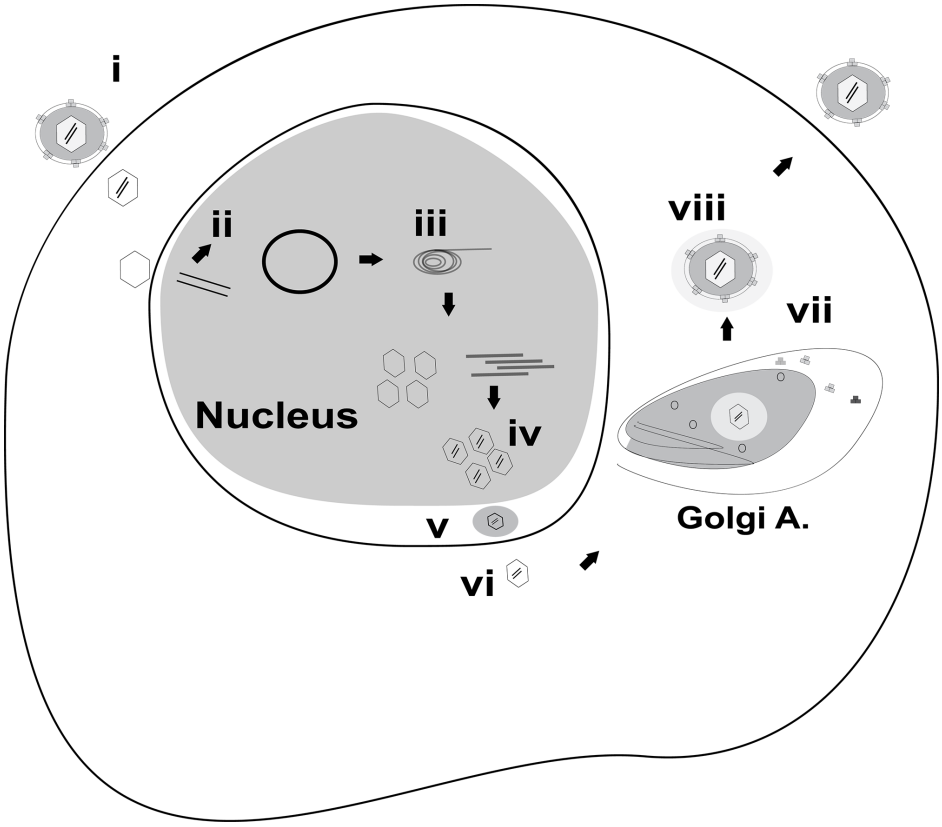


Figure 2: The herpesvirus replication cycle. A herpesvirus infection (exemplarily depicted for HSV) starts with viral entry by fusion **(i),** followed by initiation of the rolling circle replication **(ii) and (iii)**, assembly of viral particles **(vi)**, acquiring of the primary tegument **(v)**, nuclear egress **(vi)**, getting of the secondary tegument **(vii)**, and maturation of virions and release **(viii)** [23].

1.2. Marek's disease virus (MDV)

1.2.1. Historical background

In 1907, the Hungarian veterinarian Joszef Marek firstly described Marek's Disease (MD) as 'Polyneuritis' after his remarkable observation of the paralysis of wings and legs of four cockerels. A post-mortem examination showed that the enlargement of sacral plexuses was due to a massive infiltration with mononuclear cells [24]. Due to the massive expansion of the poultry industry in 1950, MD cases increased dramatically and were confused with another disease called 'Avian Leucosis'. Therefore, scientists used the term 'Lymphomatosis' to describe all cases of lymphoma in chickens [25, 26]. In 1960, the first symposium of the world veterinary poultry association declared that MD is caused by a cell-associated herpesvirus [27, 28]. In 1970, scientists developed the first MDV vaccine that was described as the first vaccine against virus-induced cancers [29]. Since that time, the intensified rate of poultry production and vaccination programs provoked MDV to evolve to more virulent strains that develop different disease symptoms from the classical MD forms [30, 31].

1.2.2. MDV genome, virulence, and evolution

Marek's disease virus 1 (MDV-1), also nominated as Gallid herpesvirus 2 (GaHV-2), is a cell-associated herpesvirus that belongs to alphaherpesvirinae. The genome of MDV belongs to class E that possess 4 isomers, depending on the orientation of the unique regions [32]. MDV genome has two unique regions, the unique long (U_L) and the unique short (U_S). These unique regions are flanked by two internal repeats: long (IR_L) and short (IR_S), and two terminal repeats: long (TR_L) and short (TR_S) (Figure 3A) [33]. MDV genome is about 175-180 kilobase pairs and harbours more than 100 open reading frames [34].

Based on virulence, MDV is classified into mild virulent (mMDV), virulent (vMDV), very virulent (vvMDV), and very virulent + (vv+MDV) strains [35]. According to species, MDV can be oncogenic or non-oncogenic [36]. Oncogenic MDV strains are RB-1B, Md5, GA, HPRS16, and CVI988. RB-1B strains cause vvMDV pathotypes while CVI988 strains cause mMDV pathotypes that are similar to the classical form of MD [37-39].

Non-oncogenic MDV species include the SB-1 and HVT (MDV-3) vaccinal strains [40]. SB-1 and HVT strains are used to protect chickens from oncogenic strains because they do not develop lymphomas in chickens and can produce antigenically related antibodies [41]. Interestingly, the oncogenic serotypes include genes that are not encoded by non-oncogenic serotypes. For instance, the major oncogene MDV EcoQ (meq), the phosphoprotein 38 (pp38), the viral telomerase RNA (vTR), and the viral interleukin 8 (vIL8) [41, 42].

Vaccines are used in the veterinary field to improve the animal health sector, prevent infectious zoonotic diseases, and reduce drug residues in the food chain [43]. However, vaccines do not always supply sterile immunity, allowing the evolution of more virulent strains into the environment. These escape mutants can produce disease in the unvaccinated flocks [44]. MD was firstly controlled in the early 1970s by the introduction of the HVT vaccine [29], which was the first example of a vaccine-controlled cancer. However, MDV evolved into more virulent strains over the last decades. The arms race between MDV and vaccination started in the late 1970s when a new MDV variant (vvMDV) appeared in the HVT-vaccinated flocks. Therefore, the MDV second-generation vaccine that combined HVT and SB-1 strains was produced in the early 1980s. However, 10 years later, MDV evolved into a more virulent vv+MDV strain that led to the production of a third-generation vaccine. CVI988 known as “Rispens” was introduced into MDV vaccination programs in the 1990s and is now considered as the “gold standard” vaccine. This vaccine does not develop sterile immunity in chickens, but it can prevent tumour formation [44, 45]. Hence, alternative interventions that can control MD in chickens and develop sterile immunity are strongly needed [44].

1.2.3. Clinical signs and pathogenesis of Marek's disease

Marek's disease (MD) is a clinical syndrome that affects chickens [46]. MD mortality rates reach up to 100% in unvaccinated flocks [40]. An immunosuppression state is a characteristic feature of MD due to the lytic replication of MDV in B cells that leads to an atrophied bursa. Immunosuppressed chickens are vulnerable to invasion by secondary bacterial infections [47]. A predominant sign in MD clinical picture is the developed lymphomas that appear in the skin and visceral organs [30]. Lymphoblastoid cells infiltrate to the peripheral nervous system leading to prominent nervous signs ranging from transient ataxia to recumbency [48].

Chickens acquire the infection by inhalation of MDV-contaminated aerosols [30]. Afterward, MDV diffuses to the lymphatic system by macrophages and dendritic cells in the respiratory tract. In the lymphatic system, MDV starts a lytic phase of replication in infected B cells. This phase is known as the early cytolytic phase that starts 2-7 dpi [31, 49]. The lytic infection of immune cells activates CD4+ T cells as a first cell-mediated immune response. Nonetheless, MDV establishes a latent phase in the activated CD4+ T cells 6-7 days post-infection [50]. Of note, resting T cells are refractory to infection [49, 51, 52]. During latency, MDV stays dormant in the host chromosomal telomeres and expresses only a few viral genes known as latency-associated transcripts (LATs) [31, 53, 54]. One of these LATs is the major oncogene *meq* which is a trans-activator protein that belongs to c-Jun/Fos family. Meq is thought to play an essential role in blocking apoptosis and maintaining the expression of other LATs as vIL-8 splice variants, some microRNAs, and vTR [55, 56]. MDV integrates into host telomeres to establish latency [53, 54]. MDV integration facilitates viral genome maintenance into the host and genome mobilization during reactivation [57]. Latently infected T cells migrate to the skin to reactivate and start another 'late productive phase' in the epithelium of feather follicles. Cell-free virions shed from feather follicles into the environment to infect another host. Transformed T cells disseminate to different visceral organs causing multiple tumours at 21-28 dpi [45]. These lymphomas are the main cause of the clinical symptoms and death of chickens.

1.2.4. MDV oncogenesis

MDV transforms $CD4^+$ $CD8^-$ T cells after a latent phase of infection [52]. Transformed cells most closely resemble the phenotype of the activated memory Th-2 or T-regulatory cells [58]. These cells express high levels of Meq and the cellular factor CD30, which is thought to have an important role in hyperproliferation and abrogation of apoptosis in infected cells [58-60]. Intriguingly, MDV-induced lymphomas mainly consist of few transformed T $CD4^+$ cells in the middle, which are surrounded by a mixture of different cells including macrophages and non-transformed T cells as a part of the body defence mechanism against tumour formation [58, 60, 61]. Therefore, it is thought that the transformation process is an early event as the transformed T cells are diffused to tissues and then proliferated. Transformed cells are then surrounded by lytically infected cells that were recruited to keep the pro-oncogenic environment. The exact mechanism by which the cell-mediated immunity contributes to MDV pathogenesis is not fully understood or clear. Nonetheless, $CD8^+$ T cells were able to prevent the expansion of transformed T cells in the tissues of MDV-resistant chickens at 5-7 days post-infection [60].

1.2.5. Viral interleukin-8 (vIL-8)

Modulation of the host immune system creates a favourable condition for viruses for efficient replication and spread to a new host. Accordingly, viruses developed several strategies to subvert the host immune system including antigenic variability, targeting the host antiviral immune responses, and mimicry of the host genes [62]. Viral chemokines (virokines) are one of the best examples of viral mimicry to host genes [63, 64]. Chemokines are soluble, small, and structurally related cytokines that mediate chemotaxis, inflammatory responses and cell migration [65-67]. Herpes- and poxviruses encode some virokines in their large DNA genome [63, 68]. These virokines boost the host immune system for the benefit of viral infection [64]. For instance, human cytomegalo herpesvirus (HCMV) and mouse cytomegalo herpesvirus (MCMV) are betaherpesviruses that express a few viral chemokines agonists and antagonist. The HCMV UL146 attracts neutrophils to the site of infection, while the MCMV MCK-1/2 is a chemoattractant to monocytes, increasing the monocyte-associated viremia *in vivo* [63].

MDV genome includes a viral chemokine known as viral interleukin 8 (vIL-8) [31, 67]. Except for MDV, alphaherpesviruses do not encode viral chemokines [69]. This unique alphaherpesviruses virokine was termed vIL-8 as it was initially thought to be a homolog of avian IL-8, a prototype of CXC chemokine family [67, 70]. However, it has been later recognized as a CXCL-13 ortholog [71]. vIL-8 has three exons that are spliced together to produce nearly a 0.7-kb transcript that expresses a true late kinetics vIL-8 protein [67]. The N-terminal 21 amino acids encoded by exon I are mostly hydrophobic residues that act as a signal peptide, which is cleaved to release the secreted virokine [67]. Exons II and III contain four conserved cysteine residues that code for the receptor-binding domain of vIL-8 [67]. Despite the structural similarity between vIL-8 and members of CXC chemokines, vIL-8 harbours an unusual long C-terminal extension that is thought to mediate an important role in chemotactic functions [72].

The secretory vIL-8 is a chemoattractant for avian peripheral blood mononuclear cells. Moreover, it is thought to antagonize chicken chemokines by binding to their receptors to recruit lymphocytes to migrate to the site of infection, which enhances viral replication and pathogenicity [67, 72, 73]. Also, vIL-8 is essential for recruiting B cells for lytic infection and T-cells for latent infection and transformation [6]. Nonetheless, the mechanism by which vIL-8 mediates its function or its binding to the target receptors has not been fully characterized.

The early *in vivo* studies on vIL-8 showed that the complete deletion of vIL-8 gene significantly reduced virus replication, especially in the early lytic phase. However, virus replication in feather follicles or transmission to naïve contact animals did not seem to be reduced [72]. In addition, MDV tumour incidence was severely reduced to nearly 90% [67, 72]. Interestingly, vIL-8 deletion delayed Meq expression and impaired the expression levels of pp38 in lymphoid organs, which suggests the importance of vIL-8 exons and/or introns for the upstream genes [56, 74]. In 2007, Jarosinski and Schat discovered a number of splice variants within MDV repeat regions that were spliced to vIL-8 [56]. These vIL-8 splice variants were identified *in vitro* and *in vivo* and included vIL-8 exons II and III (Figure 3) [56]. Therefore, it is likely that the vIL-8 deletion mutant showed a phenotype that combined the effects of disruption of the secretory virokine and the splice variants. This hypothesis was furtherly supported when the disruption of secretory virokine led to only a 50% reduction in tumour formation [6]. So, more studies are needed to address the role of only vIL-8 splice variants in MDV pathogenicity and oncogenicity.

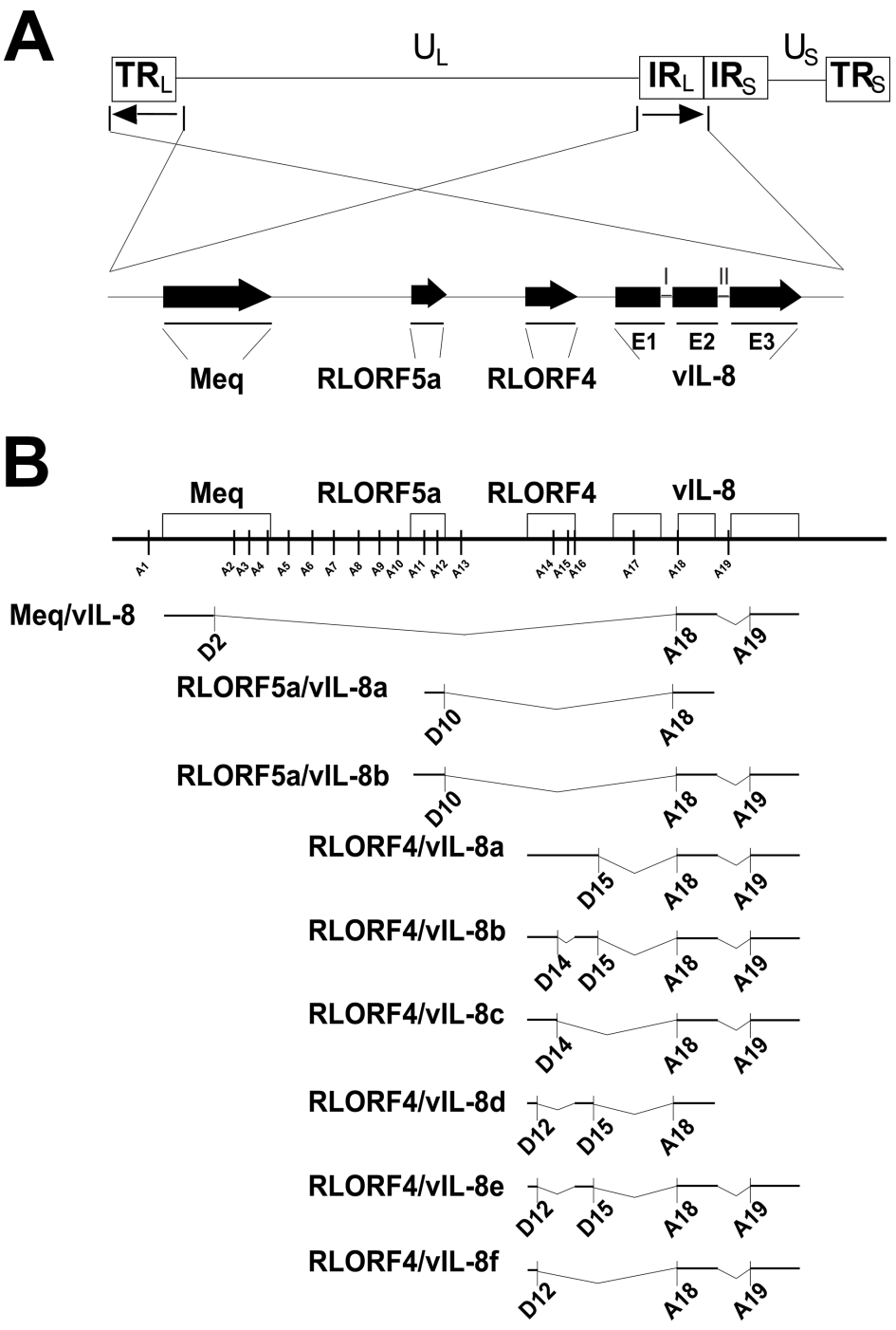


Figure 3: Overview of MDV vIL-8 splice variants . (A) Schematic representation of MDV genome showing important genes within TR_L and IR_L regions. **(B)** Map of the previously-identified acceptor (A), donor (D) sites, and vIL-8 splice variants within these genomic regions [56].

1.3. MDV and RNA splicing

1.3.1. Splicing of MDV transcripts

Herpesviruses are large DNA viruses that replicate and transcribe their genes within the nucleus [16]. The majority of herpes viral proteins are expressed from unspliced transcripts. Nonetheless they express a sustainable number of spliced proteins, mainly during latency or the early lytic phase [56, 75]. Like other herpesviruses, spliced viral transcripts were identified within MDV transcriptome *in vitro* and *in vivo* [56, 76]. A recent analysis of MDV transcriptional landscape identified a surprisingly novel splice junctions that were predicted to affect multiple viral genes [76] . This indicates that the splicing of MDV transcripts is more common than previously assumed and also contradicts the paradigm that RNA splicing is a rare event in alphaherpesviruses [76] .

1.3.2. Historical background on RNA splicing

In 1941, the paradigm, ‘one gene, one protein’ was strongly supported by some data from Beadle and Tatum [77]. This model continued till researchers deeply studied the human genome and noticed that the number of protein-coding genes is significantly lower than expected. In the late 1970s, the RNA splicing mechanism was firstly discovered in adenoviruses [78]. Intriguingly, an amazing sequence arrangement was observed at the 5` terminus of adenovirus messenger RNA (mRNA) [78]. Comparison of adenoviruses mRNA with genomic sequences revealed that before export to the cytoplasm, specific viral sequences were removed from the pre-mRNA and the remaining sequences were joined together [79]. One year after, a novel mechanism termed as alternative splicing (AS) was uncovered and experimental confirmations quickly followed [80, 81]. After that, it was quickly discovered that most mammalian-polymerases II transcripts are spliced. During RNA splicing, only small fractions of the pre-mRNA transcript are joined together while the intervening sequences, known as “introns” remain in the nucleus and finally degraded [82]. Alternative splicing is involved in gene regulation and a plethora of cellular biological functions [83].

1.3.3. Mechanism of RNA splicing

RNA splicing is a complex process that is involved in the assembly of gene exons, depending on specific splicing junctions and other splicing enhancers or silencers [84]. During maturation of mRNA, the pre-mRNA undergoes splicing where the non-coding regions known as introns are removed and the coding regions known as exons are linked together to form a mature mRNA.

Removal of the non-coding introns is a common feature of all eukaryotic lifeforms. Differential splicing of gene exons is called AS, which forms multiple proteins from a single gene, allowing proteome diversity and other regulatory functions [85]. Upon synthesis of the nascent mRNA by polymerase II in the nucleus, the spliceosome elements start to scan for exons. The spliceosome is a macromolecular complex composed of nearly 170 proteins and 5 small nuclear RNAs (snRNAs). Spliceosomes define exons by three major features that are indispensable for splicing. First, the 5` GU splice site that is known as donor site (5` end of the intron). Moreover, the conserved adenine rich splice site, known as branch point (near 3` of the intron). Finally, the 3` AG splice site that is known as acceptor site (3` end of the intron) [83, 85]. Upon identification of exons, spliceosome proteins assemble on pre-mRNA in a sequential stepwise manner and finally remove the non-coding sequences. Subsequently, the processed mRNA recruits cellular export factors to shuttle to the cytosol. Alternative splicing commonly occurs via different mechanisms as exon skipping, alternative donor or acceptor sites, or intron retention [86, 87].

1.3.4. Functions of alternative RNA splicing

The overall function of AS is to increase proteome diversity and maximize the net output from a single gene. Experimental analysis of alternatively spliced protein isoforms revealed that AS regulates protein binding, localization, and interaction with membranes or ligands. Therefore, changes in alternative splicing can lead to functional positive or negative impacts [88]. For instance, the loss of DNA binding domain of FOXP2 variant, due to changes in AS, resulted in downregulation of the transcription levels of the full-length protein [89]. Moreover, intron retention of the erythropoietin receptor changed the membrane-bounding properties and resulted in a secreted protein form that was released to the blood and created a state of erythropoietin resistance in end-stage kidney diseases [90]. In addition, changes in the alternatively spliced exons of the migration and invasion inhibitory protein (MIIP) resulted in changes in the C-terminus that lead to protein degradation [91]. Indeed, AS is involved in many other biological functions including changes in channel proteins [92], the enzymatic properties of proteins as in case of cellular kinases [83], and the interaction of proteins with other proteins as shown in insulin receptors where skipping of exon 11 enhanced the interaction of protein to the insulin-like growth factor II (IGF-II) [93].

1.4. Genome engineering technologies and custom nucleases

Targeted modification of gene/genome, epigenetics marks, or transcripts refers to genome engineering [94, 95]. Homologous recombination (HR) is one of the genome editing tools that enabled researchers to generate several knockout and knock-in animal models [96]. However, due to the complexity of eukaryotic genomes that contain billions of DNA molecules, the efficiency of HR-mediated gene editing is very low (1 to 10^{6-9}), which restricts the use of this approach for large-scale applications [96]. Therefore, scientists developed DNA nucleases systems that can disrupt eukaryotic gene functions by inducing DNA double-strand breaks (DSBs). These DSBs are repaired by homology-dependent repair (HDR) or non-homologous end joining (NHEJ), depending on the availability of the exogenous DNA templates [97-103].

Three major custom nucleases have been invented including the microbial genetic elements-derived mega nucleases [104], zinc finger (ZF) nucleases [105, 106], and transcription activator-like effectors (TALES) [107-110]. These techniques recognize targeted DNA sequences via protein-DNA interactions. ZFs and TALEs protein arrays together with FokI nuclease perform a precise nuclease activity. Unfortunately, all these custom nucleases have several technical challenges including specificity, labour intensity, and costs [111-114]. Currently, CRISPR/Cas9 is used as a powerful precise custom nuclease tool for plenty of biomedical applications as treatment of cancers, and some genetic and viral diseases [94, 115-120]. This is owing to CRISPR/Cas9 high efficiency, specificity, and precise RNA-guided nuclease activity in a wide diversity of organisms. Sequences of DNA within any endogenous genome can be simply modulated based on the guiding of Cas9 endonuclease to any specific target site by a string of short RNA [94].

1.5. CRISPR/Cas9

1.5.1. Nomenclature and historical background

CRISPR/Cas9 is a prokaryotic adaptive immune system that protects the host against recurrent rounds of infections. CRISPR are bacterial loci with multiple, short, and direct repeats of DNA sequences. These “spacer DNA” repeats are about 30 bp and act as unique fingerprints of earlier viral infections. These spacers are called CRISPR RNA (crRNA) [121, 122]. Recurrent phage infections of bacterial cells activate the CRISPR/Cas system that recognizes the foreign DNA by the aid of memory short spacer sequences. This recognition or hybridization between crRNA and the invading DNA recruits Cas nucleases to cleave the invading DNA/RNA in a very precise manner. The story of CRISPR/Cas9 started almost two decades ago when such repeats were firstly discovered in prokaryotes [116, 123]. In 2002, these repeats were officially named as CRISPR [117]. Nearly one decade after, the mystery of CRISPR repeats was completely uncovered and CRISPR/Cas9 was engineered for the first time to be applied in eukaryotic cells in 2013 [124]. Since then, enormous applications have been established, starting from the inactivation of oncogenic or harmful mutations in the human genome to the elimination of the HIV genome from infected mice [125-127].

1.5.2. Components of CRISPR/Cas9

The CRISPR/Cas systems are classified into three main types, depending on the components of Cas genes. The most popular and powerful system is type II, termed as CRISPR/Cas9 [128, 129]. Four main components are essential for CRISPR/Cas9 (1) Cas9, a DNA endonuclease bilobed protein; (2) protospacer adjacent motif (PAM), any NGG trinucleotides within the targets; (3) crRNAs, short sequences of RNA that are similar to the target loci; and (4) tracrRNAs, scaffold RNAs that are necessary for the maturation of crRNAs and association with the Cas9 complex (Figure 4) [130].

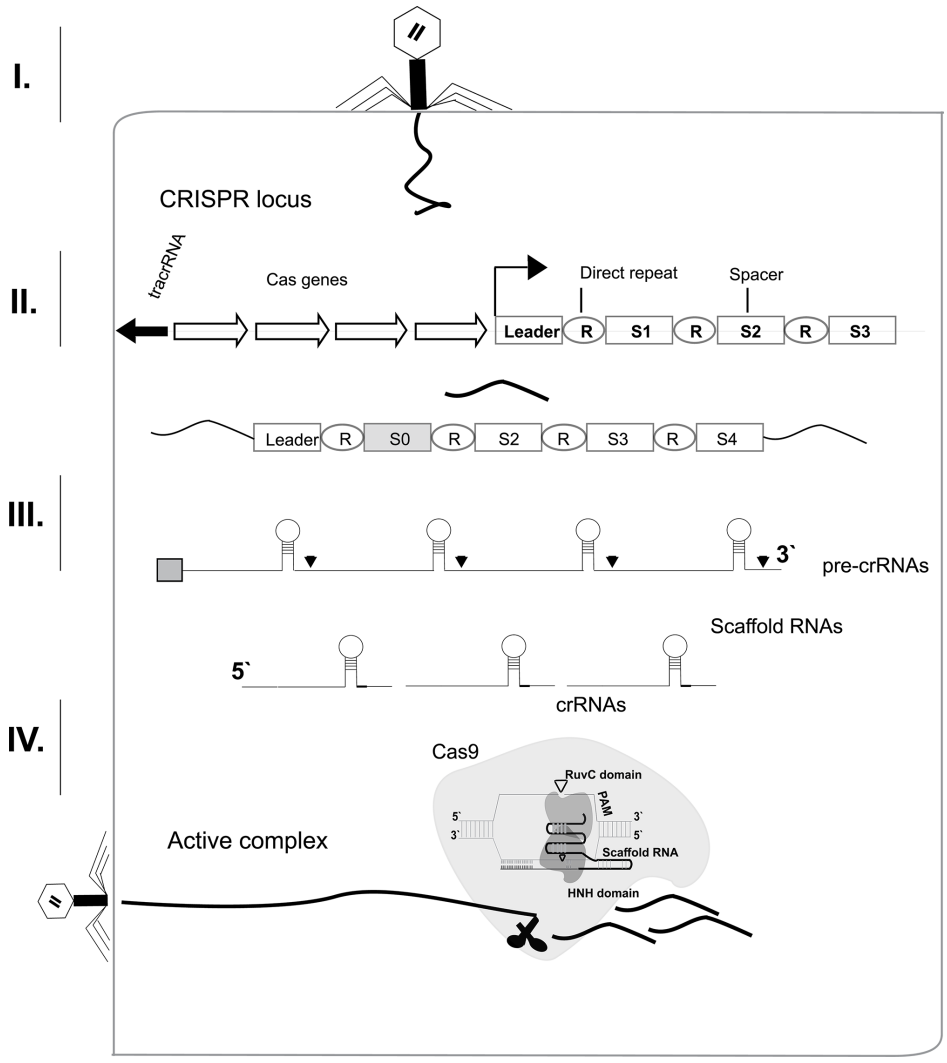


Figure 4: The bacterial adaptive CRISPR/Cas9 immune mechanism. Invasion by bacteriophage **(I)**. Spacer acquisition **(II)**. CRISPR RNAs processing **(III)**. Degradation of the foreign DNA by the CRISPR/Cas9 active complex **(IV)** [94].

1.5.3. Structure and conformation of Cas9

Cas9 protein consists of two nuclease domains, HNH and RuvC. HNH is a single nuclease domain whereas RuvC is a triplet domain that includes RuvC I in the N terminus and RuvC II/III flanking both sites of HNH [131]. Different structural conformations have been found within Cas9 before and after binding to the RNA-DNA heterocomplex. The unbound form has an autoinhibited conformation due to the blockage of the HNH active site by RuvC, rendering it positioned away from the recognition (REC) lobe [132]. This conformation makes the apoCas9 unable to bind or cleave the target DNA. Upon activation of Cas9 with the crRNA/tracrRNA complex, a specific cascade of conformational changes is activated leading to the formation of channels that accommodate the RNA-DNA duplex [132, 133]. In addition, tracrRNAs act as scaffolds in which Cas9 protein can fold and assemble its various domains [133]. The REC lobe of Cas9 protein facilitates binding to the target DNA as it harbours an arginine-rich Bridge Helix (BH) domain that contacts the first 8-12 nucleotides of the RNA-DNA duplex [133]. Therefore, the first 8-12 nucleotides of guide RNA sequences are considered the seed sequences [119, 124, 134-136]. Due to the unique structure of Cas9 protein, a series of Cas9 mutant variants have been optimized for different purposes including the DNA cleavage and binding. The REC2 domain is less conserved in different Cas9 orthologs, which enabled researchers to engineer different Cas9 mutants that lack the REC2 domain and have a reduced overall size of Cas9 without affecting the functional efficiency [133].

1.5.4. Molecular mechanisms of CRISPR/Cas9

CRISPR/Cas9 depends on two crucial steps to cleave the target DNA; the recognition phase that is mediated by the trans-activating guide RNAs complex (tracrRNA: crRNA); and the cleavage phase that is mediated by Cas9. crRNAs are short sequences of RNA that guide Cas9 to the specific-targeted DNA loci upon maturation. TracrRNAs are crucial for the maturation of crRNAs and the association with Cas9 protein. The recognition step occurs in a very specific manner and requires a protospacer matching RNA (crRNA) and the PAM [137]. After recognition of the target sequences, Cas9 undergoes conformational changes that allow the protein to mediate the DSBs [138]. HNH nuclease domain initiates a DNA cut in the single strand that matches the crRNA whereas RuvC domain precisely cleaves the non-complementary strand, three nucleotides upstream to the PAM [119]. Upon DNA cleavage, an immanent cellular DNA repair mechanism begins either via homology-dependent repair (HDR) or error-prone non-homologous end joining (NHEJ) [99, 119, 139, 140]. However, HDR is more complex and inefficient while NHEJ results in unique fingerprints “indels” that are small insertions, substitutions, or deletions in the targeted sequences. The CRISPR/Cas9-induced indels create in-frame mutations that may lead to the formation of a premature stop codon, and subsequently abrogating protein functions [137, 141-143].

1.5.6. CRISPR/Cas9 specificity

CRISPR/Cas9 specificity is important because the Cas9-induced DNA cleavage is permanent and irreversible [133]. Previously, several attempts have been performed to characterize the specificity of TALENs and ZFNs and it was difficult to synthesize large quantities of specific protein libraries [144]. But, the exceptional Watson-Crick base pairing of CRISPR/Cas9 enabled scientists to easily track specificity [133]. For example, a seed sequence model has been established to follow the specificity of Cas9 cleavage and the first 8-12 nucleotides proximal to PAM were the major determinants for CRISPR/Cas9 specificity [119, 124]. However, Cas9 may tolerate mismatches in gRNA sequences in a way that is sensitive to position, number, and distribution. These mismatches will affect only the cleavage but not the binding of Cas9 [134-136]. Hence, Cas9 may have some off-target binding sites, but the cleavage ability is only restricted to a small number of them [145]. Notwithstanding, several *in silico* prediction tools have been developed to predict Cas9 off-targets by using unbiased ways for the scanning of genomic sequences that have high similarity with the desired target locus [133].

1.5.7. CRISPR/Cas9 applications

CRISPR/Cas9 has been extensively applied in genome editing either via silencing, enhancing or modification [121, 122]. This technology has been harnessed in genetic engineering by delivering a plasmid containing the Cas9 gene together with specific gRNA sequences to cells with only one limitation, the availability of PAM sequences (NGG), close to the targets [137]. CRISPR/Cas9 was applied to edit genomes of plants [146], mice [147], zebrafish [148], HIV and herpes viruses as well (Figure 5) [149-151]. Furthermore, the catalytic domains have been inactivated to convert Cas9 into an RNA-driven guiding cassette, known as deactivated Cas9 (dCas9). Cas9 and dCas9 have been exploited into many applications (Figure 6) [133]. For instance, the wild-type Cas9 is being used for gene editing, generation of transgenic animals, genome-scale screening of genes knockout, gene therapy, modelling of diseases, rearrangement of chromosomes, and elimination of viral diseases (Figure 6A) [115, 120, 133, 152-154]. Moreover, dCas9 is used for the repression or activation of certain genes, epigenetic editing, and genome live cell imaging (Figure 6B-D) [133, 155-158].

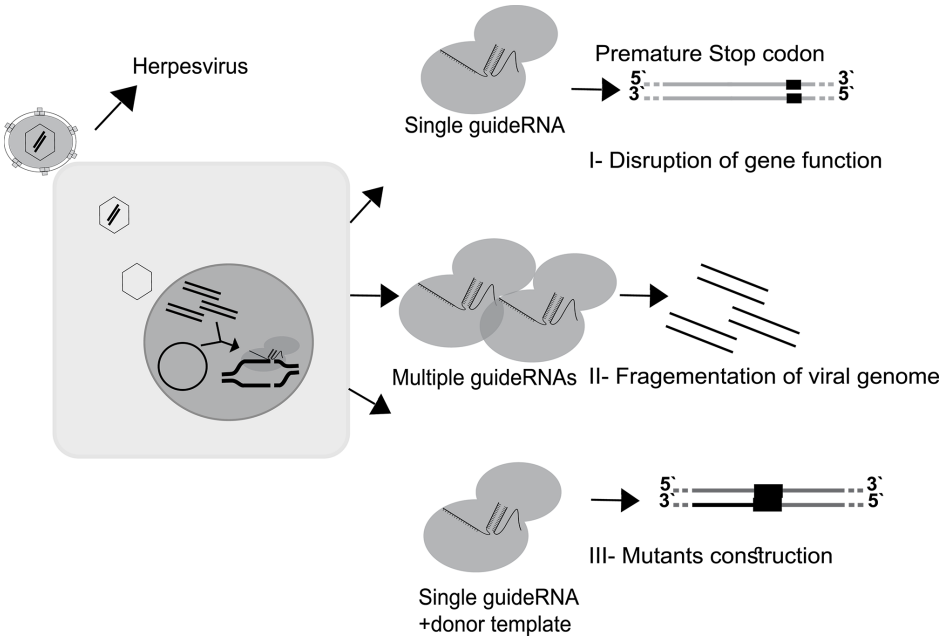


Figure 5: CRISPR/Cas9 applications in herpesviruses. CRISPR/Cas9 is used in herpesviruses research as a tool for the disruption of genes function **(I)**, fragmentation of viral genome **(II)**, and construction of recombinant viruses **(III)** [149, 151].

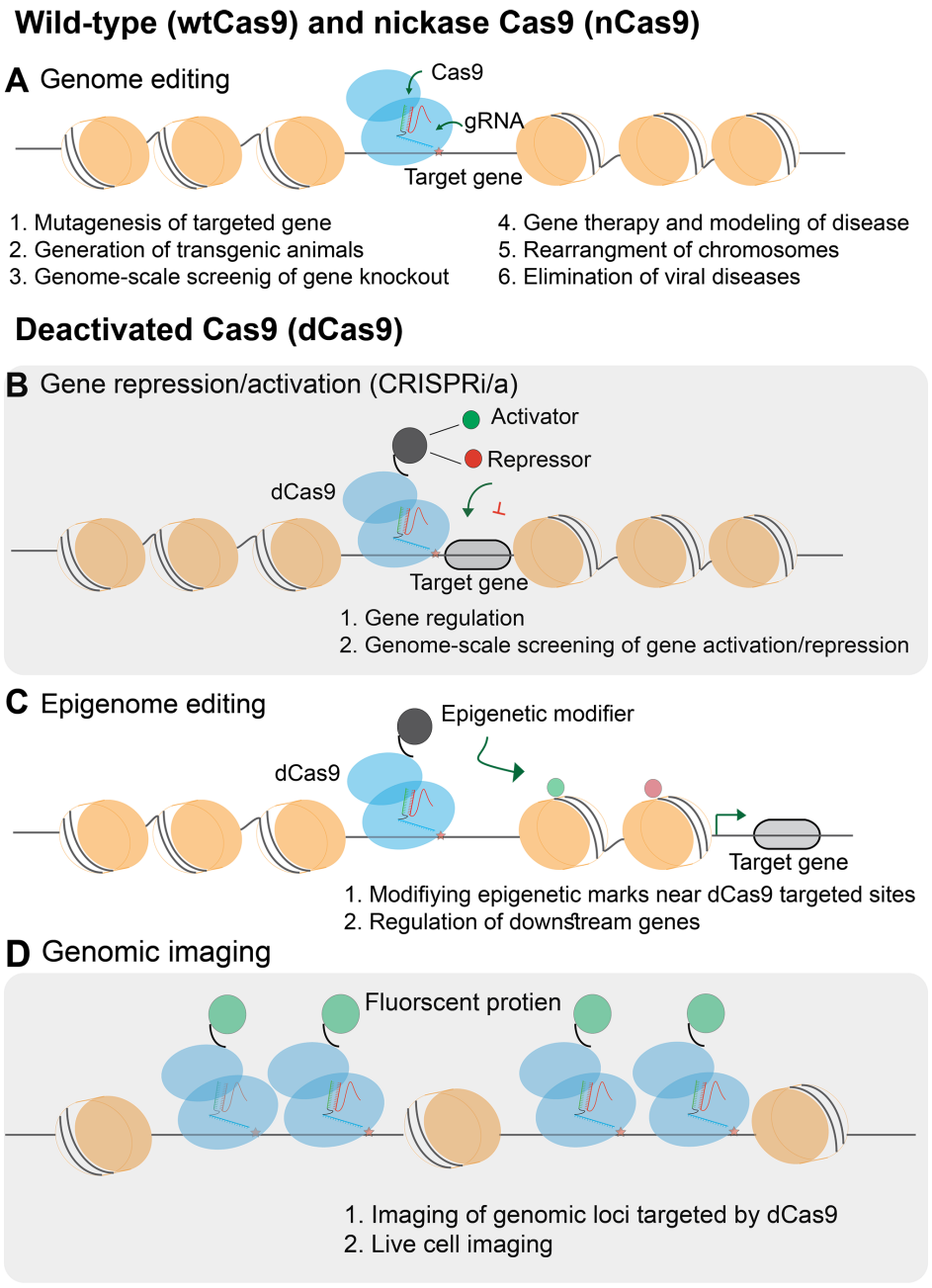


Figure 6: CRISPR/Cas9 applications in molecular biology. (A) Applications of the wtCas9 and nCas9 in genome editing. **(B)**, **(C)**, and **(D)** Applications of the dCas9 in gene repression/activation, epigenome editing, and genomic imaging respectively [159].

1.6. Projects outline

1.6.1. Alternative splicing of vIL-8

The viral interleukin-8 (vIL-8) is essential for the pathogenesis of MDV as it recruits B cells for lytic infections [6]. Multiple vIL-8 splice variants are encoded in vIL-8 gene with unknown function to date (Figure 3) [56]. These splice forms are generated by alternative splicing, a highly regulated process during gene expression that allows a single gene to express multiple proteins [160]. Unfortunately, the existence of these splice variants complicates the clear understanding of the vIL-8 role in the MDV pathogenicity. Interestingly, vIL-8 introns harbour major splice junctions that are essential for the expression of splice variants [56]. In this project, it has been hypothesized that interventions with vIL-8 introns or splice junctions could abrogate the splicing of vIL-8 without affecting virus replication or protein secretion *in vitro*. The main objectives were: (1) to delete vIL-8 introns and evaluate their importance for virus replication and protein secretion; (2) to test if vIL-8 express unknown protein variants and abrogate their expression (if any) by a splice site mutagenesis; (3) to assess the role of vIL-8 splice variants in virus replication and protein secretion. This knowledge would lay the foundation for future studies to dissect the role of vIL-8 splice variants in MDV pathogenesis and tumour formation.

1.6.2. CRISPR/Cas9

Prokaryotic organisms own an ancient, but remarkably effective, immune system called CRISPR/Cas9. Like the adaptive immune system, CRISPR/Cas9 provides an immunological memory for bacteria against subsequent rounds of bacteriophage infections. Once infected, short sequence fragments of the viral genome are kept within the bacteria. At the time of a second infection this sequence fragments are recruited to guide Cas9 to the bacteriophage DNA [161]. Recently, this system has been used to edit the genomes of many cells and has been widely used against multiple human and animal viruses [149, 162-165]. In this project, it has been hypothesized that CRISPR/Cas9 could be a potential tool to control MDV replication. The main goals were: (1) to test if single or multiple targeting of MDV genome by CRISPR/Cas9 can protect cells from virus replication; (2) to test if MDV could evade CRISPR/Cas9 upon serial passage of the infected cultures. This knowledge would provide proof-of-concept and the basis for future studies to generate MDV resistant chickens.

2. Material and methods

2.1. Material

2.1.1. Chemicals

Chemical (Identification code)	Manufacturer
Acrylamide 30% (A124.2)	Carl-Roth, Karlsruhe
Agar bacteriological (2266.2)	Carl-Roth, Karlsruhe
Agarose-Standard Roti® grade (3810.4)	Carl-Roth, Karlsruhe
Ammonium persulfate APS (K38297601)	Merck, Darmstadt
Bromophenol blue (A512)	Carl-Roth, Karlsruhe
Bovine serum albumin [BSA] (A6588.0100)	AppliChem, Darmstadt
Chloroform (411 K3944831)	Merck, Darmstadt
Dimethyl sulfoxide [DMSO] (1.02952.2500)	Merck, Darmstadt
dNTP Mix 10 mM (BIO-39053)	Bioline, Luckenwalde
EDTA (A2937, 1000)	AppliChem, Darmstadt
Ethanol 99%, denatured (A5007)	AppliChem, Darmstadt
Ethidium bromide 1% (2218.2)	Carl-Roth, Karlsruhe
FACS Clean (identification code)	Beckman Coulter GmbH, Germany
FACS Rinse (identification code)	Beckman Coulter GmbH, Germany
Glycerol 99% (A2926, 2500)	AppliChem, Darmstadt
Isopropyl alcohol 2-propanol (A0892)	AppliChem, Darmstadt
L (+) Arabinose (A11921)	Alfa Aesar, Karlsruhe
Lipofectamine® 2000 (11668027)	Life Tech., Carlsbad
Paraformaldehyde (P6148)	Sigma-Aldrich, St. Louis
Sodium dodecyl sulphate [SDS] (75746)	Sigma-Aldrich, St. Louis
ß-mercaptoethanol (28625)	Serva, Heidelberg
TEMED (2367.3)	Carl-Roth, Karlsruhe
Trisaminomethane [Tris] (A1086,5000)	AppliChem, Darmstadt
Triton X-100 (8603)	Merck, Darmstadt
Trypsin 0.05%/PBS (L 2103-20G)	Biochrome, Berlin
Tween-20 (9127.2)	Carl Roth, Karlsruhe
Ultrapure water by DEPC (T143)	AppliChem, Darmstadt

2.1.2. Consumables

Consumable (Features)	Manufacturer
Bacterial Petri dishes (100 mm)	Sartsedt, Nümbrecht
Cell culture dishes (6-well, 100 mm)	Sarstedt, Nümbrecht
Cell culture flasks (25-, 75 ml)	Sarstedt, Nümbrecht
Cryotubes (1.8 ml)	Nunc, Kamstrupvej
Electroporation cuvettes (90 µL)	VWR, Darmstadt
Eppendorf tubes (1.5 and 2 ml)	Sarstedt, Nümbrecht
Parafilm® M	Bems, Neenah
PCR tube (0.2 ml)	VWR, Darmstadt
Pipette tips (1000, 200, 100 and 10)	VWR, Darmstadt
Pipettes (5, 10, 25 ml)	Sartsedt, Nümbrecht
PVDF membrane (0.45) (T830.1)	Carl-Roth, Karlsruhe
Test tubes (conical) (15 ml, 50 ml with and without feet)	Sartsedt, Nümbrecht
Transfection polypropylene tubes (17.1 x 105)	TPP, Trasadingen
Whatman blotting paper (WM 3MM)	GE Healthcare, Freiburg

2.1.3. Equipment

Equipment (Features)	Company/Cat. No.
Bacterial incubator	Binder, Turtlingen/07-26860
Bacterial incubator shaker	New Brunswick Scientific, NewJesey/shaker Innova 44
Bunsen burner	Usbeck, Radevormwald/type 1020
Centrifuges (5424/ 5804R)	Eppendorf, Hamburg
CO2 cell incubators	New Brunswick Scientific, New Jersey/Excella ECO-1
Electrophoresis power supply (Power Source TM 250V)	VWR, Darmstadt
Electroporator (Genepulser Xcell)	Bio-Rad, Munich
CytoFLEX flow cytometer	Beckman Coulter GmbH, Germany

Freezer (-20°C)	Liebherr, Bulle
Freezer (-80°C)	GFL, Burgwedel
Galaxy mini centrifuge	VWR, Darmstadt
Gel electrophoresis chamber (SUB-Cell GT)	Bio-Rad, München
Ice machine	Scotsman, Vernon Hills
Imaging system (Chemismart 51000)	Peqlab, Erlangen
Magnetic stirrer (RH basic KT/C)	IKA, Staufen
Microwave oven (Clatronic700 W)	Severin GmbH, Germany
Mini-gel chambers (Protean 2D)	Bio-Rad, München
Nanodrop 1000	Peqlab, Erlangen
Neubauer counting chambers	Assistent, Sondheim/Rhön
Nitrogen tank	Air liquide, Düsseldorf/ARPEGE70
Orbital shaker	Peqlab, Erlangen/0S-10
pH-meter	Inolab, Weilheim/RHBKT/C WTW pH L1
Pipetboy INTEGRA	IBS Integrated Biosciences, Fernwald
Pipetman (P1000-P200-P100-P10)	VWR, Darmstadt
StepOneTM Real-time PCR system	Applied BiosystemsTM /4376357
Sterile Laminar flow chambers (BSL-2)	Bleymehl, Inden
Thermocycler Flexcycler	ThermoFlex, Analytik Jena, Jena
Thermocycler (Biometra TRIO)	Analytik Jena AG
Thermocycler (T-Gradient)	Biometra, Göttingen
Thermomixer	Eppendorf, Hamburg
UV transilluminator (Bio-Vision-3026)	Peqlab, Erlangen
Vortex Genie 2™	Bender&Hobein AG, Zurich
Water bath shaker (C76)	New Brunswick Scientific, New Jersey
Water baths (TW2 and TW12)	Julabo, Seelbach

2.1.4. Microscopes

Microscope	**Supplier**
Fluorescence microscope Axio-observer. Z1	Carl Zeiss MicroImaging, Jena
Fluorescence microscope Axiovert S100	Carl Zeiss MicroImaging, Jena
Inverted microscope AE20	Motic, Wetzlar

2.1.5. Software and online tools

Name	Supplier or Reference
Axiovision v4.8/Zeiss microscopes	Carl Zeiss MicroImagi, Jena
BLAST	NCBI, Bethesda, USA.
Chemi-Capt	Vilber-Lourmat, Eberhardzell
CHOPCHOP (version 3)	https://chopchop.cbu.uib.no/ [166]
CytExpert (version 2.3)	Beckman Coulter GmbH, Germany
Finch TV (version 1.4.0)	Geospiza, Inc
Graphpad Prism v8	Graphpad Software Inc, La Jolla
Image J (version 1.41)	NIH, Bethesda
NA copy number and dilution calculator	Thermo Fisher Scientific, UK.
ND-1000 (version 3.0.7)	PeqLab, Erlangen
StepOnePlusTM (version 2.3)	Applied Biosystems by Thermo Fisher, UK
Vector NTI (version 9)	Invitrogen, Grand Island
Vision-Capt	Vilber-Lourmat, Eberhardzel

2.1.6. Enzymes and markers

Name (Cat. No.)	Manufacturer
BamHI (R0136)	New England Biolabs, Ipswich
BsmB-I (10043216)	New England Biolabs, Ipswich
Calf intestinal phosphatase CIP (M0290S)	New England Biolabs, Ipswich
DpnI (ER1701)	New England Biolabs, Ipswich
EcoRI (R0101)	New England Biolabs, Ipswich
GeneRuler 1kb Plus DNA Ladder (SM1331)	Thermo Scientific, Darmstadt
HindIII (R0104)	New England Biolabs, Ipswich
PageRuler pre-stained plus marker (26619)	Thermo Scientific, Darmstadt
Proteinase K (7528.2)	Thermo Scientific, Darmstadt
Q5 high fidelity DNA Pol (M0491L)	New England Biolabs, Ipswich
RNase A (7528.2)	Carl-Roth, Karlsruhe
RNase-free DNase (19253)	Qiagen, Hilden
SaL-I HF (0141709)	New England Biolabs, Ipswich
T4 DNA ligase (M02025)	New England Biolabs, Ipswich

T4 quick ligase (10050901)	New England Biolabs, Ipswich
Taq DNA polymerase (01-1020)	PeqLab, Erlangen
XhO-I (R0146S)	New England Biolabs, Ipswich

2.1.7. Antibodies

Antibody	**Dilution (Purpose)**	**Manufacture /Cat. No.**
Rabbit anti-vIL-8	1:1000 (WB)	Cui et al. [167]
Mouse anti-FLAG epitope	1:500 (IF) and 1:1000 (WB)	ABM Biozol/AP9414
Mouse anti-glycoprotein C (clone 1A6), monoclonal	1:100 (WB)	Tischer et al [168]
Alexa goat anti-mouse IgG (H+L) 568	1:5000 (IF)	Invitrogen, Grand Island
Alexa goat anti-mouse IgG (H +L), 488	1:10000 (IF)	
Goat anti-rabbit HRP	1:5000 (WB)	Cell Signalling, Boston
Goat anti-mouse HRP IgM	1:10000 (WB)	

2.1.8. Kits

Kit	**Manufacturer/Cat. No.**
ECL Prime WB Detection Reagents	GE Healthcare, UK/12124052
GF-1 AmbiClean (Gel and PCR)	Vivantis, Malaysia/GF-GC-200
Hi Yield PCR/Gel DNA Extraction	SLG, Gauting/HYDF100-1
Enhanced Avian HS RT-PCR Kit	Sigma Aldrich, St. Louis/HSRT100
MAXIscript® T7 In vitro Transcription	Ambion, USA
Roti-Prep Plasmid Mini	Carl-Roth, Karlsruhe/HP29.2
Qiagen Plasmid Midi	Qiagen, Hilden/12145
RTP® DNA/RNA Virus Mini	Stratec M. GmbH, Berlin/1040100300

2.1.9. Antibiotics

Name	Dilution	(Cat. No.)/Manufacturer
Ampicillin	100 µg/ml	(K029.2)/Carl-Roth, Karlsruhe
Chloramphenicol	30 µg/ml	(3886.3)/Carl-Roth, Karlsruhe
Hygromycin	200 µg/ml	(1287.1)/Carl-Roth, Karlsruhe
Kanamycin sulphate	50 µg/ml	(T832.4)/Carl-Roth, Karlsruhe
Penicillin G	100 IU/ml	(HP48.3)/Carl-Roth, Karlsruhe
Puromycin	1 µg/ml	(0240.1)/Carl-Roth, Karlsruhe
Streptomycin	100 µg/ml	(0236.3)/Carl-Roth, Karlsruhe

2.1.10. Bacteria

Name	Genotype description	Reference or supplier
GS1783	DH10B λcI857 ∆ (cro-bioA) >araC-PBAD, I-SceI	[169]
Stbl3	F- glnV44 recA13 mcrB mrr hsdS20 (rB-,mB-) ara-14 galK2 lacY1 proA2 rpsL20xyl-5 leu mtl-1	Invitrogen, Carlsbad

2.1.11. Cells

Name	Description	Reference
CR	Carina Retina (duck embryo retina-derived cell line)	[170]
293T	Human epithelial kidney cell line, SV-40 T-antigen	ATCC CRL-11268
CECs	Chicken embryo cells/ fibroblasts from SPF chicken eggs (VALO Biomedia, Germnay)	Primary cells [171]

2.1.12. Viruses

Name	Description	Reference
RB-1B	Bacterial artificial chromosome (BAC) clone of the RB-1B GFP reporter vvMDV strain	[76, 172]

2.1.13. BACs

BAC Name	Description	Reference
∆IR_L_RB-1B	Bacterial artificial chromosome (BAC) clone with deleted IR_L in RB-1B vvMDV strain	[173]
∆intron I-vIL-8 (∆I)	∆IR_L_RB-1B with deletion of vIL-8 intron I	This study
∆intron II-vIL-8 (∆II)	∆IR_L_RB-1B with deletion of vIL-8 intron II	This study
∆intron I+II-vIL-8 (∆I+II)	∆IR_L_RB-1B with deletion of vIL-8 introns I+II	This study
FLAG-E3`	∆IR_L_RB-1B with insertion of FLAG tag in E3`of vIL-8	This study
FLAG-E3`mut	FLAG-E3` with a point mutation (G→A) in the acceptor site (A19`) of E3`	This study
E3`mut	∆IR_L_RB-1B with a point mutation (G→A) in the acceptor site (A19`) of E3`	This study

2.1.14. Plasmids

Name	Description	Reference
pEP Kan-S	Addgene Cat. No. 41017	[174]
pSicoR-CRISPR-PuroR	Cas9 gene, N-terminally fused to PuroR via a T2A-ribosome skipping sequence and expressed under the control of the human EF1A promoter	[149, 175]
pLKO5.sgRNA.EFS.PAC	Addgene Cat. No. 57825 but modified by exchanging PuroR cassette with HygroR using the flanking BamHI and MluI sites.	[176]

2.1.15. Buffers and gels

Name	Composition
0.8% Agarose Gel	80 mM Agarose + 1x TAE buffer + 5 µL Ethidium bromide 10 mg/ml
10x SDS-page running buffer	25 mM Tris-HCL+ 190 mM Glycine + 0.1% SDS
1x PBS with Tween 20 (PBST)	PBS + 0.05% Tween 20
1x Phosphate buffered saline (PBS)	2 mM KH2PO4 + 10 mM Na2HPO4 + 137 mM NaCl + 2.7 mM KCl pH 7.4
1x Tris acetate EDTA (TAE)	40 mM Tris + 1 mM EDTA + 20 mM Acetic acid (pH 8.0)
6x DNA loading buffer	0.2% Bromophenol blue + 60% Glycerol + 60 mM EDTA
6x SDS sample loading buffer (Lämmli buffer)	0.35 mM Tris-HCl (pH 6.8) + 10% SDS + 30% glycerol + 0.1% β-mercaptoethanol + 0.6% bromophenol blue

RIPA I buffer	50 mM Tris-HCl (pH 7.4) +150 mM NaCl + 0.1% (w/v) SDS + 0.01% Triton X100 + 6 mM sodium deoxycholate + 1x mini-EDTA-free protease inhibitor tablet
RIPA II buffer	RIPAI + 0.5 M EDTA
Western blot transfer buffer	100 ml 5x running buffer + 100 ml Methanol + 100 ml water
Western blot washing buffer	PBS + 0.05% Tween 20

2.1.16. Bacterial media and supplements

Media	**Composition**
LB agar	10 g/L tryptone + 5 g/L yeast extract + 10 g/L NaCl + 15 g/L Bacto™ agar
LB medium	10 g/L tryptone + 5 g/L yeast extract + 10 g/L NaCl
SOC medium	20 g/L tryptone + 5 g/L yeast extract + 0.584 g/L NaCl + 0.186 g/L KCl + 2 g/L MgCL2 *6H2o + 2.5 g/L MgSO4 *7H2o + 3.6 g/L glucose

2.1.17. Plasmid-preparation buffers

Buffer name	**Composition**
Buffer (P1)	50 mM Tris-HCL + 10 mM EDTA + 100 µg/ml RNase A (pH 8.0)
Lysis buffer (P2)	200 mM NaOH + 1% (w/v) SDS
Genomic DNA lysis buffer	10M Tris-HCl (pH 8.0) + 0.1 M EDTA (pH 8.0) + 0.5% SDS + 20 ug/ml RNase A
Neutralization buffer (P3)	3 M K-acetate (pH 5.0)

2.1.18. Media and supplements for the cultivation of cells

Name	Company/Cat. No.
Dulbeco's MEM/Ham's F-12 (1:1)	Biochrom, Berlin/FG 4815
RPMI 1640	PAN biotech, Berlin/P04-18500
Minimal Essential Media (MEM) Eagle	PAN biotech, Berlin/P04-09500
Foetal bovine serum (FBS)	Biochrom, Berlin/S 0415
OptiMEM	ThermoFisher, Darmstadt/31985070
Trypsin 0.05%	Biochrom, Berlin/L 2103-20G

2.1.19. Primers

Primers were designed using the Vector NTI AdvanceTM 9.1 software package (Life Technologies, CA, USA) and were purchased from the Integrated DNA Technologies (IDT, Coralville, USA). Sequencing reactions were performed by (LGC Genomics GmbH, Berlin, Germany).

Primers used for the construction of vIL-8 mutants

Construct	Sequences (5` → 3`)	Direction
∆intron I-vIL-8 (∆I)	GTTCTATTCATAGTACAGATCTATTTGTTGCCTGGAAATGG CATATCACTGGAGAGTCTCTAGGGATAACAGGGTAATCGA TTT	forward
	TTGCACCTCTTGTCGACAGCGAGACTCTCCAGTGATATGC CATTTCCAGGCAACAAATAGGCCAGTGTTACAACCAATTAA CC	reverse
∆intron II-vIL-8 (∆II)	TGATACCACCGGGTATACACTGCAGGAGGACTGAAATTAT CTTTGCTCTCAAGAAGAACATAGGGATAACAGGGTAATCG ATTT	forward
	AGGGTCCACACATACCTTCCTGTTCTTCTTGAGAGCAAAG ATAATTTCAGTCCTCCTGCAGCCAGTGTTACAACCAATTAA CC	reverse
FLAG-E3`	ACATACCTTCCTGTTCTTCTTGAGAGCAAAGCTACAAAAGC TTATCGTCGTCATCCTTGTAATCGCTGCCGCCAGTGTTACA ACCAATTAACC	forward
	GTAGTGTCTGGCTGTAAAGCTAATTTGGTTAAGGTTTTCCG GCAGCGATTACAAGGATGACGACGATAAGTAGGGATAACA GGGTAATCGATTT	reverse
FLAG-E3` mut	CTTCCTGTTCTTCTTGAGAGCAAAGCTACAAAAGGGAAAA CTTTAACCAAATTAGCTTTACAGCCAGTAGGGATAACAGG GTAATCGATTT	forward
	CTTAGGTGTAGTGTCTGGCTGTAAAGCTAATTTGGTTAAAG TTTTCCGCCAGTGTTACAACCAATTAACC	reverse
E3`mut	GCTACAAAAGCTTATCGTCGTCATCCTTGTAATCGGAAAAC TTTAACCAAATTAGCTTTACAGCCAGTAGGGATAACAGGGT AATCGATTT	forward
	CTTAGGTGTAGTGTCTGGCTGTAAAGCTAATTTGGTTAAAG TTTTCCGCCAGTGTTACAACCAATTAACC	reverse
vIL-8 sequencing [6, 7]	CCGTATCCCTGCTCCATCCAATAGC	forward
	GGTCTCCAATATCACGTGTTGGTGG	reverse

Primers used for the construction of single and multiple guide RNAs

Gene/s	Construct/s	Direction	Sequences (5` → 3`)
	1	For	CACCGTTAGGATTACTGATGGCCA
UL6		Rev	AAACTGGCCATCATATATCCTAAC
	2	For	CACCGTAATTCGGGAAGGCAAGCG
		Rev	AAACCGCGTTGCCTTCCCGAATTAC
UL19	3	For	CACCGCACTTCAGATATAATGCGA
		Rev	AAACTCGCATTATTATCTGAAGTGC
UL27	5	For	CACCGGGTTCGGACATTTTCGCGG
		Rev	AAACCCGCGAAAATGTCCCGAACC
	6	For	CACCGTATGGTAGATACATTGCAC
		Rev	AAACGTGCAATCGTATCTACATAC
UL30	7	For	CACCGAATGGCTTATCATTTTCCAC
		Rev	AAACGTGGAAAATGATAGCCATTC
	8	For	CACCGATGTTCACAACGATAGAAG
		Rev	AAACCTTCGTATCGTTGTGAACATC
UL49	9	For	CACCGGACGTTTCGTCTACCACCCG
		Rev	AAACCGGGTGGTAGACGAAAGTCC
	10	For	CACCGTCTGAACGTACAAGAGCGG
		Rev	AAACCCGCGTCTTGTACGTTCAGAC
ICP4	11	For	CACCGGAGGCAATTGGCAGTACGG
		Rev	AAACCCGTATCTGCCAATTGCCTCC
	12	For	CACCGGTTGTTGTTCACATTCCCGA
		Rev	AAACTCGGAATGTGAACAACAACC
	(5+6) (8+11)	Rev	CCCGTTGCGAAAAAGAACG
UL27, UL30,	(4x)		
UL49+ICP4	(5+6) (8+11)	For	CTGTCGACTTTCCCATGATCCTTCATATTTG
	4x	For	TGTCGACGGCAAGTTTGTGGAATGGTTTAAC

2.2. Methods

2.2.1. Construction of mutagenesis primers for virus mutants

Primers were designed based on the sequences of ΔIR$_L$_RB-1B vvMDV strain [6]. Primers included homologous sequences upstream and downstream to the target site and a sequence duplication that allows removal of the positive selection marker (PSM). Primer arms allowed the modification of the target site either by deletion, insertion, or point mutation as previously described (section 2.1.19.) [177]. Primers were used to construct six recombinant viruses as following: (1) Δintron I-vIL-8 (ΔI), (2) Δintron II-vIL-8 (ΔII), (3) Δintron I+II-vIL-8 (ΔI+II), (4) FLAG-E3` with an inserted FLAG tag; 89 bp downstream to 5` of vIL-8 intron II, (5) FLAG-E3`mut and (6) E3`mut in which a point mutation (G→A) was introduced 82 bp downstream to 5` of intron II (Figures 7 and 9).

2.2.2. Preparation of recombination and electro-competent E. coli

The E. coli strain GS1783 containing the infectious MDV RB-1B BAC (clone 1232) was set to be recombination and electro-competent. Briefly, 5 ml LB media with 30 µg chloramphenicol were inoculated with the respective BAC clone and grown overnight at 32°^{C}. Next, 1 ml was inoculated into another 50 ml of fresh LB media and furtherly incubated for 3h/32°^{C} to have an optical density 600 (OD600) of 0.5-0.7, which ensures that the bacteria were in the logarithmic growth phase. Subsequently, cells were heat-shocked at 42°^{C}/15 min to express the Red-recombination system and then were immediately cooled on an ice-cold water bath for 20 min with continuous shaking [174]. Cells were then washed two times by sterile ice-cold 15% glycerol to remove residues of LB medium and excess salts. Finally, bacteria were resuspended in 500 µl 15% ice-cold glycerol, aliquoted, and frozen in -80°^{C} till further use.

2.2.3. Two-step Red-mediated mutagenesis

The pEPkanSI vector was used as a template to amplify the PSM aphAI-I-SceI using a set of specific primers (section 2.1.19.) and a two-step PCR protocol (Table 1) [174, 177, 178]. Next, PCR products were gel purified (Vivantis technologies, Malaysia) and 100 ng were electroporated into the recombination and electro-competent E. coli. Afterward, bacteria were recovered using 1 ml of LB media without antibiotics and grown for 2h/32°^{C} and plated into LB plates with 30µg/ml chloramphenicol and 50µg/ml kanamycin for the selection of recombinant clones.

Digested BACs were then separated on 0.8% agarose gel O/N at 60 V., stained with ethidium bromide, and visualized with the UV transilluminator (PeqLab, Erlangen). L-arabinose 1% was used to induce the expression of I-Sce-I to remove the PSM from the intermediate clones. After, 100 µl of an overnight culture from the intermediates was added to 2 ml of LB without antibiotics and furtherly grown for 3h/32°C. Another 2 ml of LB media with 1% L-arabinose were added to the culture and incubated for 1h/32°C. Subsequently, the culture was incubated at 42°C for 30 min to induce the expression of Red recombination. Bacteria were recovered by incubation at 32°C/4h and plated at 10^{-5} dilution on agar plates having 30µg/ml chloramphenicol and 1% arabinose and incubated for 48h/32°C. At least 10 clones were double plated on Cam and Cam/Knm agar plates. Successful modification of the target sites was confirmed by Sanger sequencing (LGC Genomics GmbH, Berlin, Germany). The integrity of the viral genome was confirmed by next-generation sequencing (NGS). Positive final clones were stored as glycerol stocks in -80 until further use.

Table 1. Two-step TaqMan-based mutagenesis PCR.

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	94	5 min	
First step			
Denaturation	94	20 s	
Annealing	50	20 s	10
Extension	68	45 s	
Second step			
Denaturation	94	20 s	
Annealing	68	1 min 5 s	25
Extension	72	45 s	
Final extension	72	7 min	

2.2.4. Isolation of BAC DNA (Mini-preps)

The standard alkaline lysis protocol was followed to isolate a small-scale amount of BAC DNA. Briefly, bacterial pellets were resuspended in 300 µl P1 buffer and 300 µl of P2 buffer were added, gently mixed, and incubated for 5 min at room temperature. To neutralize the reaction and precipitate the protein, 300 µl of P3 buffer were furtherly added to the mixture, gently mixed, and incubated on ice for 10 min. To eliminate cellular debris, residues of proteins, and bacterial genomic DNA, 900 µl of phenol: chloroform mixture were added to the solution and mixed by inversion for at least 50 times. Next, the mixture was centrifugated at 10,000 rpm/10 min. The aqueous phase was mixed with 550 µl of ice-cold isopropanol and mixed 10 times by inversion. DNA pellets were precipitated by centrifugation at 15000 rpm/30 min/4°C. Bacterial DNA pellets were then washed two times with 800 µl 70% ethanol, incubated at 37°C/5 min, dissolved in 45 µl TE-RNAse, and incubated 37°C/20 min.

2.2.5. Isolation of BAC DNA (Midi-preps)

The standard protocol from QIAGEN Plasmid Midi Kit was followed to isolate a medium-scale amount of BAC DNA. In brief, bacterial pellets were resuspended in 4 ml of P1 buffer by vigorous vortexing. Another 4 ml of P2 buffer was added and incubated for 5 min at room temperature after 5-6 times of shaking. Also, 4 ml of P3 buffer were added and the mixture was incubated on ice for 20 min and centrifuged at the maximum speed at 4°C/20 min. Later, supernatants were transferred to QIAGEN-tip columns, which were then washed two times with 10 ml washing buffer. DNA was eluted in 5 ml of prewarmed elution buffer, precipitated with 3.5 ml isopropanol, and centrifuged at 15,000 xg/30 min/4°C. Pellets were washed with 70% ethanol, centrifugated at 15,000 xg/10 min/4°C, and finally dissolved in 40 µl TE-RNase after evaporating ethanol residues. Of note, DNA quality and quantity were assessed by Nanodrop 1000 (PeqLab, Erlangen).

2.2.6. Preparation of primary chicken embryo cells (CECs)

Chicken embryo cells (CECs) were prepared from 11-days old specific-pathogen-free fertile eggs (VALO Biomedia, Germany) as previously described [179]. Head, extremities, and internal organs of embryos were removed, and remnant tissues were washed in sterile PBS and furtherly dissected into tiny pieces. Next, soft tissues were mechanically dissociated into PBS using a magnetic stirrer for 10 min. To release the single embryonic cells, PBS was discarded, and tissues were digested using 100 ml PBS 0.05% Trypsin for 3 times, each for 10 min. Sterile gauze membranes were used to filter cell suspensions and cells were collected into complete MEM and then harvested by centrifugation at 300 xg for 10 min at room temperature. After, cell pellets were resuspended into 150 ml MEM with 10% heat-inactivated FBS and cellular aggregates were settled down for 20 min at room temperature. Finally, cells were plated into tissue culture flasks.

2.2.7. Reconstitution of recombinant MDV BACs

BACs were dissolved into 10 mM Tris of pH 7.5 and molecular-grade water to a volume of 438 µl and incubated for 30 min at room temperature. Further, 62 µl from 2 M CaCl2 was added to the mixture in a drop-wise manner while gently shaking and then incubated overnight/4°C. Next, 500 µl from the 2x HBS was added to each transfection mixture and incubated for 15 min at room temperature. After, 500 µl from the transfection mixture was delivered to cells in a drop-wise manner and incubated for 3h/37°C. Cells were gently washed with PBS and incubated with 1 ml of warm 1x HBS 15% glycerol for 2.5 min and then gently washed with PBS 2-3 times. MEM complete were added to cells that were furtherly incubated for 6 days/37°C and daily observed for plaques formation [179, 180]. Reconstituted BACs were propagated into fresh CECs, stored in liquid nitrogen, and titrated before use as previously published [181].

2.2.8. Propagation of viruses

To propagate the recombinant viruses, infected cells were maintained for 3-4 passages into fresh CECs. In brief, infected and uninfected cells were co-seeded together at appropriate density into 100 mm dishes and grown to 95% confluence using MEM with reduced serum 1% for up to 6 days. Highly infected cells were detached using 0.05 % trypsin for 2 min at 37°C, resuspended into MEM complete to inactivate trypsin, aliquoted into freezing medium having 50% serum and 10% DMSO, and stored in liquid nitrogen till further use. For titration of viral stocks, 10^{-3} and 10^{-4} dilutions from the infected cells were co-seeded with uninfected cells into 6-well plates into triplicates and plaques were counted at 6 dpi. Viral titers were calculated as pfu as previously described [181].

2.2.9. Plaque size and multi-step growth kinetics assays

CECs were infected with 100 pfu from each recombinant virus together with the respective controls. Five days post-infection (5 dpi), plaque areas of 50 randomly selected plaques were calculated using the ImageJ software (NIH; https://imagej.nih.gov/ij/) as previously described [182]. Plaque diameters were calculated and compared to the controls to determine the replication and spread properties of virus mutants. A minimum of three independent experiments were performed for each recombinant virus. Replication properties were furtherly assessed by multi-step growth kinetics as previously shown [183]. After an infection of 100 pfu, $1x10^6$ of CECs were harvested at 0, 1, 2, 3, 4, and 5 dpi and viral DNA were extracted from cells at each time point. MDV genome copy numbers from at least three independent experiments were evaluated by qPCR as previously described [184].

To test if the CRISPR/Cas9 system can protect cells from MDV replication, 100 pfu from the RB-1B GFP reporter virus were inoculated into CRISPR/Cas9 cells. Viral replication properties were monitored by plaque numbers and plaque sizes that were calculated as above described. In addition, 10,000 pfu were inoculated into CRISPR/Cas9 cells that were serially passaged for six passages in a ratio of 1:15. For each passage, MDV genome copy numbers from three independent experiments were evaluated by qPCR as previously described [184].

2.2.10. Extraction of viral DNA from MDV-infected cells

Viral DNA was extracted from infected cells at 5 dpi using the RTP DNA/RNA Virus Mini Kit (Stratec Molecular, Germany). Briefly, cells were trypsinized and resuspended into MEM complete and subjected to thawing and freezing before centrifugated at 300 xg for 5 min at room temperature. Further, 200 µl of cell pellets were then mixed with 200 µl ddH2O and transferred to the Kit-provided extraction tubes, which were vigorously vortexed and incubated at 65°C/15 min and 95°C/10 min respectively under continuous shaking. After, 400 µl from the binding solution were mixed to lysates and transferred to the Kit-provided spin filters, which were centrifugated at 11,000 rpm/2 min. Spin filters were washed two times using 500 µl wash buffer R1 and 700 µl wash buffer R2 and residuals of ethanol were removed by centrifugation at maximum speed for 4 min. Viral DNA was eluted into 35 µl of prewarmed ddH2O.

2.2.11. Western blot (WB)

To examine vIL-8 secretion in the soup of cultures infected with 500 or 10,000 pfu from the recombinant viruses, 30 µl of soup were collected, mixed with 5 µl Lämmli buffer, denatured at 95°C/5 min, and immediately cooled. Samples were separated by 12% SDS-PAGE for 20 min at 75 V. and 40 min at 150 V. and then transferred to a polyvinylidene difluoride (PVDF) membrane (Carl-Roth, Karlsruhe, Germany) using the Biometra semi-dry blotting system for 1h/7V. PVDF membranes were probed by the rabbit polyclonal anti-vIL-8 antibody or the mouse monoclonal anti-gC antibody in PBS-T 5% skim milk and incubated at 4°C overnight as previously described [72]. Next, membranes were washed 3 times using PBS with 0.1% Tween-20 (PBST) before further staining with the secondary antibody. The goat anti-rabbit HRP-conjugated and anti-mouse-IgM antibodies were used as secondary antibodies for vIL-8 and gC respectively (section 2.1.7.) (Figures 8 and 12). Finally, membranes were visualized using the enhanced chemiluminescence (ECL) plus western blot detection reagents and protein signals were visualized by the Chemi-Smart 5100 detection system (Peqlab, Erlangen). To investigate the expression of the intracellular viral proteins, infected and uninfected CECs were harvested and lysed using the radioimmunoprecipitation assay buffer (RIPA) and separated by SDS-PAGE as previously mentioned. Using the mouse monoclonal α-D tag antibody (ABM, Canada) as a primary antibody and the goat anti-mouse HRP IgM secondary antibody (Cell Signalling, USA), intracellular proteins were recorded.

2.2.12. Fluorescence microscopy

Fluorescence microscopy was used to examine the ability of FLAG-E3` and FLAG-E3`mut viruses to express spliced proteins. Briefly, cells were infected with 100 pfu and at 5 dpi were fixed with PFA 4% and then air-dried for 20 min. Non-specific cellular antigens were blocked with 3% BSA in PBS for 30 min. Subsequently, cells were stained with the mouse anti-FLAG epitope antibody (ABM, Canada) of dilution 1:500 into 1% PBS-BSA and incubated for 45 min at room temperature. After 2 times of washing with PBS, cells were probed with Alexa goat anti-mouse IgG (H+L) 568 antibody of dilution 1:5000 in 1% PBS-BSA and incubated at room temperature for 45 min. After 3 times of PBS washing, cells were furtherly stained with DAPI stain (5 mg/ml) of dilution 1:1000 in PBS and stained cells were examined under the fluorescence microscope.

In addition, the immunofluorescence assessment was furtherly used to examine the expression of Cas9 into CR cells. Cas9-transduced and wild-type cells were stained using the same protocol except for using the Alexa goat anti-mouse IgG (H+L) 488 antibody of dilution 1:10000 for the secondary probing. Stained cells were examined under the fluorescence microscope and images were processed using the ImageJ software (NIH; https://imagej.nih.gov/ij/).

2.2.13. Construction of CRISPR/Cas9 guide RNAs

To construct guide RNAs that target essential MDV genes, the online algorithm tool (http://chopchop.cbu.uib.no/) was used [185, 186]. Guide RNAs with the best scores and no off-targets were selected. Two independent gRNAs were designed for the following essential viral genes: minor capsid protein (UL6), major capsid protein (UL19), glycoprotein B (UL27), polymerase subunit (UL30), tegument protein (UL49), and the infected cell protein 4 (ICP4) (Table 2). Forward and reverse oligos that harbour unique BsmB-I cutting sites were annealed together using 1x duplex buffer and gradually cooled from 95°^{C} to 30°^{C} by 0.6$^{\circ C}$/min by a gradient PCR. Using T4 DNA ligase, the phosphorylated oligos were ligated to dephosphorylated, BsmBI-digested, and linearized pLKO5.sgRNA.EFS.PAC vectors. *Stbl3* E. coli strain was chemically transformed, and several clones were mini-prepped and subjected to Sanger sequencing (LGC Genomics, Berlin, Germany) using the primer 5`-ATTTCTTGGGTAGTTTGCAG-3`. Analysis of sequences was performed using the Vector NTI AdvanceTM 9.1 software package and positive clones were stored as glycerol stocks in -80$^{\circ C}$ upon further use.

Table 2. gRNA target sequences.

Construct	gRNA target sequence	MDV target gene
1	TTAGGATATACTGATGGCCA	Minor capsid protein (UL6)
2	TAATTCGGGAAGGCAACGCG	
3	CACTTCAGATAATAATGCGA	Major capsid protein (UL19)
4	GGTTCGGGACATTTTCGCGG	Glycoprotein B (UL27)
5	TATGGTAGATACGATTGCAC	
6	AATGGCTTATCATTTCCAC	DNA polymerase (UL30)
7	ATGTTCACAACGATACGAAG	
8	GACGTTTCGTCTACCACCCG	Tegument protein (UL49)
9	TCTGAACGTACAAGACGCGG	
10	GAGGCAATTGGCAGATACGG	Infected cell protein (ICP4)
11	GTTGTTGTTCACATTCCCGA	
gRNA control	GGAGTAGTGTTTGACGGCCA	HHV6 tegument protein (UL25)

2.2.14. Multiplexing of gRNAs

For multiplexing of the vectors (5+6) and (8+11), a set of primers (section 2.1.19.) was used to amplify single gRNA cassettes (~750 bp) by Taq polymerase according to manufacturer's protocol. Amplified bands were purified (Vivantis technologies, Malaysia), SaL-I and XhO-I-digested (New England Biolabs, Ipswich, UK), and ligated to dephosphorylated linearized XhO-I-digested vectors. Ligated vectors were transformed into chemically competent *Stbl3* E. coli cells. After sequences confirmation, glycerol stocks were made and stored in -80°C till further use. To construct the 4x vector, the Q5 high-fidelity DNA polymerase was used to amplify the 2x cassette (5+6) (~1300 bp) that was fused to the (8+11) vector using SaL-I and XhO-I.

2.2.15. Maintenance of cells and virus propagation

In this study, the primary chicken embryo cells (CECs), duck embryo retina-derived cell line known as Carina Retina (CR), and 293T human embryonic kidney cells were used [170]. CECs were prepared from 11-day-old (Valo Biomedia, Germany) specific-pathogen-free (SPF) embryos [181]. MEM Eagle (PAN biotech, Germany), Dulbecco's MEM/Ham's F-12 (1:1, Biochrom, Germany), and RPMI 1640 (PAN biotech, Germany) media were used for CEC, CR, and 293T cells respectively. All media were supplemented with glutamine and NaHCO3 and furtherly complemented with 10% heat-inactivated FBS and 1% 100 U/ml penicillin and 100µg/ml streptomycin (AppliChem, Germany). Cells were maintained by incubation at 37$^{\circ C}$ and 5% CO2. The very virulent recombinant viral strain RB-1B GFP reporter was propagated in fresh CECs. Virus stocks (passage 7) were frozen at liquid nitrogen, titrated in CR cells before using for all CRISPR/Cas9 infections [76, 172, 181].

2.2.16. Establishment of CRISPR/Cas9 cell lines

To establish CRISPR/Cas9 cell lines, the pSicoR-CRISPR-PuroR lentiviral transfer vector and two third-generation lentiviral packaging plasmids were used to transfect 293T cells following the standard lentiviral production [187]. Briefly, the packaged lentiviruses were harvested at 48 hpi and later used to transduce CR cells via spin infection at 1200 xg for 2 hours at room temperature. Two days post-infection, cells were subjected to puromycin selection (1 µg/ml, Carl-Roth, Germany) for 3-4 days. Next, cells were transfected with single or multiplexed vectors using lipofectamine 2000 (Invitrogen, MA, USA) according to the manufacturer's protocol. After, hygromycin (200 µg/ml, Carl-Roth, Germany) was used to select the CRISPR/Cas9 cells for 6 days. CRISPR/Cas9 cells were then expanded and frozen in liquid nitrogen containers upon further use.

2.2.17. Quantitative PCR (qPCR)

MDV viral DNA was extracted from infected cells at 5 dpi using the RTP® DNA/RNA Virus Mini Kit (Stratec Molecular, Germany) according to the manufacturer's instructions and as described in section 2.2.10. Next, MDV genomic replication was measured using a set of specific primers and a probe that targets ICP4. The inducible nitric oxide synthase (iNOS) was used for the normalization of MDV ICP4 copy numbers as previously published [184, 188].

2.2.18. Flow cytometry

The RB-1B GFP reporter virus was used to infect CRISPR/Cas9 cells with 10,000 pfu. At 5 dpi, the infected living cells were subjected to analysis by flow cytometry (CytoFLEX S; Beckman Coulter, CA, USA) to analyse the percent of expression of the GFP reporter in infected and control culture. At least 10000 events were counted for each independent experiment and collected data were analysed by the CytExpert software (version 2.3; Beckman Coulter, CA, USA).

2.2.19. CRISPR/Cas9 escape mutants

CRISPR/Cas9 cells were infected with 10,000 pfu and serially passaged at a ratio of 1:2 every three days up to 33 dpi to ensure that no virus escape mutants can emerge and bypass the multiplexed gRNAs. Viral DNA was extracted, and genomic gRNA targets were amplified and subjected to Sanger sequencing (LGC Genomics, Berlin, Germany). Sequences were analysed using the Vector NTI AdvanceTM 9.1 software package.

2.2.20. Statistical analysis

Data were statistically analysed using GraphPad Prism (version 8; GraphPad Software, Inc., CA, USA). Initially, data sets were checked for normal distribution, and then analysed using the one-way analysis of variance (ANOVA) and Bonferroni correction. P values <0.05 were considered as significant.

3. Results

3.1. Introns of vIL-8 are dispensable for virus replication

The replication and spread properties of the ΔI, ΔII, and ΔI+II recombinant viruses were measured by plaque size and multi-step growth kinetics assays (Figure 8). Virus mutants showed no significant differences in plaque diameters when compared to the wild type as shown by plaque size assays (Figure 8A). Moreover, no significant differences in MDV genome copy numbers were seen between the wild type and the recombinant viruses as shown by multi-step growth kinetics assays (Figure 8B). Together, these data show that vIL-8 introns have no role in virus replication or spread properties *in vitro*.

3.2. Introns of vIL-8 are indispensable for efficient protein secretion

Next, the outcomes of the deletion of vIL-8 introns on the secretion of vIL-8 were tested. Cells were infected with 500 pfu from the ΔI, ΔII, and ΔI+II recombinant viruses. At 5 dpi, the supernatants of infected cells were subjected to SDS-PAGE. Interestingly, vIL-8 was efficiently secreted in cells infected with the ΔI virus mutant and the protein secretion levels were not significantly changed when compared to the wild type (Figure 8C-D). Interestingly, secretion levels were significantly lowered in cells infected with the ΔII mutant (Figure 8C-D). Strikingly, secretory levels were extremely reduced up to ~50% in cells infected with the ΔI+II mutant (Figure 8C-D). Together, these data suggest a potential role of intron II in the secretion of vIL-8 and confirm that the deletion of the two introns impairs the efficient secretion of vIL-8. Of note, next-generation sequencing of virus mutants ensured the integrity of the viral genome and confirmed that the observed phenotype was due to the solely introduced mutations.

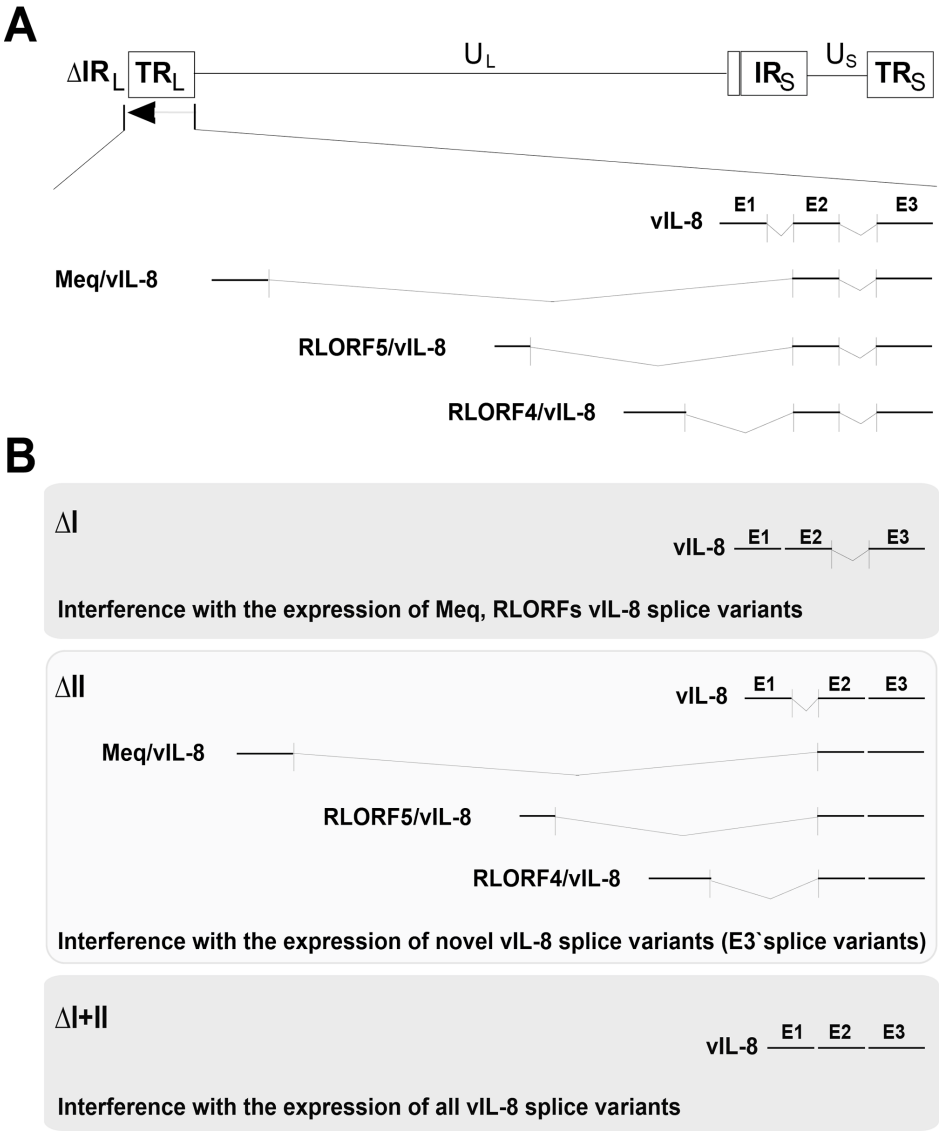


Figure 7: Overview of Δintrons vIL-8 virus mutants. (A) Diagram of the genome of the ΔIR_L pRB-1B wild-type recombinant virus encoding vIL-8 and splice variants as described before [56]. **(B)** Schematic representation of vIL-8 virus mutants that were constructed in this study by Red recombination. ΔI stands for a mutant with deletion of vIL-8 intron I that harbours an essential acceptor site (A18) for the expression of Meq and RLORFs splice variants. ΔII stands for a mutant with deletion of intron II that harbours an essential acceptor site (A19`) for the expression of novel vIL-8 splice variants (E3`splice variants). ΔI+II is a mutant with deletion of both vIL-8 introns.

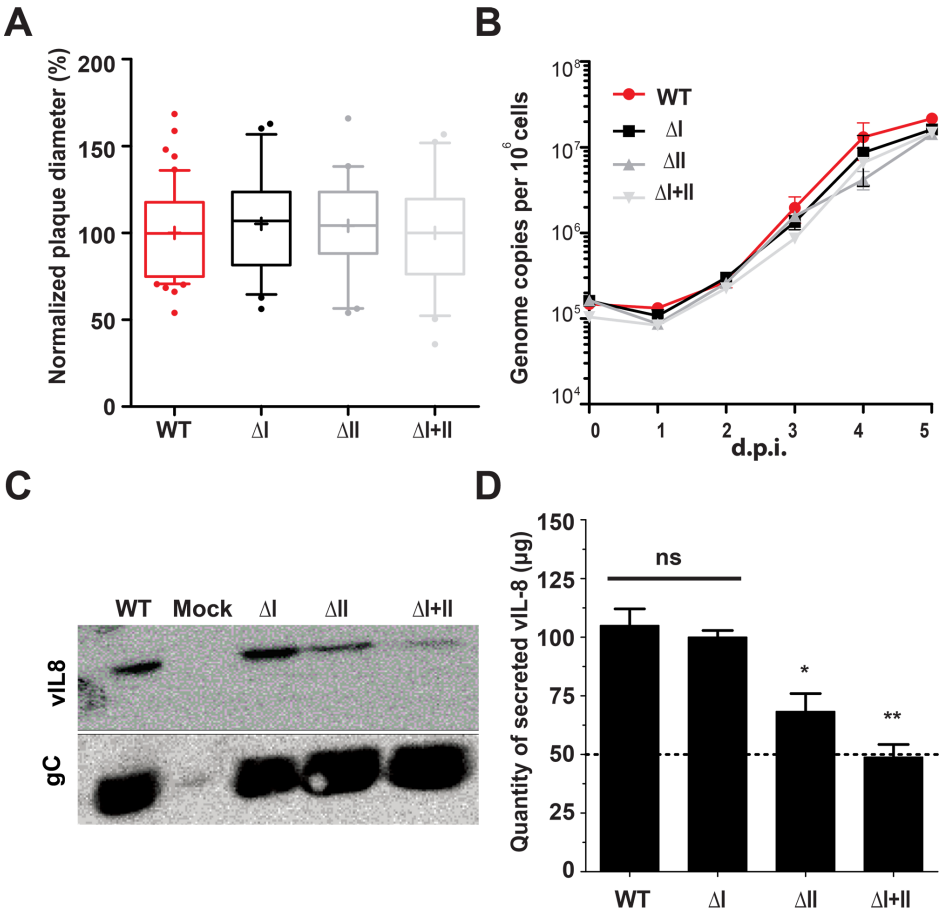


Figure 8: Efficient virus replication and inefficient vIL-8 secretion in ΔIntrons mutants. **(A)** and **(B)** Plaque size and qPCR-based growth kinetics assays of the RB1-1B GFP reporter MDV and three mutants of vIL-8. **(C)** Western blot for assessment of the secretion efficiency of vIL-8 and gC proteins in the supernatants of infected cells with the WT and vIL-8 recombinant viruses. **(D)** Relative quantification of vIL-8 secretory levels in the WT and mutant viruses. Significant differences between the WT and the mutants are shown with asterisks (**p≤0.01). At least three independent experiments were performed. Data sets were analysed by the one-way analysis of variance (ANOVA) with Bonferroni correction and error bars are the standard deviations.

3.3. Novel vIL-8 splice variants

Here vIL-8 intron II was tested for encoding novel vIL-8 isoforms. Based on previous RNA-Seq data and using the *in silico* prediction, vIL-8 intron II was found to encode an acceptor site (A19`) that produces an ORF expresses a truncated-vIL-8 isoform [76]. This ORF includes the first two exons and the last 16 bp of vIL-8 intron II (E3`). Therefore, the FLAG-E3` virus mutant was constructed using the Red recombination system (Figure 9). Fresh CECs were infected with 10,000 pfu from the FLAG-E3`and the wild-type RB1-1B viruses. After, cell lysates were immunoblotted by the mouse monoclonal α-D tag antibody (ABM, Canada). Strikingly, three novel vIL-8 splice variants of different molecular weights were expressed (Figure 10). A splice variant of ~15 kDa was strongly expressed in the FLAG-E3`-infected cells (Figure 10). This novel splice variant might correspond to the predicted truncated-vIL-8 isoform. In addition, another two novel splice variants of ~28 and ~55 kilodaltons were also detected (Figure 10).

In the light of these findings, it seems that the last 16 bp of vIL-8 intron II stands for a novel exon (E3`) that is alternatively spliced to form multiple vIL-8 splice variants. Hence, abrogation of the expression of these novel isoforms by mutagenesis of the acceptor splice site (A19`) was the next goal. Hence, the FLAG-E3`mut was constructed by Red recombination (Figure 9). After reconstitution, fresh cells were infected with 100 pfu from the FLAG-E3` and FLAG-E3`mut recombinant viruses together with the respective controls. At 5 dpi, cells were stained with the mouse monoclonal α-D tag antibody (ABM, Canada) and the secondary goat anti-mouse IgG Alexa Fluor 568 antibody (Invitrogen, Carlsbad, CA, USA) and analysed by fluorescence microscope. Strikingly, the immunofluorescence assessment showed that the expression of vIL-8 splice variants was completely abrogated in cells infected with FLAG-E3`mut (Figure 11). These findings confirm that these novel splice variants were expressed as a part of vIL-8 alternative splicing and the acceptor splice site A19` was essential for this mechanism. In addition, they prove that a splice site-directed mutagenesis is a successful tool to abrogate the expression of the spliced gene products.

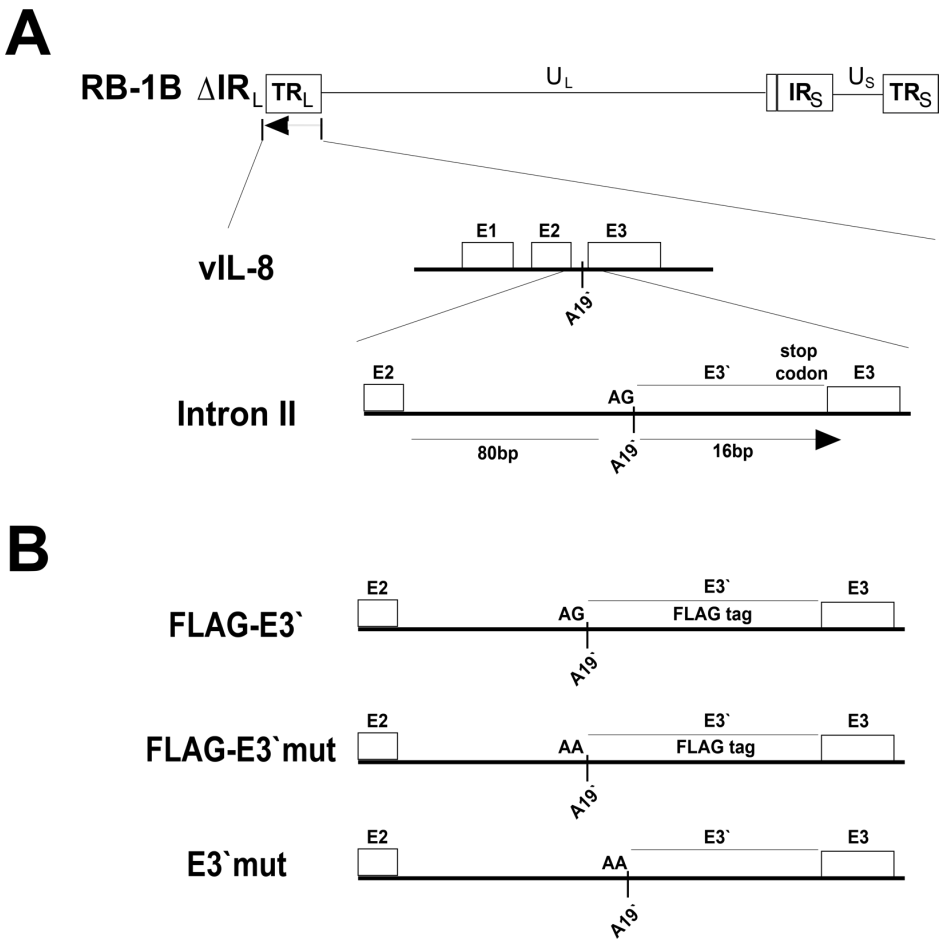


Figure 9: Overview of novel vIL-8 splice variants virus mutants. (A) Diagram of the genome of ΔIR_L pRB-1B recombinant virus, showing the composition of vIL-8 gene and intron II. **(B)** Overview of the mutagenesis strategy used in this study to detect the novel vIL-8 splice variants. FLAG tag is an insertion of the FLAG-tag amino acid sequence (DYKDDDDK) at position 89 bp downstream to 5` of vIL-8 intron II. The point mutation (G→A) was introduced 82 bp downstream to 5` of intron II. A19` stands for a novel splice acceptor site, E stands for "Exon", and E3` is the novel vIL-8 exon that was discovered in this study.

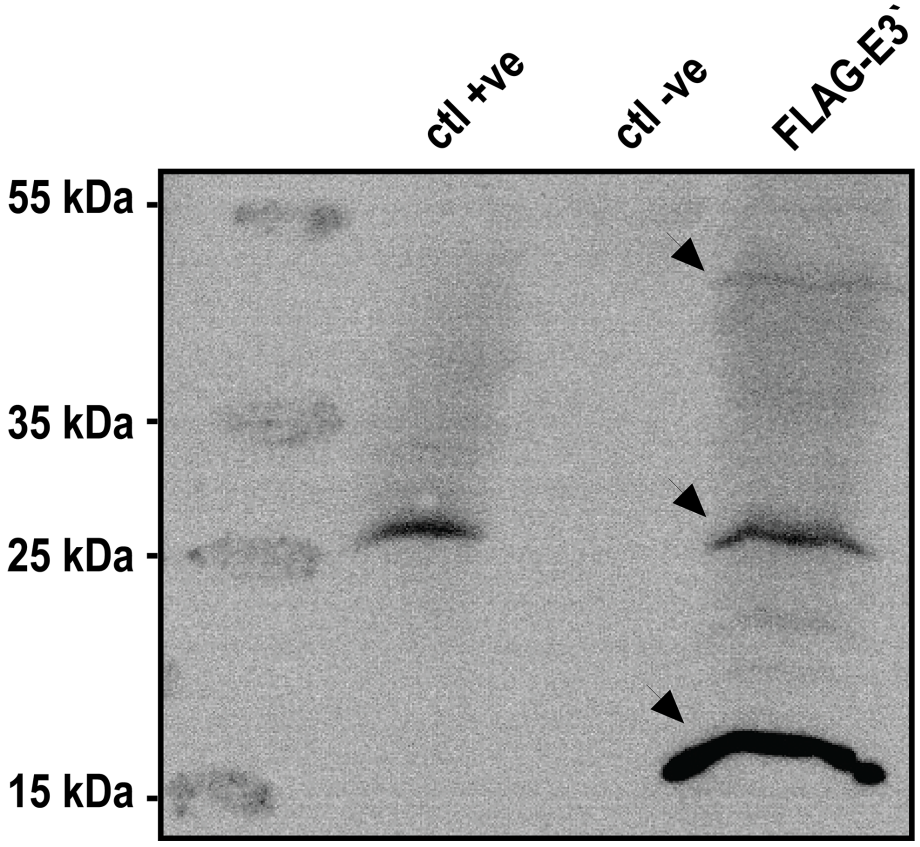


Figure 10: Western blot of novel vIL-8 splice variants (E3` splice variants). Cells were infected with 10,000 pfu and lysates were harvested using the RIPA I buffer. Lysates were subjected to SDS-PAGE and then immunoblotted with the mouse monoclonal α-D tag antibody (ABM, Canada) and the secondary anti-mouse IgM HRP-conjugated antibody (Cell signalling, Boston, USA). Ctl+ve stands for lysates from cells infected with a positive control FLAG-tagged recombinant virus. Ctl-ve is a loading negative control. FLAG-E3` is a virus mutant with a FLAG tag downstream to vIL-8 A19`. The novel vIL-8 splice variants of ~15, ~28, and ~55 kDa in FLAG-E3`-infected cells were pointed by (arrows).

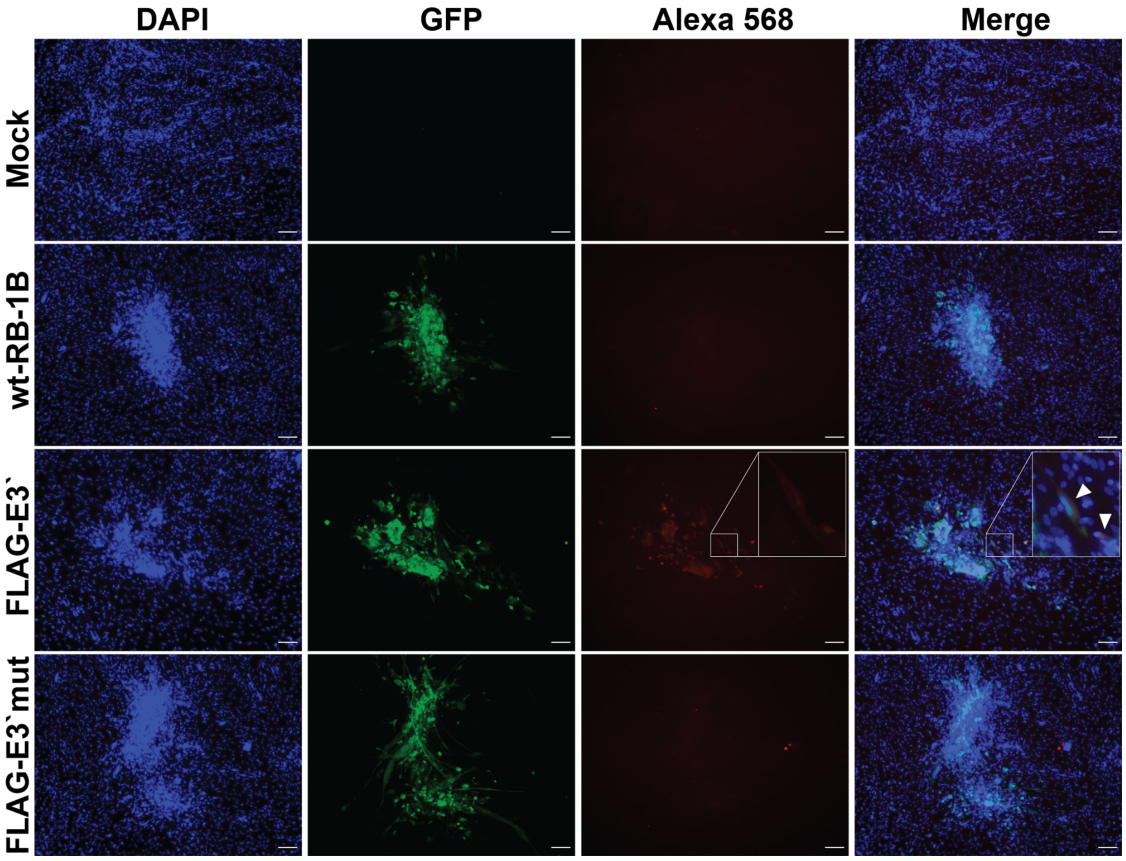


Figure 11: Fluorescence microscopy of novel vIL-8 splice variants. Cells were infected with mock and 100 pfu from the wild-type RB-1B GFP reporter virus, FLAG-E3`, and FLAG-E3`mut recombinant viruses. Cells were fixed at 5 dpi and stained with the mouse monoclonal α-D tag antibody (ABM, Canada) and the secondary goat anti-mouse IgG Alexa Fluor 568 antibody (Invitrogen, Carlsbad, CA, USA). Expression of the FLAG-tag protein in infected cells was pointed by (arrows). Images were processed using the ImageJ software. The scale bars correspond to 100μM.

3.4. The novel variants are dispensable for virus replication and protein secretion

Here the aim was to assess the role of novel vIL-8 splice variants in virus replication and vIL-8 secretion. The virus mutant E3`mut that harbours only a point mutation in vIL-8 intron II was constructed. Importantly, this point mutation abrogated the expression of vIL-8 protein variants as shown in (Figure 11). After reconstitution, CECs were infected with 100 pfu from the wild-type RB-1B virus and the E3`mut recombinant virus. Infected cells were analysed by plaque size and multi-step growth kinetics assays. The recombinant virus E3`mut showed no significant difference in virus replication and spread properties when compared to the control (Figure 12A-B). Next, CECs were infected with 10,000 pfu and supernatants were subjected to SDS-PAGE. Interestingly, the E3`mut was able to efficiently secrete the putative chemokine to a comparable level to the wild-type virus (Figure 12C). Next-generation sequencing of E3`mut BAC ensured the integrity of the viral genome. Together, these data clearly show that the novel vIL-8 splice variants are non-essential for virus replication or protein secretion *in vitro*. Nonetheless, their role in MDV pathogenesis and tumorigenesis stays as an intriguing future study.

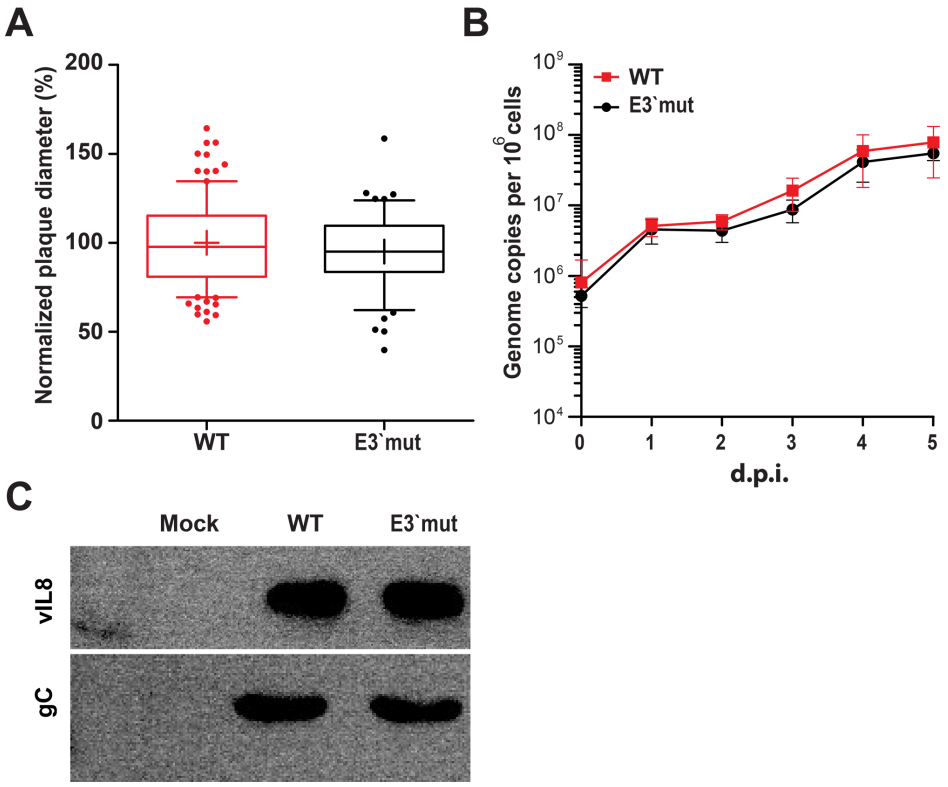


Figure 12: Characterization of the novel vIL-8 splice variants virus mutant. (A) and **(B)** Plaque size and qPCR-based growth kinetics assays of the wild-type RB1-1B MDV and the E3`mut recombinant virus. **(C)** Western blot for the evaluation of vIL-8 and gC secretions in the supernatants of cells infected with 10,000 pfu from the wild-type RB-1B and E3`mut recombinant viruses.

3.5. CRISPR/Cas9 impairs virus replication

To test if CRISPR/Cas9 can impair MDV replication. The lentiviral system was used to deliver Cas9 to CR cells and the Cas9 expression levels were measured by immunofluorescence and flow cytometry. Cas9 was expressed in polyclonal CR cells and the expression levels were more than 90% in monoclonal cells (Figure 13). In addition, the individual guide RNAs that target six MDV essential genes were transfected to cells. The targets were the minor capsid protein (UL6), the major capsid protein (UL19), the glycoprotein B (UL27), the polymerase (UL30), the tegument protein (UL49), and the infected cell protein (ICP4) (Figure 14A). The selected target sequences were aligned to multiple MDV genomes and showed high sequence conservation. Next, cells that express Cas9 and individual gRNAs were infected with 100 pfu of the RB1-1B GFP reporter virus. At 5 dpi, plaque diameters were measured to analyse the impact of CRISPR/Cas9 on virus replication. Interestingly, the system significantly impaired virus replication and spread (Figure 14B). Remarkably, targeting the essential genes UL27, UL30, UL49, and ICP4 by CRISPR/Cas9 reduced plaque diameters by more than 50% ($p<0.001$, Figure 14B-C), which reflects the ability of CRISPR/Cas9 to halt or impair MDV replication.

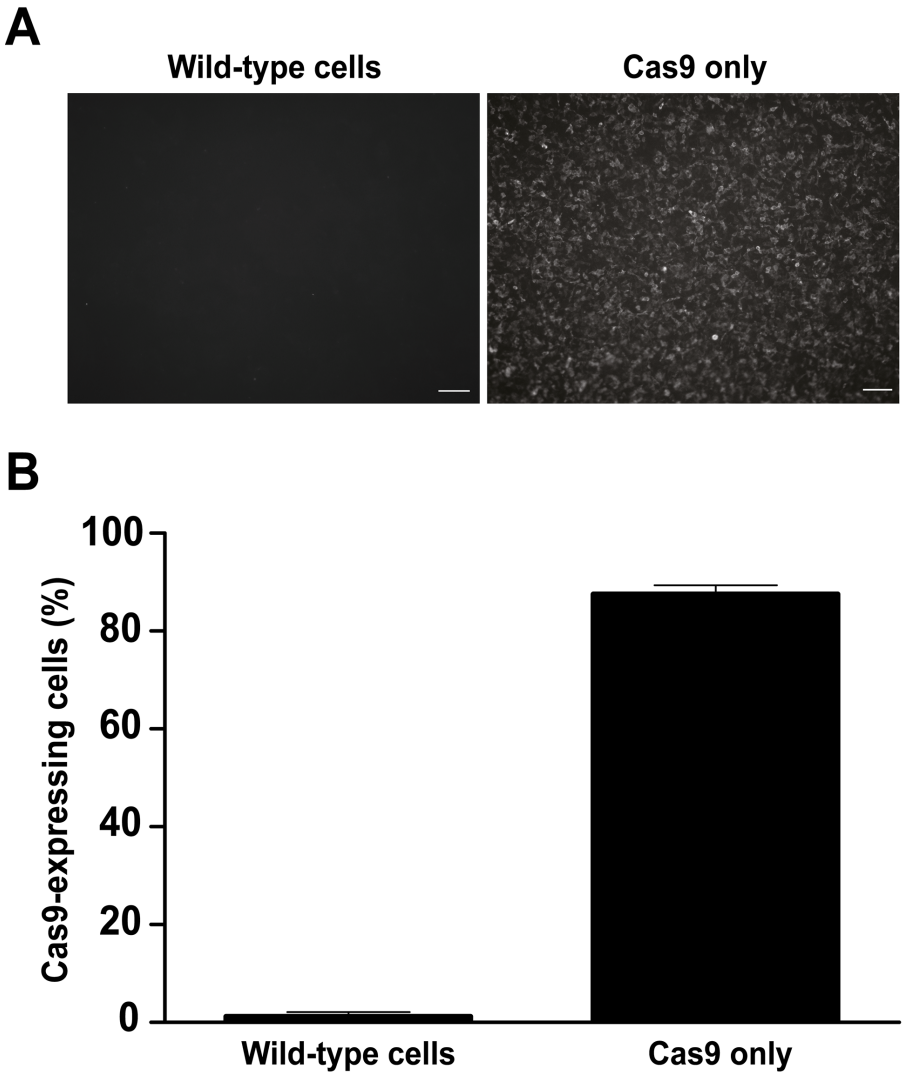
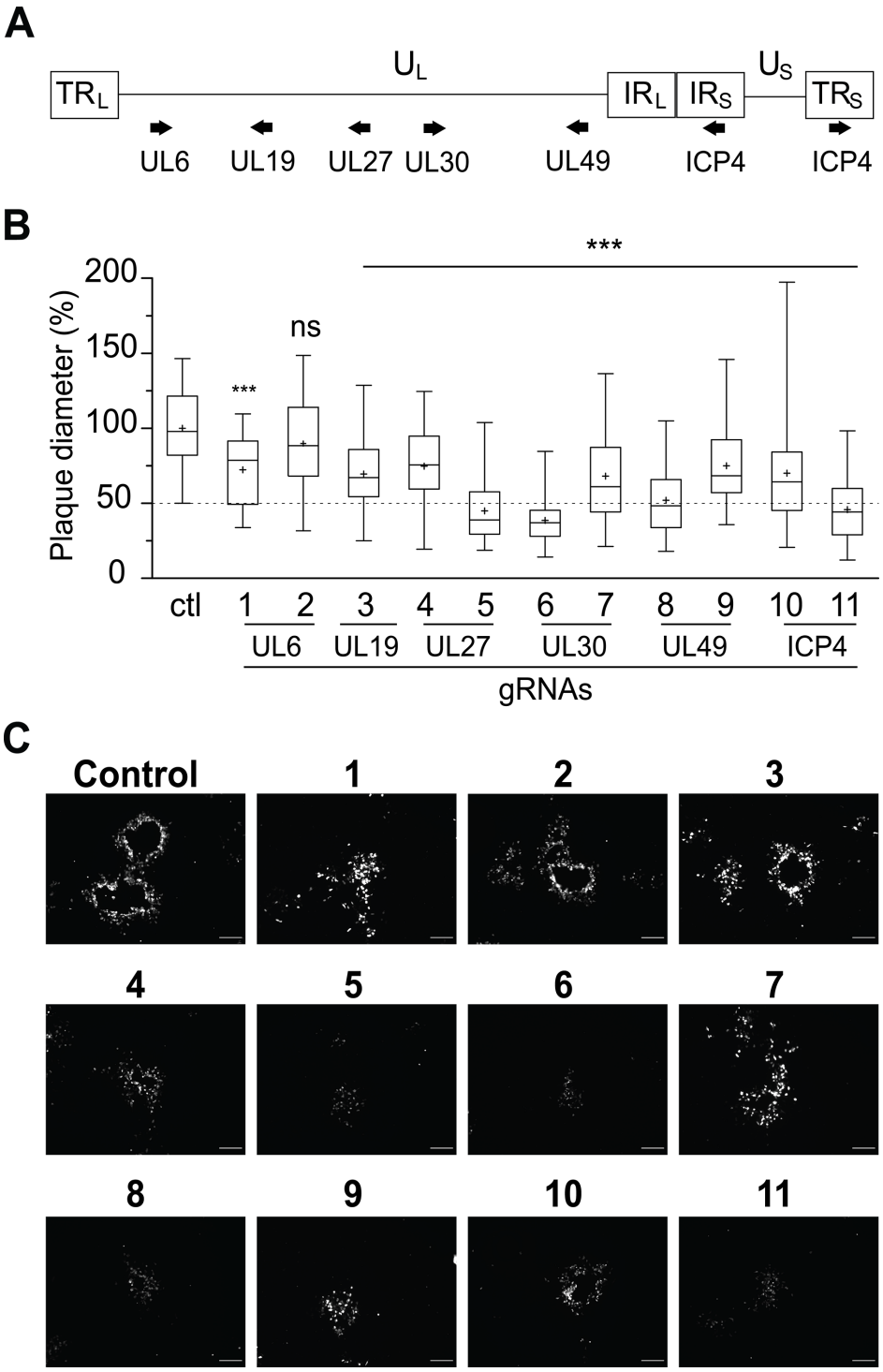


Figure 13: Cas9 expression in CR cells. (A) Immunofluorescence assessment of control CR cells and Cas9-expressing polyclonal CR cells. The scale bars correspond to 100µM. **(B)** FACS analysis of Cas9 expression in control CR cells and Cas9-expressing representative monoclonal CR cells (n=3). Error bars are standard deviations [189].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Plaque diameter (%)
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150
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Figure 14: CRISPR/Cas9 impairs virus replication. (A) Overview of MDV genome showing the target genes. **(B)** Plaque size assays of 11 different gRNAs targeting 6 different MDV essential genes; gRNAs 1 and 2 target the minor capsid protein (UL6, 5' and 3'); gRNA 3 targets the major capsid protein (UL19, 5'); gRNAs 4 and 5 target the glycoprotein B (UL27, 5' and 3'); gRNA 6 and 7 target the polymerase protein UL30 (5' and 3'); gRNAs 8 and 9 target the tegument protein (UL49, 5' and 3'); and gRNAs 10 and 11 target the infected cell protein (ICP4, 5' and 3'). Data were analysed by the one-way analysis of variance (ANOVA) with Bonferroni correction and error bars are the standard deviations (***p≤0.001). **(C)** Representative plaques images from RB1-1B-infected CR cells without (control) and with showed gRNAs (1-11). The scale bars correspond to 1000µM. (Modified from [189])

3.6. CRISPR/Cas9 completely abrogates virus replication

To inhibit MDV replication by multiple targeting of MDV genome, the efficient gRNAs were combined either in pairs or all in one vector (4x) and delivered to Cas9 cells. Next, cells that expressed single gRNA against HHV-6 genome or multiple gRNAs against MDV were infected with 100 pfu of the RB1-1B GFP reporter virus. At 6 dpi, plaque numbers, diameters, and genome copy numbers were measured (Figure 15A-C). Interestingly, only very few plaques were detected in cells that expressed two or four gRNAs (Figure 15A). Moreover, plaque diameters were significantly reduced by more than 90% in comparison to controls ($p<0.001$, Figure 15B). Additionally, the corresponding net increase in MDV genome copy numbers was significantly decreased by six logs when compared to the control ($p<0.001$, Figure 15C). To challenge the system by high-dose infections, cells that expressed multiple gRNAs were infected with 10,000 pfu and viral spread and replication were measured by flow cytometry and qPCR. A significant reduction in virus spread was seen after using multiple gRNAs as shown by flow cytometry (Figure 15D). Additionally, the net viral genome copy numbers were significantly reduced by eight logs in comparison to the control ($p<0.001$, Figure 15E). Together, these data show the ability of multiple gRNAs to completely abrogate MDV replication and in infected cultures.

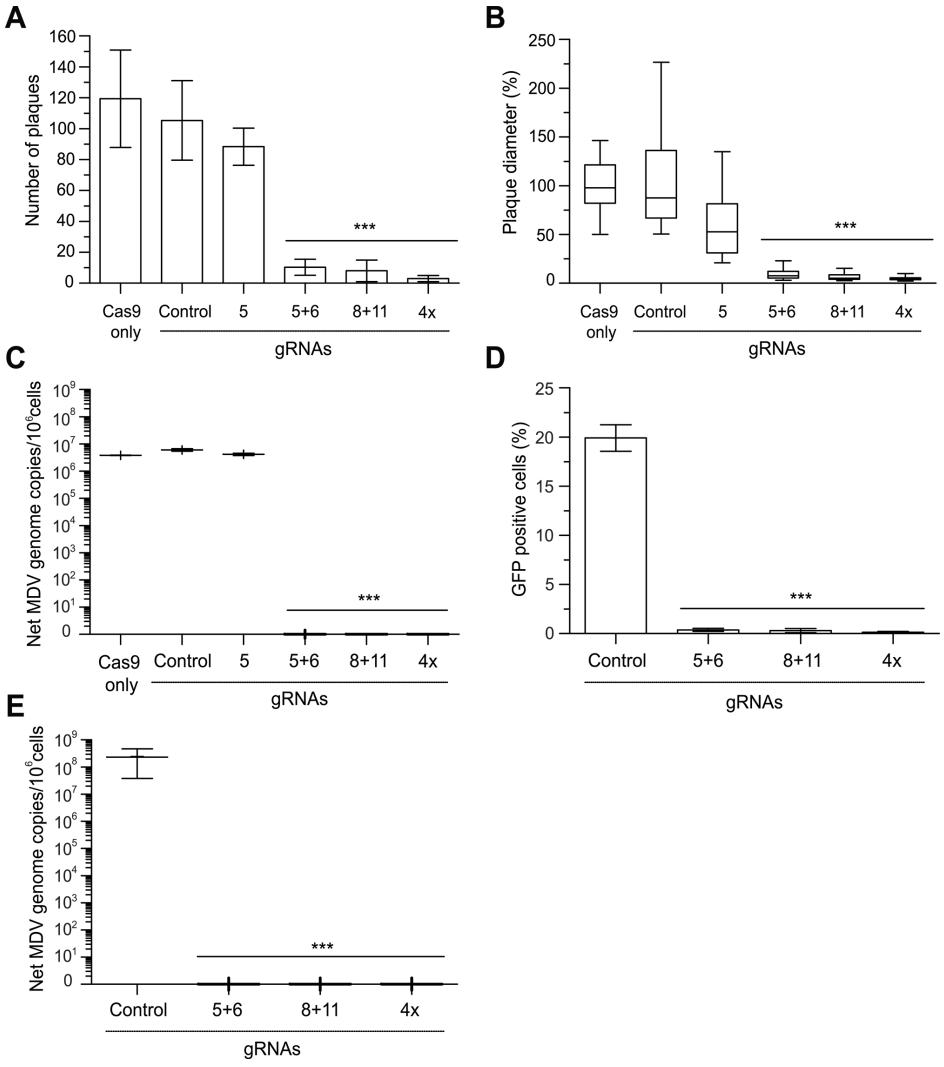

Figure 15: CRISPR/Cas9 efficiently abrogates MDV replication. (A) Plaque numbers and **(B)** sizes after infection with 100 pfu of the very virulent RB-1B MDV strain. **(C)** Corresponding net increase in MDV genome copies between 0 and 6 days after infection with 100 pfu. **(D)** Percent of MDV-infected cells detected by flow cytometry and **(E)** relative genome copies detected by qPCR at 5 days post-infection with 10,000 pfu. The significant differences between the controls, single gRNAs, and the multiplexed gRNAs are showed with asterisks (***p≤0.001). At least three independent experiments were performed. Data set was analysed by the one-way analysis of variance (ANOVA) with Bonferroni correction and error bars stand for the standard deviations [189].

3.7. CRISPR/Cas9 blocks virus escape

To determine whether the system prevents the development of virus escape mutants upon serial passaging, CRISPR/Cas9 cells were infected with 10,000 pfu and cultured for up to six passages (33 days). MDV genomic replication in control, single, and multiple gRNAs was measured by qPCR at each passage. Interestingly, virus escape mutants evolved in cells expressing individual gRNAs that targeted the 3` ends of UL27 and ICP4 genes. These mutants were able to restore virus replication properties at comparable levels to the wild-type virus (Figure 16A). Nonetheless, no escape mutants were detected in cultures expressing single gRNAs targeted the 5` ends of UL30 and UL49 genes. Importantly, CRISPR/Cas9 combining two or four gRNAs was able to efficiently block virus escape and no evolved mutants were observed in infected cultures (Figure 16A). To confirm that no evolved mutants arose upon using the multiplexed CRISPR/Cas9, cells were infected with a high dose, splitted only in a ratio of 1:2, and propagated for 10 passages. The virus showed no plaques in cells expressing multiple gRNAs while it was able to restore the wild-type replication properties in cells expressing single gRNA against 3` ends of UL27 and ICP4 genes. To examine the pattern by which MDV escaped the Cas9 cleavage, the gRNAs target sites of the RB-1B wild-type virus stock and the escape mutants were analysed by sanger sequencing. Interestingly, while no mutations were detected in virus stocks, mutations in the cleavage site of the gRNAs targeted UL27 and ICP4 were detected. These mutations resulted in one or two amino acid substitutions that allowed the virus to keep the efficient replication properties as shown in (Figure 16B-C). Based on this evidence, it was concluded that single gRNA could allow the emergence of escape mutants in certain cases while multiplexed CRISPR/Cas9 completely blocked virus escape.

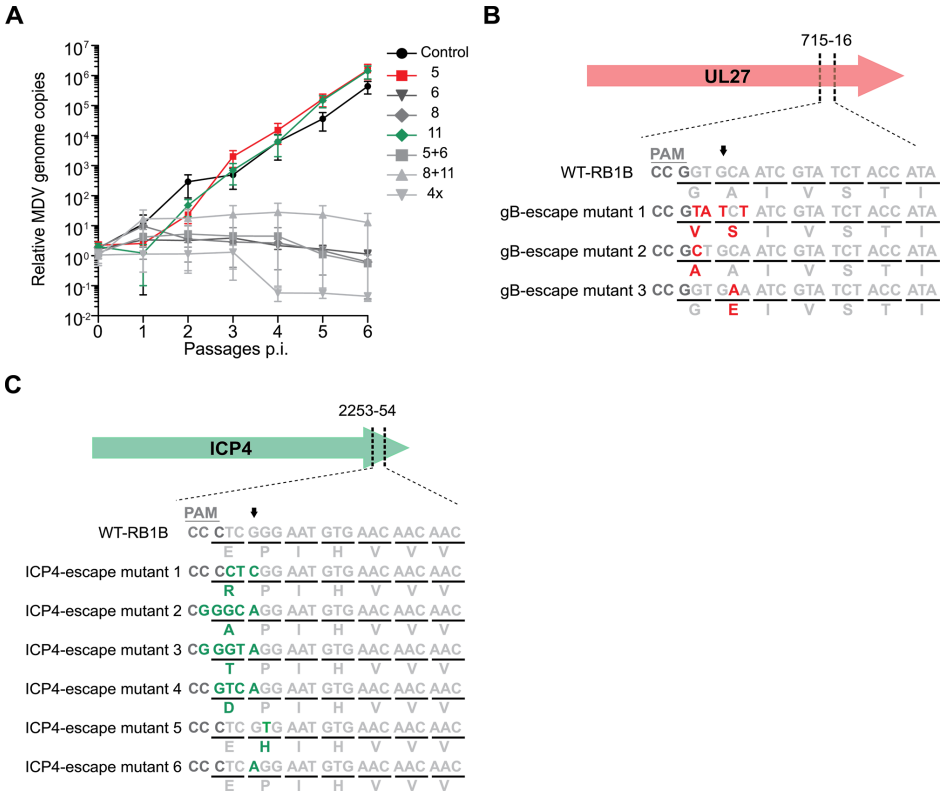

Figure 16: Emergence of MDV escape mutants that evade inefficient single gRNAs. (A) qPCR-based multiple-step growth kinetics of MDV in different CRISPR/Cas9 expressing cells upon prolonged infection for up to six passages (33 days). Data are shown as an average of three independent experiments and error bars are the standard deviations ($p \leq 0.001$, ctl vs. 6, 8, 5+6, 8+11 and 4x; Kruskal-Wallis test). **(B)** Analysis of sequences of the MDV variants detected in the single gRNA 5 and **(C)** gRNA 11, both targeting the 3' ends of UL27 and ICP4, respectively. The sequences on the top correspond to the wild-type (WT) RB-1B sequences and at the bottom to sequences of detected CRISPR/Cas9 escape mutants. The number above the arrow shows the positions of the amino acid substitutions in the respective open reading frame. Arrows at +3 positions after the protospacer adjacent motif (PAM) site refer to the Cas9 cleavage site. (Modified from [189])

4. Discussion

The specific goals of this dissertation were (1) to analyse the importance of vIL-8 introns for virus replication and vIL-8 secretion; (2) to unravel whether the vIL-8 gene encodes novel protein variants; (3) to examine the impact of CRISPR/Cas9 on virus replication; and (4) to test if MDV can evade from the CRISPR/Cas9 system.

4.1. The importance of vIL-8 introns

Introns are non-coding sequences that are ubiquitously distributed in eukaryotic and some viral genomes and yet their significance is underappreciated or poorly understood. However, they have been recognized as mediators of several biological functions [190, 191]. In this study, one or all vIL-8 introns were removed, and MDV replication and vIL-8 secretion of the recombinant viruses were examined. Replication and spread properties of virus mutants were comparable to the wild-type virus (Figure 8A-B). Previously, complete deletion of the vIL-8 gene did not interfere with MDV replication *in vitro* albeit to a much lower extent *in vivo* [67]. Therefore, it is conceivable that vIL-8 introns are non-essential for virus replication.

MDV vIL-8 is a true late kinetics protein that is expressed by the splicing of three exons [67]. Interestingly, cellular spliceosomes define exons by major splice junctions that are often encoded within introns [85]. In this study, intron I deletion did not interfere with vIL-8 secretion (Figure 8C-D). Nonetheless, intron II deletion significantly reduced the amount of vIL-8 secretion by ~30% (Figure 8C-D). Besides, the deletion of both introns extremely reduced the secretion by more than 50% (Figure 8C-D). Which essential role for secretion intron II fulfils is currently unknown. Nonetheless, one possibility is that intron II could encode specific sequences for vIL-8 secretion. Moreover, the presence of intron II could be essential to ensure enough expression of vIL-8. This agrees with a recent study on murine cytomegalovirus (MCMV) that emphasized the importance of the presence of intron I, but not its specific sequence, for sufficient expression of p87 protein [75].

Splicing is known to promote rapid and efficient translocation of mRNA from the nucleus to cytoplasm [192]. Therefore, an explanation of the additive effect of removal of both introns is that vIL-8 in this case was encoded from unspliced transcript. Unspliced transcripts are poorly shuttled from the nucleus to cytoplasm that would lead to a reduced translation [75, 192].

4.2. Uncovering novel vIL-8 splice variants

A recent analysis of MDV transcriptome by RNA-Seq identified novel splice junctions within MDV genome, indicating that the splicing of MDV transcripts is more complex than previously thought [76]. Indeed, herpesviruses encode a substantial number of spliced genes in their genome as a part of proteome diversity [75]. These spliced products are expressed during latency or the very early lytic phase, more likely to ensure the expression of critical proteins during times of absence of mRNA export factors [56, 75, 192]. Furthermore, these spliced products might be a part of a regulatory mechanism for protein levels or activities [75, 88]. In this study, intron II was spliced to novel vIL-8 splice variants of different molecular weights (Figure 10). Moreover, immunofluorescence images confirmed that the protein variants were expressed due to vIL-8 alternative splicing (Figure 11). These findings clearly show that vIL-8 is alternatively spliced more than previously thought and agree with previous studies that identified vIL-8 as an importantly spliced gene [56, 193, 194].

In contrast to other CXC chemokines, it has been hypothesized that vIL-8 possesses unusual long C-terminal extension that modulates binding to receptors or polyglycans [67]. In this study, a truncated vIL-8 isoform of ~15 kDa was detected (Figure 10). Whether a discovery of this shorter vIL-8 isoform could refute this hypothesis needs further clarification. Previously, a variant of Meq protein (Meq-sp) of 28-kDa was identified by Peng and colleagues [193]. Also, another study detected another Meq splice variant, indicating that Meq has more than one splice variant [56]. Meq-sp has been proposed to be a potential negative regulator for Meq oncogenic properties[193, 194]. In this study, novel vIL-8 isoforms of ~28 and ~55 kDa were detected (Figure 10). Whether these isoforms can be linked to Meq or other upstream genes variants remained to be determined.

Splice gene products are well-known to regulate protein levels or activities [75, 88]. Therefore, the role of novel vIL-8 isoforms in virus replication and protein secretion was examined. Neither MDV replication nor vIL-8 secretion were seen to be significantly changed after abrogating the expression of the spliced products (Figure 12). Complete vIL-8 deletion did not interfere with virus replication and therefore it is understandable that vIL-8 isoforms are also non-essential for replication. Moreover, the E3`mut still harbours the complete sequence of intron II and a point mutation in A19` is unlikely to interfere with the splicing of the parental vIL-8. Indeed, most of the spliced herpesviruses genes were found to be expressed in latency or the very early lytic phase [56, 75]. Therefore, the regulatory functions of these protein variants may be seen in MDV latency or transformation. Hence, the E3`mut virus could serve as a promising candidate for *in vivo* characterization of the function of vIL-8 novel splice variants in MDV pathogenicity and oncogenicity.

4.3. The impact of CRISPR/Cas9 on virus replication

CRISPR/Cas9 is an adaptive immune system that protects bacteria from invading viruses [121, 131]. Here, this system was applied to protect cells from MDV replication. Different CRISPR/Cas9 cell lines that target MDV essential genes were established and analysed for their ability to impair virus replication (Figure 14A). Interestingly, individual gRNAs were able to impair virus replication by up to 50% where some gRNAs performed better than the others (Figure 14B). Indeed, gRNAs are known to have different targeting efficiencies and the reason for that is poorly understood [195, 196]. Strikingly, targeting the MDV genome by multiple gRNAs completely blocked virus replication (Figure 15).

Previous studies showed that CRISPR/Cas9 can be harnessed to interfere with the replication cycle of herpesviruses in different ways [149]. First, CRISPR/Cas9 can induce DNA double-strand breaks (DSBs) in the viral genome, which impair the packaging of intact viral genomes. In addition, the expression of targeted essential proteins can be disrupted. Finally, CRISPR/Cas9 is likely to have indirect impacts on virus replication, as the introduced DSBs are often repaired by the non-homologous end joining (NHEJ) DNA repair mechanism. It is well known that NHEJ often produces harmful mutations that can hamper the following rounds of viral infections [149]. Nonetheless, combining multiple gRNAs produces an additive effect because of the loss of large parts of the viral genome [149]. This could shift the balance between the Cas9 cleavage and the DSB repair leading to a severe loss of genome integrity due to the formation of fragments that cannot replicate [197].

Moreover, disrupting the expression of essential viral proteins prevents the formation of infectious viral particles [149, 165]. Taken together, these data are in agreement with a previous study that showed an inhibited herpesviruses replication using combinatorial CRISPR/Cas9 [149].

4.4. The impact of CRISPR/Cas9 on virus escape

Only a few data are available on the ability of multiple gRNAs targeting to prevent virus escape. However, few studies reported the ability of multiple CRISPR/Cas9 targeting to prevent or delay the emergence of HIV escape mutants, which is known to have high evolutionary rates [164, 165]. In this study, CRISPR/Cas9 was examined to prevent the development of virus escape mutants. Multiple targeting of MDV genome by CRISPR/Cas9 did not develop any viral breaks in more than 30 days in culture, which could be attributed to the high efficiency of multiplexed gRNAs and the low evolutionary rates of MDV.

Intriguingly, the wild-type virus replication levels were restored after single CRISPR/Cas9 targeting of the MDV genome. Virus escape mutants were able to emerge over two of the individual gRNAs that targeted UL27 and ICP4 respectively (Figure 16). It is tempting to speculate that the error prone NHEJ DNA repair mechanism was exploited by MDV to mutate the targets without disrupting the function of the essential viral proteins. Therefore, virus escape mutants have been strongly selected and rapidly accumulated over time leading to the restoration of the wild-type replication levels. Indeed, CRISPR/Cas9 identifies the targets in a highly-specific manner and non-specific bindings especially in the “seed sequences” are known to be transient and short-lived [198]. Consequently, nucleotides substitutions in the protospacers or the protospacers adjacent motif (PAM) (Figure 16B-C) would interfere with the recognition phase of CRISPR/Cas9 [198]. Importantly, targeting non-coding sequences or non-essential genes would likely accelerate the emergence of escape mutants due to the rapid repair and escape from the negative selection [199]. Therefore, targeting the essential genes would minimize the likelihood of the emergence of escape mutants, as only mutations that keep the protein function are tolerated by the virus. Together, these data agree with previous studies that showed an accelerated virus escape upon inefficient CRISPR/Cas9 targeting [149, 200, 201].

4.5. Conclusions and outlook

Overall, multiple vIL-8 virus mutants and CRISPR/Cas9 cell lines were developed and characterized during this dissertation and the following can be concluded:

1) vIL-8 introns are non-essential for virus replication *in vitro*, but they are indispensable for efficient protein secretion.
2) vIL-8 is a highly spliced gene that does not express only one protein, but also expresses multiple protein variants that have not been described before.
3) Novel vIL-8 protein variants are non-essential for virus replication or protein secretion *in vitro*, but whether they have a role in MDV latency and transformation remained to be determined.
4) CRISPR/Cas9 can be used as an efficient system to abrogate the replication of a cell-associated oncogenic herpesvirus.
5) CRISPR/Cas9 prevents virus escape and can be used for the long-term elimination of MDV from infected cultures especially when two or more gRNAs are used.
6) MDV escape mutants can develop over CRISPR/Cas9 targeting especially when single gRNAs are used.

MDV genome encodes important genes within MDV repeat regions including meq, RLORF5a, RLORF4, and vIL-8 (Figure 3). Several studies have been published to address the importance of each gene within this region. For instance, the deletion of meq gene did not affect viral replication but severely reduced cellular transformation [202]. Additionally, RLORF5a was non-essential for both replication and transformation. Interestingly, deletion of RLORF4 resulted in a virus with an attenuated phenotype [203]. Moreover, vIL-8 deletion reduced tumour incidence by ~90% [67]. Nonetheless, this gene-knockout strategy resulted in MD phenotypes that combined the effects of deletion of the gene of interest and the splice products. Therefore, it is of great importance to analyse the function of only the splice products especially in regions with such complex splicing patterns [56].

In 2007, Jarosinski and Schat discovered multiple splice products between exons II and III of vIL-8 and the upstream genes (Figure 3B) [56]. Interestingly, most of these splice variants rely on the acceptor site (A18) within intron I (Figure 3B) [56]. Thus, the deletion of intron I is expected to imbalance the splicing pattern between vIL-8 and upstream genes without interfering with virus replication or protein secretion (Figures 7 and 8). In addition, mutation of the acceptor site A19` abrogated the expression of novel vIL-8 splice variants without disrupting virus replication or protein secretion (Figures 11 and 12). Hence, ∆intron I-vIL-8 and E3`mut recombinant viruses are forthcoming and promising candidates to dissect the role of vIL-8 protein variants in MDV pathogenesis and tumorigenesis.

The recent ability to culture and genetically modify chickens primordial germ cells (PGCs) represent one of the milestones in developing transgenic chickens [204]. PGCs tolerated the insertion of foreign DNA that did not affect their ability to migrate to gonads after injection into the vasculature of white leghorn embryos [205]. CRISPR/Cas9 and the germline transmission ability of PGCs brought the *in vivo* gene editing in chickens to “golden age” and provided the basis for developing transgenic chickens that are resistant to infectious diseases [204]. Hence, CRISPR/Cas9 is of further scientific interest to protect chickens from MDV. Right now, a collaborative project is ongoing to develop transgenic chickens that express Cas9 and the multiple gRNAs construct (4x) that was constructed and characterized in this study.

All in all, these data do not only supply promising mutant candidates for *in vivo* analysis of the function of vIL-8 splice variants, but also lay the foundation for future studies to generate MDV resistant chickens using CRISPR/Cas9.

Virus der Marek‘schen Krankheit: von neuartigen viralen Interleukin-8 (vIL-8)-Spleißvarianten bis zur Hemmung mit CRISPR/Cas9

Zusammenfassung

Das Virus der Marek‘schen Krankheit (Marek’s Disease Virus, MDV) ist ein strikt zellassoziiertes Herpesvirus, das in seinem natürlichen Wirt tödlich und onkogen wirkt und bei ungeimpften Herden eine Sterblichkeit von bis zu 100 % aufweist. Neben der wirtschaftlichen und landwirtschaftlichen Bedeutung ist es ein natürliches Virus-Wirt-Modell für die Untersuchung von virusinduzierten Lymphomen. Lymphome sind die Hauptursache für MDV-induziertes enormes Leiden und Sterben bei Hühnern. MDV beherbergt ein lineares doppelsträngiges Genom mit etwa 180 Kilobasenpaaren, bestehend aus zwei einzigartigen Regionen zusammensetzt, der einzigartigen langen (UL) und der einzigartigen kurzen (US). Die einzigartigen Regionen werden von weiteren Wiederholungen flankiert, die einen Satz von Genen aufweisen, der zur Pathogenität und Onkogenität des MDV beiträgt. Diese Gene sind das Hauptonkogen meq, die offenen Leseraster (ORF) RLORF5a und RLORF4 und das Chemokin-Homolog vIL-8. Darüber hinaus kodieren diese Gene für eine Reihe von Spleißvarianten, von denen angenommen wird, dass sie zur MDV-Pathogenese und Karzinogenese beitragen. Es ist jedoch unklar, ob alle oder einige dieser Spleißvarianten für ein exprimiertes Protein kodieren. Interessanterweise sind diese Varianten an das vIL-8-Gen gespleißt, das aus drei Exons und zwei Introns besteht.

Introns sind nicht-kodierende Sequenzen und dennoch wird ihre Bedeutung unterschätzt oder schlecht verstanden, obwohl sie wichtige Knotenpunkte enthalten, die das Spleißen vermitteln. In dieser Studie wurden ein oder alle Introns von vIL-8 entfernt, um das Spleißen von vIL-8 aufzuheben und die Bedeutung der Introns für die Virusreplikation und die Proteinsekretion zu charakterisieren. Interessanterweise waren vIL-8-Introns für die Virusreplikation nicht essentiell, aber für die Proteinsekretion waren sie jedoch unverzichtbar. Darüber hinaus wurde festgestellt, dass Intron II ein neues Exon (E3`) beherbergt, das gespleißt wird, um neue vIL-8-Proteinvarianten zu exprimieren, die für die Virusreplikation oder Proteinsekretion nicht essentiell waren. Diese Studie ist die erste, in der berichtet wird, dass Alphaherpesviren neuen Chemokin-Varianten exprimieren, und sie bildet die Grundlage für zukünftige Studien zur Aufklärung der Rolle von vIL-8-Spleißvarianten in der MDV-Pathogenese und Tumorbildung.

CRISPR/Cas wurde bei Bakterien und Archaeen als ein adaptiver antiviraler Immunmechanismus entdeckt. Streptokokken-Pyogene haben CRISPR/Cas9 entwickelt, welches das bisher am besten untersuchten Beispiel für das CRISPR/Cas-System ist. CRISPR/Cas9 wurde kürzlich in verschiedenen Anwendungen eingesetzt, welche die grundlegende Molekularbiologie revolutioniert haben. Im Prinzip rekrutieren Leit-RNAs Cas9, um die Zielobjekte auf sehr präzise Weise zu spalten.

In dieser Studie wurde CRISPR/Cas9 verwendet, um MDV-wichtige Gene anzuvisieren und die Virusreplikation zu hemmen. Interessanterweise beeinträchtigten einzelne gRNAs die Virusreplikation in unterschiedlichem Ausmaß, aber mehrere gRNAs die MDV-Replikation vollständig inhibierten. Darüber hinaus wurde CRISPR/Cas9 für die langfristige Eliminierung von MDV aus infizierten Kulturen getestet, die seriell passagiert wurden. Interessanterweise zeigten die einzelnen gRNAs MDV-Fluchtmutanten, die sehr schnell auftraten, doch die kombinierten gRNAs produzierten kein MDV, das CRISPR/Cas9 ausweichen kann. Die Sequenzierung der Fluchtmutanten offenbarte spezifische Mutationen, die in den Erkennungsstellen von zwei MDV-Einzel-gRNAs gesehen wurden, was ihre Fähigkeit, die mutierten MDV-Ziele funktionell zu spalten, eliminieren würde. Alles in allem ist dies der erste Nachweis, dass CRISPR/Cas9 die MDV-Replikation und -Evasion hemmen kann, insbesondere wenn zwei oder mehr gRNAs verwendet werden. Diese Studie bildet die Grundlage für künftige Studien zum Schutz von Hühnern vor diesem tödlichen onkogenen Herpesvirus.

Marek's disease virus: from novel viral interleukin-8 (vIL-8) splice variants to inhibition with CRISPR/Cas9

Summary

Marek's disease virus (MDV) is a strictly cell-associated herpesvirus that is deadly and oncogenic in its natural host with up to 100% mortalities in unvaccinated flocks. In addition to the economic and agriculture importance, it is a natural virus-host model for the investigation of virus-induced lymphomas. Lymphomas are the main cause of MDV-induced tremendous suffering and death in chickens. MDV harbours a linear double-stranded genome of about 180-kilobase pairs composed of two unique regions, unique long (U_L) and unique short (U_S). Unique regions are flanked by further repeats that have a set of genes that contributes to MDV pathogenicity and oncogenicity. These genes are the major oncogene meq, the open reading frames (ORF) RLORF5a and RLORF4, and the chemokine homolog vIL-8. Moreover, these genes encode a set of splice variants that are thought to contribute to MDV pathogenesis and carcinogenesis. Yet it is unclear if all or some of these splice variants encode an expressed protein. Interestingly, these variants are spliced to the vIL-8 gene that consists of three exons and two introns.

Introns are non-coding sequences and yet their significance is underappreciated or poorly understood, despite they include important junctions that mediate splicing. In this study, one or all introns of vIL-8 were removed to abrogate the splicing of vIL-8 and characterize the importance of introns for virus replication and protein secretion. Interestingly, vIL-8 introns were non-essential for virus replication, but they were indispensable for protein secretion. In addition, intron II was found to harbour a novel exon (E3`) that is spliced to express novel vIL-8 protein variants that were non-essential for virus replication or protein secretion. This study is the first to report that alphaherpesviruses express novel chemokine variants and lay the foundation for future studies to dissect the role of vIL-8 splice variants in MDV pathogenesis and tumour formation.

CRISPR/Cas was discovered in bacteria and archaea as an adaptive antiviral immune mechanism. Streptococcus pyogenes have evolved CRISPR/Cas9, which is the best-studied example of the CRISPR/Cas systems until now. CRISPR/Cas9 has been recently used in various applications that revolutionized the basic molecular biology. In principle, guide RNAs recruit Cas9 to cleave the targets in a very precise manner.

In this study, CRISPR/Cas9 was used to target MDV essential genes to inhibit virus replication. Interestingly, while single gRNAs significantly impaired virus replication to different degrees, multiple gRNAs completely inhibited MDV replication. Moreover, CRISPR/Cas9 was tested for the long-term elimination of MDV from infected cultures that were serially passaged. Intriguingly, the single guides showed MDV escape mutants that appeared very quickly, yet the combined gRNAs did not produce MDV that is able to evade CRISPR/Cas9. Sequencing of the escape mutants revealed specific mutations that were seen in the recognition sites of two MDV single gRNAs, which would eliminate their ability to functionally cleave the mutated MDV targets. In all, this is the first demonstration that CRISPR/Cas9 can inhibit MDV replication and evasion, especially when two or more gRNAs are used. This study provides the basis for future studies to protect chickens from this deadly oncogenic herpesvirus.

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Selbständigkeitserklärung

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

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