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4   Abbreviations

aa	Amino-acid
APC	Antigen-presenting cell
BAC	Bacterial artificial chromosome
BSA	Bovine serum albumin
bZIP	Basic leucine zipper
CD	Cluster of differentiation
CtBP	C-terminal binding protein
CTLs	Cytotoxic T-lymphocytes
CXCL	CXC ligand
CXCR	CXC receptor
DBS	Gal4-binding sites
DC	Dendritic cell
DEFs	Duck embryo fibroblasts
DF-1	Immortalized chicken embryo fibroblast cell line
DNA	Deoxyribonucleic acid
dpi	Days post-infection
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
EMS	Ethyl-methanesulfonate
FFE	Feather follicle epithelia
GaHV	Gallid herpesvirus
Gal4	Galactose-induced genes
GM-CSF	granulocyte-macrophage colony-stimulating factor
HCMV	Human cytomegalovirus
HHV	Human herpesvirus
HSV	Herpes simplex virus
HVT	Herpesvirus of turkey
ICP	Infected cell protein
IE	Immediate early
IFN	Interferons
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRES	Intronic internal ribosome entry site
IRF	Interferon regulatory factor
IRL	Internal repeat long

IRS	Internal repeat short
kbp	Kilobase pairs
kDa	Kilodalton
KSHV	Kaposi's sarcoma-associated herpesvirus
LAT	Latency associated transcripts
MD	Marek's disease
MDV	Marek's disease virus
MeHV	Meleagrid herpesvirus
MEM	Minimal essential medium
meq	MDV Eco Q-encoded protein
MERE I	Meq response element I
MERE II	Meq response element II
MHC	Major histocompatibility complex
miRNA	Micro RNA
MSB-1	MD lymphoblastoid cell line
MYXV	Myxoma virus
MΦ	Macrophage
n	Sample size
NK cells	Natural killer cells
NO	Nitric oxide
ns	Not significant
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pfu	Plaque forming units
pp14	Phosphoprotein 14
pp38	Phosphoprotein 38
PDC4	Programmed death cell 4
Pro	proline-rich domain
PRP	Pattern-recognition receptors
PRR	Proline-rich repeats
qPCR	quantitative PCR
Rat-2	rodent fibroblast cell line
REV	Reticuloendotheliosis virus
RFLP	Restriction fragment length polymorphism

RLORF4	Repeat long open reading frame 4
RLORF5a	Repeat long open reading frame 5a
RNA	Ribonucleic acid
RT	Room temperature
SD	Standard deviation
TA	Transactivation domain
TLRs	Toll-like receptors
TMR	Telomeric repeat region
TNF	Tumor necrosis factor
TR	Telomerase RNA
TRL	Terminal repeat long
TRS	Terminal repeat short
UL	Unique long
US	Unique short
vacMeq	Vaccine *meq*
vIL8	Viral Interleukin 8
vMDV	Virulent MDV
vMeq	Virulent *meq*
vTR	Viral telomerase RNA
vv+MDV	Very virulent plus MDV
vv+Meq	Very virulent plus *meq*
vvMDV	Very virulent MDV
vvMeq	Very virulent *meq*
ZIP	Leucine zipper

5 Introduction

5.1 Herpesviruses

Most humans and animals are infected with one or several herpesviruses, which remain in their host for the rest of their lives. When the host is immunocompetent, this relationship between the host and virus is harmonious. However, when the immune status of the host is compromised due to ageing, stress, co-infections or neoplastic disease conditions – the host-herpesvirus relationship is compromised. Herpesviruses are ubiquitous and can cause disease in all classes of vertebrates, even in animals of lower taxa, including molluscs. In this thesis, the focus is onherpesviruses infecting poultry – namely Marek's disease virus (MDV). MDV is an important avian alphaherpesvirus and is the causative agent of Marek's disease (MD) in poultry with significant economic losses worldwide.

5.1.1 Taxonomy and classification of herpesviruses

The order Herpesvirales was introduced only recently by the International Committee for the Taxonomy of Viruses (ICTV) to reflect the fact that herpesviruses found in fish, frogs, and bivalve molluscs are significantly different to mammalian, reptilian and avian members of the formerly single-family Herpesviridae [1]. Herpesviruses are members of the Herpesviridae family and are characterized into alpha- (α), beta- (β), and gamma- (γ) herpesviruses. These subfamilies are based on differences in antigenic cross-reactivity, genome size, structure and capability to establish latency in certain cell types [2]. Alphaherpesviruses have a broad host range, are characterized by fast replication cycles and establish lifelong latent infections mostly in neurons [3]. Alphaherpesviruses includes the human pathogens herpes simplex virus (HSV) types 1 and 2, Human herpesvirus 3 (HHV-3) and varicella-zoster virus. Betaherpesviruses, have a more restricted host range and replicate slower than alphaherpesviruses. The Betaherpesvirinae includes important human viruses such as human cytomegalovirus, HHV-6, and HHV-7. Infection with Betaherpesviruses is often associated with cell enlargement (so named cytomegalovirus) and the establishment of latency in the monocyte lineage [4]. Members of the subfamily Gammaherpesvirinae have a restricted host range, replicate and establish latent infection in lymphocytes and contain Eppstein Barr virus (EBV) and HHV-8 as an example [5].

5.1.2 Structure of herpesviruses

Herpesviruses are spherical to pleomorphic in shape and range from 150 – 200 nm in diameter. The composition of polypeptides within the virion vary between different herpesviruses. The

virions are enveloped by a host-cell-derived lipid membrane, which contains up to 20 integrated glycoproteins forming spike structures on the surface (Fig. 1) [6]. These glycoproteins allow the attachment to the host's membrane and entry into the host cell through specific interaction with cell receptors. The icosahedral shaped nucleocapsid surrounds the genomic material and consists of 162 capsomers, of which 150 are hexametric, and 12 are pentameric [7]. The inner core contains the DNA genome and associated proteins. The herpesvirus genome consists of a linear dsDNA molecule that is infectious under appropriate experimental conditions. There is a notable degree of variation in the composition, size, and structure of herpesvirus DNA genomes. Similar to the complex genome of the large poxviruses, the genomes of herpesviruses are also large and monopartite, ranging from 125 to 295 kilobase pairs (kbp), and encode for at least 70 to around 200 proteins [1, 2].
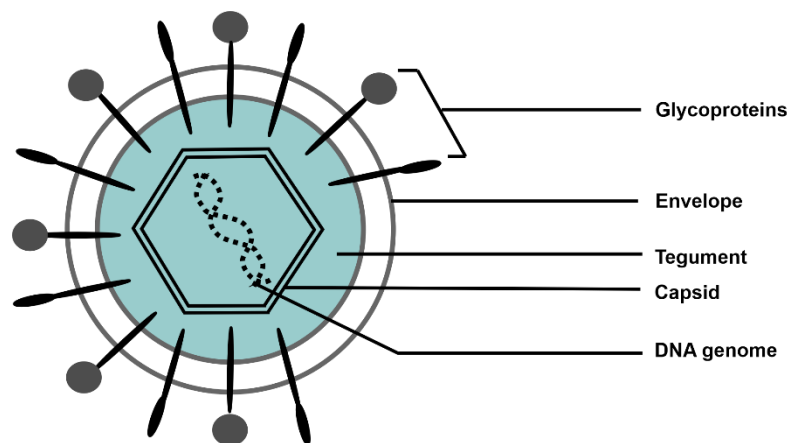
Figure 1: Schematic representation of the herpesvirus structure. The herpesvirus contains a double-stranded DNA genome, which is monopartite and has a size of 125 – 295 kbp, enclosed in the nucleocapsid. The capsid is surrounded by an amorphous protein coat called tegument. The outer layer is composed of glycoprotein spikes embedded in the lipid bilayer envelope. Members of the same family in herpesviruses share antigens, but envelope glycoproteins are specific for each species.

5.1.3   Herpesvirus genomes

Herpesvirus genomes consist of linear, double-stranded DNA molecules. The genomes range in size from about 125 to 295 kbp and in nucleotide composition from 32 to 75% G+C, depending on the virus species. Herpesviruses have complex genomes, not only are they large, but also constitute unique (U) and repeat (R) region sequences. The herpesviruses DNA genomes can be divided into six groups, designated as class A to F (Fig. 2). Five of the six genome classes harbour characteristic direct or inverted repeats that are believed to originate from their distinct virus replication rather than an evolutionary advantage of possessing

multiple copies of specific repeat-region genes [8]. Inversion of these repeats can give rise to up to four different isomers of a herpesvirus genome, which are believed to be present in equimolar proportions [8-10]. These inversions are due to herpesvirus rolling circle DNA replication and subsequent concatemer-cleavage into unit-length genomes during packaging and genome isomers have been shown to be functionally equivalent (Fig. 2) [8, 11]. However, the importance of these sequence duplications and genome isomers in *in vivo* infections, replication and pathogeneses remain mostly elusive.
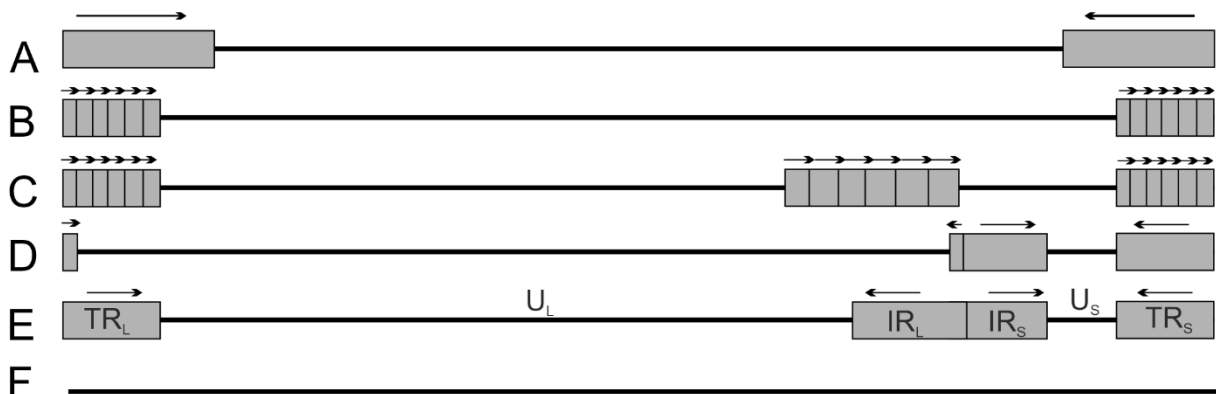
Figure 2: Classes of herpesvirus genome structures. Unique and repeat regions are shown as horizontal lines and rectangles, respectively. Arrows show the orientations of repeats. The nomenclature of unique and repeat regions. The nomenclature for terminal repeat long and short (TR_L and TR_S) and internal repeat long and short (IR_L and IR_S) are indicated for the class E genome, what MDV harbours.

5.2 Marek's disease virus

MDV is the topic of my research. MDV is an important avian alphaherpesvirus and is the causative agent of Marek's disease (MD) in poultry with devastating economic losses worldwide. The first description of MDV dates back to the beginning of the 20th century.

5.2.1 History of Marek's disease virus

József Marek first described MDV in 1907. József was a pre-eminent clinician of the Budapest veterinary school when he reported inflammation of nerves, polyneuritis, in four chickens [12]. Upon histological examination of the infected chickens, he observed that the sciatic nerves and parts of the spinal cord were infiltrated with mononuclear cells – a common observation still found today in chickens infected with MDV. In the following years a second syndrome arose, in addition to polyneuritis, which was observed in chickens infected with MDV. The second syndrome, visceral lymphomas, were proposed by Pappenheimer and colleagues in

the 1920s [13]. Finally, in 1960, it was identified that the causative agent of MD is the herpesvirus MDV [14, 15].

Today, Marek's disease virus (MDV) is classified as an alphaherpesvirus based on sequence homology with other viruses of the subfamily. MDV is part of the genus Mardivirus and classified as Gallid herpesvirus type 2 (GaHV-2) following the current ICTV nomenclature. Within the genus, two other viruses are recognized, the apathogenic Gallid herpes virus type 3 (GaHV-3, formerly MDV-2) and the Meleagrid herpesvirus type 1 (herpesvirus of turkey, HVT) [1, 16].

5.2.2 MDV pathogenesis

The MDV lifecycle is complex, and Calnek and colleagues described this and termed it 'the Cornell model of MDV pathogenesis' [17]. This Cornell model describes MDV pathogenesis in four phases of the MDV infection and also the time, day post-infection (dpi), each cycle appears (Fig. 4):

   (i)   early cytolytic (2 – 7 dpi)
   (ii)   latent (7 – 10 dpi)
   (iii)   late cytolytic and immunosuppressive phase (18 dpi)
   (iv)   transformation phase (28 dpi)

Each phase will be discussed in detail separately, together with a few dominant essential MDV genes associated with each phase that are summarized in table 1.
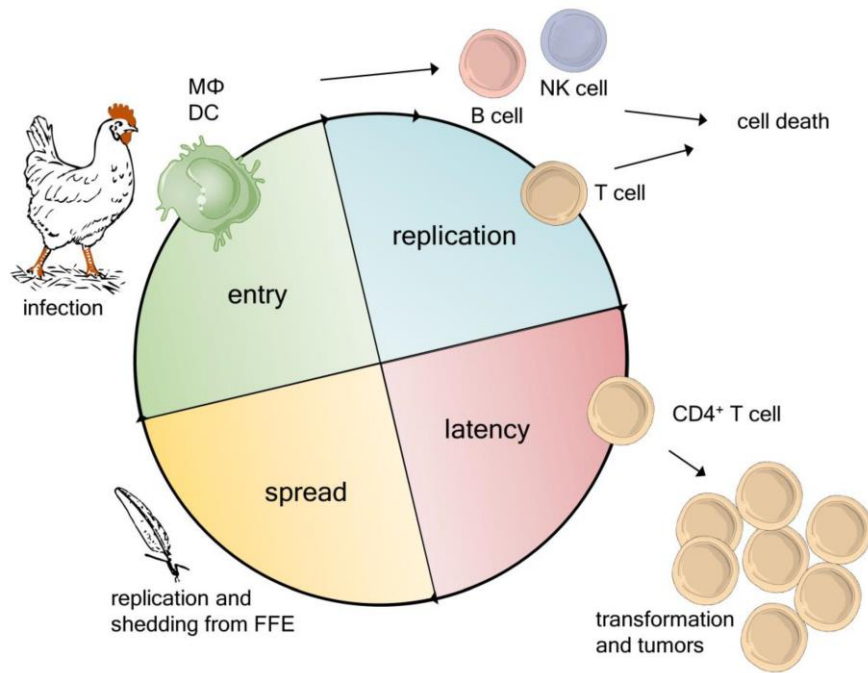
Figure 3: Schematic illustration of the MDV infection phases. MDV infection starts with the inhalation of infectious dust. Mononuclear phagocytes transfer the virus to lymphoid organs, such as the spleen, thymus, and bursa, where the virus lytically replicates in lymphocytes. MDV can establish latency in infected T cells. Latently and/or lytically infected T cells transport the virus to the skin and feather follicle epithelial (FFE), where cell-free MDV is generated. Also, MDV can transform latently infected T cells, resulting in malignant lymphomas. Figure obtained from [18].

Table 1: Important MDV factors or proteins expressed during the infectious cycle

MDV gene	Function
vIL8	A viral chemokine secreted that is involved in the attraction of target cells [19].
meq	The major oncogene and transcription factor involved in the regulation of cellular and viral genes [20, 21].
miRNAs	The non-coding RNAs that regulate gene expression at a post-transcription level [22, 23].
vTR	The viral telomerase RNA homologue that is essential for MDV lymphomageneses [24].
pp38	A unique phosphoprotein that is necessary to establish cytolytic infection in B cells [25].

5.2.2.1 Infection and early cytolytic phase

First thing first - the virus needs to get into the chicken. The natural route of infection is through the inhalation of cell-free virus particles of virus-contaminated dust and feather dander. The virus can persist in the environment for extended periods, and therefore challenging the poultry industries [17].

After the inhalation of virus particles, the virus infects parenchymal cells of the lung or is taken up by phagocytic alveolar cells - mainly macrophages and dendritic cells (DCs) [26]. These cells then deliver the virus to the primary lymphoid organs: the bursa of Fabricius, thymus and spleen. In these organs, the virus particles are transferred to mainly B cells and T cells [27], or natural killer (NK) cells [28] where primary replication takes place and production of viral progeny. The high replication, especially in the B cells, leads to immunosuppression that ultimately increases the susceptibility of the infected bird of other infections [29]. The infection of B cells results in the activation of CD4+ T cells. The activated CD4+ T cells are infected by B cells, or macrophages, directly via cell-to-cell transfer. The interplay of recruitment, activation and infection of CD4+ T cells is possible due to the virus secreting a virokine, vIL-8, that plays a important role as a chemoattractant [19]. Consequently, the virus can establish latency in the activated T cells where it integrates into the genome of host telomeres and also serves as a reservoir for latent MDV genomes [30].

5.2.2.2 Latent phase

The MDV latent phase is characterized by the integration of the viral genome into the telomeres of host chromosomes, using telomeric repeat (TMRs) arrays $(TTAGGG)_n$ at the ends of the MDV genome [12, 31, 32]. MDV enters this latent phase from approximately seven days pi. As mentioned, this phase is mainly restricted to CD4+ T cells, although in reports B cells, CD4-, CD8- T cells and CD8+ T cells containing latent MDV, have been isolated [33-35]. During the latent phase, the viral genome can be detected, but with minimal expression of viral genes and transcription of the viral genome is limited to the latency-associated transcripts (LATs) [36]. LATs are spliced RNAs that interfere with translation of the immediate-early (IE) regulatory protein and therefore maintains the balance between latent and lytic infections [37]. The MDV major oncogene, *meq,* expressed and maintained latency by activating latent gene expression and transcripts associated with oncogenicity [38]. These latently infected T cells serve as reservoirs for the virus to distribute the virus to the target organs via lymph and bloodstream.

5.2.2.3 Late cytolytic and immunosuppressive phase

The depletion of lymphocytes early during the cytolytic phase hinders both humoral and cellular-mediated immune response and leads to immunosuppression [39]. This immunosuppression phase is a significant feature of MDV infection and occurs around 18 dpi onwards. It is still unknown how the stages switch latency to late cytolytic phase. However, it has been confirmed that once the virus reactivates from latency, it replicates in the feather follicle epithelium (FEE) resulting in very high replication and production of infectious cell-free viral particles [18]. These particles are shed from feather to dust throughout the life of an infected bird which continues the viral life cycle [40].

5.2.2.4 Transformation

During the late cytolytic phase, the transformation phase of infection becomes apparent. Several factors encoded in the MDV genome are involved in the establishment and the maintenance of tumorigenesis. This includes, for instance, the phosphoprotein 38 (pp38), infected cell protein 4 (ICP4), vTR, vIL-8, and Meq (the latter discussed in detail later) were characterized in MDV tumorigenesis [41]. The deletion or interference of these proteins mentioned above results in altered lymphomagenesis, therefore, indicating the importance of these genes in tumor formation. The latent infected CD4+ T cells proliferate and give rise to lymphomas [42]. The population of viruses establishing latency are also responsible for transformation – making their presence a prerequisite for transformation and lymphomagenesis [12]. Lymphomas are observed in various organs (at around 3-4 weeks pi.), leading to organ failure and death.

5.2.3 Clinical signs of Marek's disease

The clinical picture of MDV changed over the years that leans on the infecting viral strain's pathotype, which will be discussed in detail later. Generally, chickens infected with MDV will develop one or more of the following syndromes:

i)   Neurolymphomatosis or classical Marek's disease: Involves the paralysis of one or several limbs. Torticollis can appear due to an inflammation of nerves controlling the neck, and vagal involvement will lead to dilatation of the crop. Including difficulty breathing or dilation of the crop may occur. Besides lesions in the peripheral nerves, there are frequently lymphomatous infiltration/tumors in the skin, skeletal muscle, visceral organs. Organs that are commonly affected include the ovary, spleen, liver, kidneys, lungs, heart, proventriculus and adrenals [43].

ii) Acute Marek's disease: Early-onset (four to eight weeks) of depression, paralysis and death before tumor formation. Post-mortem lesions include various degrees of oedema due to inflammation of the cerebrum, cerebellum, and brain stem [44].

iii) Ocular lymphomatosis: The infiltration of lymphocytes in the iris, unequal size of the pupils, and blindness.

iv) Cutaneous Marek's disease: Development of round and firm lesions at the feather follicle [45].

v) Immunosuppression: MD lesions target the lymphoid organs, which include the source of chicken B and T-lymphocytes (bursa of Fabricius and thymus respectively), as well as the spleen (site for immunological recognition). Therefore, affected birds become more susceptible to secondary infections [46].

vi) Non-specific: Weight loss, paleness, anorexia, and diarrhoea are also observed [44].
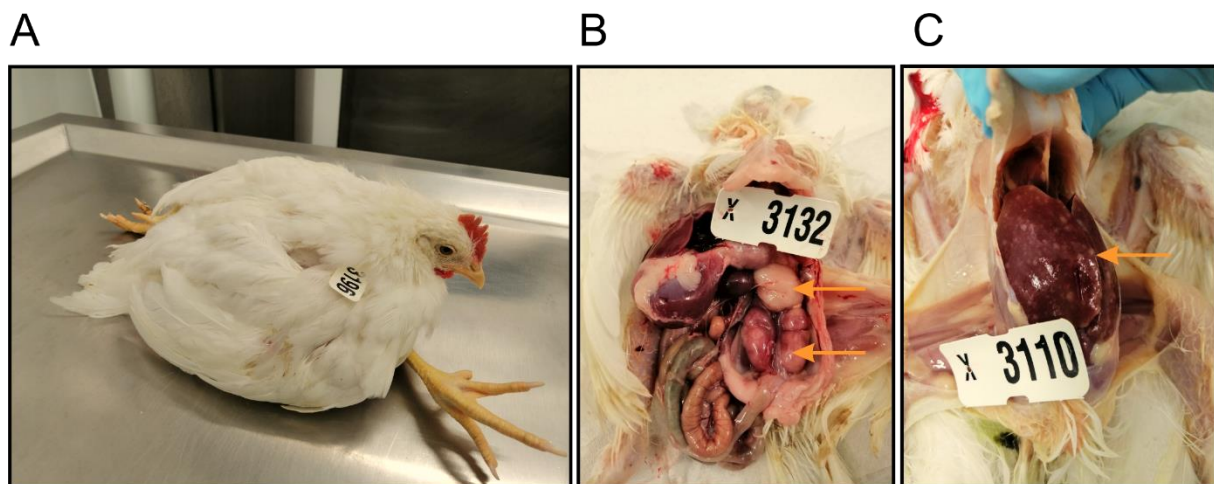
Figure 4: Clinical MD signs observed. A few cases of syndromes observed in chickens infected with MDV during my animal experiments. (A) An image of classical MD where lymphocytic infiltration into the peripheral nerves led to the paralysis of the limbs. (B) Enlargement of the kidneys and gonads (arrows) due to progressive tumor development and (C) diffused lymphomas in the liver.

5.2.4 MDV vaccines

Vaccines are critical for the protection of humans and animals against viruses. Vaccines against MDV were developed that not only protects against disease but were also the first vaccines developed against cancer. All the vaccines developed to provide protection against the disease depending on the 'pathotype'. Vaccines protect against MD, but with drawbacks

that will be discussed in detail in section 5.4.1.2. Here, only the vaccines developed against MDV and their uses will be discussed.

The first vaccine developed was the attenuated serotype 1 MDV, HPRS-16att (HPRS-16) vaccine in the 1960s. HPRS-16 was first isolated by *in vivo* transmission experiments where chickens displayed acute MD [47]. HPRS-16 was passaged in cell-culture, and at passage 31, it was no longer pathogenic and therefore used in vaccine trials. In this trial, birds were vaccinated with HPRS-16 at one day of age and challenged with MDV. At 60 weeks, 15.2% of vaccinated birds displayed MD, and non-vaccinated birds had 51.1% MD [48]. The HPRS-16 vaccine is not in use today and was quickly replaced by better vaccine candidates (Table 2). However, even when HPRS-16 was 'unsuccessful', it did open avenues by relaxing hurdles such as working with the cell-associated virus, strict storage conditions of vaccines, route of vaccination and field trials.

The next vaccine developed, was the non-oncogenic serotype 3, herpesvirus of turkey FC126 (HVT) in 1970 (Table 2). The non-pathogenic herpesvirus was found in turkeys in a field case 126, hence the abbreviation FC126 [48]. The early work from a research group in Wisconsin found that MDV and HVT shared some antigens based on serology – realizing then the potential usefulness [49]. The HVT candidate was attenuated by serial passage in tissue culture and assessed in protection studies, which resulted in HVT protecting very well against challenge with the JM strain of MDV [50]. An advantage of the HVT vaccine is that it is available as both cell-associated and cell-free lyophilized vaccines. Lyophilized vaccines can be stored at 4°C and do not need to be stored in liquid nitrogen. This feature of lyophilized HVT is useful in countries where the storage of MDV in liquid nitrogen is a problem [48]. However, the effectiveness of the cell-free HVT vaccines is reduced compared to the cell-associated vaccines due to interference from neutralization by maternal antibodies and therefore led to the emergence of MDV field strains of increased virulence [51].

Table 2: Overview of vaccine strains developed against MDV

Vaccine	Serotype	Year published	Currently used
HPRS-16att	1	1969	No
HVT	3	1970	Yes
SB-1	2	1978	Yes
CVI988	1	1972	Yes

As MDV increased in virulence, bivalent vaccines were introduced that have a synergistic effect – providing better protection [52, 53]. The bivalent vaccine consists of a mixture of a serotype two non-oncogenic MDV strain, SB-1 together with the existing HVT vaccine. The SB-1 (S-strain chickens in building B) were isolated from highly susceptible S-strain chickens (not available anymore), that displayed MDV symptoms [48]. Tissue culture adaptation of the virus resulted in attenuation and protection (at passage 25) in chickens [54]. For the higher virulent MDV strains that emerged, such as the common RB-1B strain, the HVT nor the SB-1 alone were able to provide sufficient protection [48, 52]. HVT and SB-1 are not dose-dependent since a dose of as low as 80 plaque-forming units (pfu) of SB-1 with 2 000 pfu of HVT or 80 pfu of HVT with 400 pfu of SB-1 resulted in higher protective indices than 2 000 pfu of HVT and SB-1 alone [52]. The exact reason for elevated protection and protective synergism is still not understood.

The final vaccine developed, and still, in use today, is the golden standard vaccine – CVI988 (isolate from hen 988) also called Rispens (I will call it 'CVI' in the rest of the thesis). The CVI vaccine (attenuated serotype one strain) was developed in response to the evolution of MDV to even higher virulence and widely used since the 1990s [55]. CVI was isolated as a virus from flocks without a history of clinical MD that was tested positive [55]. The CVI had low pathogenicity and was further attenuated by 25-35 serial passages in duck embryo fibroblasts (DEFs). This vaccine is now widely used in the US and Europe. It is considered the "gold standard" of MD vaccines that provide superior protection against the highest virulent MDV strains – for which HVT and bivalent vaccines, are mostly ineffective [56, 57]. CVI is today's vaccine of choice and reduced substantial losses in the poultry sector and protects a variety of chickens, from long-lived layers to breeders.

Even after 40 years, scientists still attempt to develop vaccines that are safer and provide better protective immunity than the CVI vaccine. For example, two other attenuated MDV serotype one viruses, BH16 and Md11/75c/R2/23 have been licensed for use as vaccines [58]. Upon protective studies, both the vaccines are regarded as safe; however, their protective efficacies are either less or compared to the CVI vaccine [59, 60]. A recombinant virus, RM1, was derived by the co-cultivation of the JM/102W strain of MDV with the reticuloendotheliosis virus (REV) [61]. RM1 resulted in the generation of a recombinant MDV containing the REV long terminal repeat (LTR) named the RM1 strain of MDV. This strain was highly attenuated for oncogenicity but induced severe bursal and thymic atrophy, making them unsafe for further use and unable to be licensed [61].

Researchers have identified essential genes involved in pathogenesis to target and generate improved vaccines to control MD. Several deletion mutant viruses constructed by the deletion of pathogenicity associated genes, such as vIL-8, vTR, *meq,* miRNA, have been tried as

vaccines with varied success [24, 62, 63]. One success story of a candidate is the rMd5ΔMeq, derived from rMd5 strain, where both copies of the *meq* gene were deleted by using overlapping cosmid clones. Vaccine challenge experiments consistently provided superior protection of rMd5ΔMeq compared to CVI [64] and as a follow-up study later [65] showing that the ΔMeq mutant significantly decreased immunosuppression - making it a plausible candidate [64]. These laboratory trials should be followed up with larger-scale trials where vaccines are tested against early contact challenge in commercial chickens and then compared to the most efficacious of the commercially available MD vaccines.

5.2.5 Immunity to Marek's disease

The chicken relies on two major pillars to defend itself against MDV: First, the innate immunity, general immune system, and the adaptive immunity, specialized immune system. The innate immunity responses are first and are early responses emerging immediately after infection, whereas, the adaptive immunity, also known as acquired immunity, is detectable around 5-7 dpi [66]. The cells involved in the innate and adaptive immunity are summarized for simplification (Fig. 5).
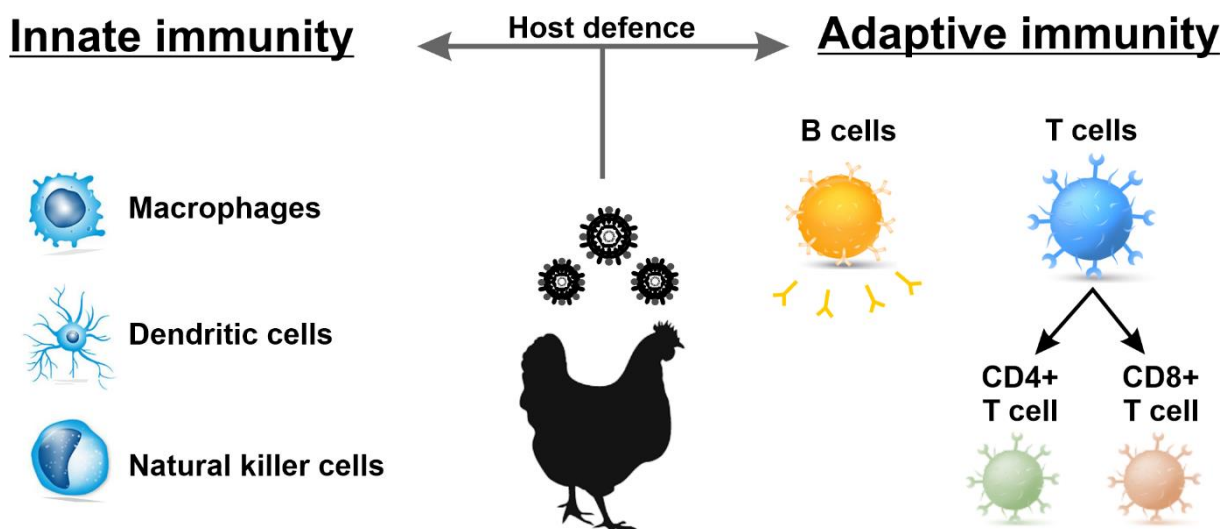
Figure 5: Summary of the cells involved during the immune response of the host upon MDV infection. The host defences are divided in the first line of defence, innate immunity, and then the adaptive, or acquired immunity line of defence.

5.2.5.1 Innate immune responses

The innate responses are initiated upon the detection of MDV by various host pattern-recognition receptors (PRP) that recognize conserved pathogen-associated molecular patterns and trigger the production of type I interferons (IFN – α/β), inflammatory cytokines and chemokines against MDV [67].

The interferon family can be classified into three main types of cytokines: type I, type II and type III IFNs. IFN-α and IFN-β belong to type I IFN family, while the type II IFN family includes only one cytokine: IFN-γ, which also exhibits antiviral activities [68]. The third type of IFNs is the IFN-λ family. In mammals, plasmacytoid dendritic cells (pDCs), monocytes, epithelial cells and fibroblasts are the leading producers of type I IFNs [69]. As chickens are infected with MDV from infectious dust, the virus is taken up by phagocytic cells such as macrophages or dendritic cells. Here, the virus may be recognized by toll-like receptors (TLRs), such as TLR21 (recognizing unmethylated CpG DNA), leading to the initiation of protein signalling cascaded which stimulates the expression of type I interferons (α and β), shown to be involved in antiviral defence. It has been shown that the administration if IFN-α reduces MDV viral replication *in vitro* [70, 71] and activates other immune cells such as NK cells [72]. More recently, and of our importance, it has been shown that MDV has the ability to evade the cGAS-STING DNA sensing pathway [73]. They show that Meq delayed the recruitment of TANK-binding kinase one and IFN regulatory factor 7 (IRF7) to the STING complex, thereby inhibiting IRF7 activation and IFN-β induction. The overexpression of Meq reduced antiviral responses and in contrast, a ΔMeq elevated MDV-triggered induction of IFN-β and downstream antiviral genes [74].

Macrophages and dendritic cells play an essential role in the innate immunity. They function as antigen-presenting cells (APCs) – involved in the initiation of immune responses against MDV in the respiratory system of chickens [75, 76]. Both macrophages and dendritic cells (DCs) in the end play a role in linking to adaptive immunity. It has been found that macrophages support cytolytic replication of MDV as they express three herpesvirus antigens, ICP4 (immediate early), pp38 (early), and gB (late) (Table 3) [77]. Macrophages can transfer MDV to other cells [78] and also have the ability to inhibit viral replication. The latter has been demonstrated where macrophages had been depleted in splenocytes and resulted in an increase of MDV replication [79]. Macrophages are activated by IFN-γ and tumor necrosis factor-alpha (TNF-α). Activated macrophages can exert their antiviral activities through the production of nitrogen oxide (NO) that is induced by inducible nitric oxide synthase (iNOS). The production of NO has been reported in the spleen, lungs and brains of MVD-infected chickens [80, 81] and has been shown to inhibit MDV replication and play an essential role in the control of MDV replication *in vivo* [82]. Another function of macrophages is their ability to

kill tumor cells, and it is believed that activated chicken macrophages can lyse MDV-derived tumor cells *in vitro* [83].

DCs play an essential role in initiating the adaptive immune responses, as mentioned before for macrophages as well, by presenting antigens to T cells [84]. There is still not much known of how DCs present MDV-antigens to the T cells. However, it has been shown that the upregulation of two interleukin cytokines, IL-12 and IL-18, are critical for polarizing and activating T-helper cells (Th) 1 cells [85, 86]. However, it is unclear whether DCs or other APCs secrete these cytokines and how these cytokines shape T cell-mediated immunity after MDV infection or vaccination.

NK cells are another critical population of cells fundamental for the innate immune responses. NK cells can respond to stimuli and produce antiviral cytokines such as IFN-γ, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) [87]. They have the ability to recognize virus-infected cells via ligation of death receptors and the release of granules. Increased activity of NK cells is observed during infection of MDV [88], chickens resistant to MDV [89] and vaccination of chickens with SB-1 or CVI [28, 90]. Higher NK activity as a response to vaccination may explain how MDV vaccine can provide protection in vaccinated chicks as early as three days post-vaccination [88]. NK cells also play a fundamental role as an anti-tumor sense through downregulation of cell surface markers such as major histocompatibility complex class 1 (MHCI) - a prominent characteristic of herpes viral infection. Surprisingly, it has been shown that the *meq* gene contributes to the enhanced NK-cell activity and IFN-γ production *in vitro* [28]. This comes to no surprise as the Meq oncogene forms homodimers and heterodimers with specific intracellular signalling proteins, that in turn modulate the host cell cycle. This will be discussed in section 5.3.

5.2.5.2 Adaptive immune responses

The key components involved in the adaptive immune responses are the B- and T-lymphocytes that recognize antigens. Because MDV is a cell-associated virus, the humoral immune response was not considered to be significant compared to the cell-mediated responses. It has been cleared where it has been shown that antibodies provide protective immunity against MDV, by perhaps blocking virus entry or by antibody-dependent cell-mediated cytotoxicity (ADCC) of infected cells [76]. The importance of the antibody immune response is strengthened by showing that maternal antibodies delay the development of clinical signs and tumor formation [66]. After the inhalation of MDV-contaminated dust, and the phagocytosis by macrophages and DCs, the B and T cells are recruited to the lungs by MDV-encoded viral IL-8 (vIL8). vIL-8 is a functional orthologue of chemokine CXCL13L1 but distinct

from chicken IL-8 which recognizes the C-X-C chemokine receptor type 5 (CXCR5) on B and certain T cells and induces chemotaxis [91]. B and T cells become infected and B cells, showing the highest cytolytic replication, become apoptotic and depleted. In the interim, the virus is transferred from B to T cells, leading to the establishment of latency and transformation [26]. Antibodies, against glycoproteins anti-gB, -gE and -gI, (Table 3) have been detected in MDV infected chickens, generated by B cells showed neutralizing activity and thus could play a role in blocking the entry of MDV [84, 92]. Nonetheless, it is still thought that antibodies produced by B cells play a minimal role and that the strength lies in the T cell mediated immunity [84].

It is well established how cytotoxic CD8+ T cells and cytokine-producing CD4+ helper T cells mediate antiviral immunity to other herpesviruses, such as human herpesvirus [93] and herpes simplex virus [94]. However, how these T cells mediate antiviral and/or anti-tumor immunity against avian herpesvirus MDV is poorly understood. Earlier studies by Ross et al. and Sharma et al. showed that T cells produced against MDV inhibit plaque formation [95] and specifically killed the MD lymphoblastoid cell line (MSB-1) [96], together indicating that 'killing' was T cell dependant. Studies have shown the occurrence of CD8+ T cells produced against MDV antigens such as: gB, Meq, pp38 and ICP4 (Table 3) [76]. Omar and Schat showed that cytotoxic T lymphocytes (CTLs) in the spleen at 7 dpi or post-vaccination were CD8+ TCRαβ+ T cells, not CD4+ or TCR1+ (γδ T) cells [97, 98]. The depletion of CD8+ cells led to higher titres in the CD4+ cells, suggesting CD8+ cells exert a substantial antiviral effect that influences the course and outcome of the disease [99]. However, the role of CTL in conferring long term immunity, generation of memory cells, in genetically resistant chickens is unknown [100], and the role of cell-mediated immunity in vaccine-induced protection has not been determined. The function of CD4+ T cells prior to MDV infection or vaccination remains unclear. Morimura et al. attempted to study the role of CD4+ T cells post-vaccination with CVI by depleting CD4+ T cells [99]. Their role remains undetermined in vaccine-induced protective immunity, possibly because depletion of CD4+ T cells may also result in the shortage of lymphoma cells that are transformed from MDV infected CD4+ T cells after challenge.

More studies are required to validate the exact role of CD4+ and CD8+ T cells in the control of MDV replication and tumor growth. The availability of CD4+ and CD8+ knockout chicken could provide valuable tools to study the role of these cells in vaccine-induced protective immunity against viral replication and tumor growth.

Table 3: Summary of MDV genes involved in immune modulation

MDV gene	Function	Antigenic potential	Infection stage
gB	Hypothetical function: Virion membrane protein; facilitate viral fusion with host membrane	Yes	Lytic replication
gI	Forms complex with gE. Important for cell-to-cell spread	Yes	Lytic replication
gE	Forms complex with gI. Important for cell-to-cell spread	Yes	Lytic replication
pp38	An early protein expressed during cytolytic infection and phosphorylated by Us3p	Yes	Lytic replication
ICP4	Viral gene transactivation function	Yes	Lytic replication
*Meq	Major oncogene; Regulates cellular and viral genes	Yes	Latency and transformation

* Detailed functions in the following section (Section 5.3)

5.3   The *meq* oncogene

The *meq* gene, named after its location in the MDV genome (**M**arek's **E**coRI **Q** fragment), is one of the most important genes for MDV. There are two copies of the *meq* gene located in the repeat regions (IR_L and TR_L) and is expressed in both the lytic and latent/tumor phase of the infection [101]. The 339-amino acid protein has been identified as the principal oncoprotein of MDV and has nuclear localization [20, 102]. Meq is a versatile protein and has been extensively studied to dissect the various functions which include transactivation, DNA binding, chromatin remodelling and transcriptional regulation [12].

5.3.1   Meq dimerization partners

Meq is a basic leucine zipper protein (b-ZIP) and shares many properties with other viral oncoproteins, including E6 and E7 of human papillomaviruses, large T antigen of SV40, E1a and E1b of adenoviruses and EBNA3C of Epstein-Barr virus (EBV) [103-106]. The structure of *meq* consists of N- terminal and C-terminal domain. The N-terminal domain includes a proline-rich domain (Pro), basic region (basic) and a leucine zipper domain (ZIP) that closely

resembles those found in c-Fos and c-Jun [107]. The C-terminal domain includes a transactivation domain (TA) that is characterized by proline-rich repeats (Fig. 6).
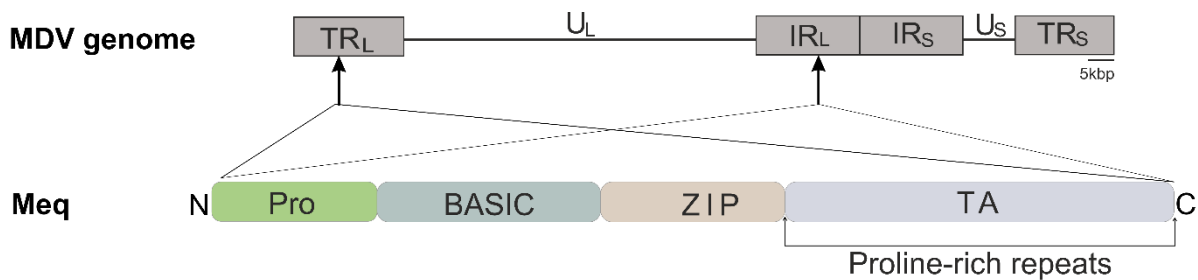
Figure 6: Illustration of the MDV genome and the *meq* oncogene. A schematic diagram of the MDV genome that encodes two copies of the *meq* gene within TR_L and IR_L regions, respectively. The following domains characterize the 339-aa Meq protein: The N-terminal domain includes a proline-rich domain (Pro), basic region (basic) and a leucine zipper (ZIP). The C-terminal domain includes a transactivation domain (TA) that is characterized by proline-rich repeats.

Meq acts as a regulator for transcription that can regulate cellular and viral genes. Through its bZIP domain, it can homodimerize with itself, Meq binds to Meq, or form functional heterodimers, Meq binds to cellular c-Jun, or other partners such as B-Jun, c-Fos, c-myc, ATF and CREB (Fig. 7) [108]. The Meq/Jun heterodimers can bind with high affinity to DNA sequences (resembling tetradecanoyl phorbol acetate response elements and cyclic AMP-dependent response elements) called Meq-responsive elements (MERE I; 5′-GAGTGATGACGTCATC-3′) and can activate linked reporter genes in *in vitro* assays through them [109]. The Meq/Meq homodimers bind to the second class of binding site, called MERE II (5′-ACACACA-3′), and it appears to act as repressors of transcription in *in vitro* assays (Fig. 7A) [110, 111]. Several genes have been identified that are regulated by Meq, through microarray analysis of DF-1 cells constitutively expressing Meq [20] and more recently by ChIP-seq experiments [112]. Some of the essential pathways regulated by Meq are (i) apoptosis, (ii) cell-cycle, (iii) cell proliferation, and (iv) cell migration. Some of the essential genes regulated by Meq are summarized in figure 7.
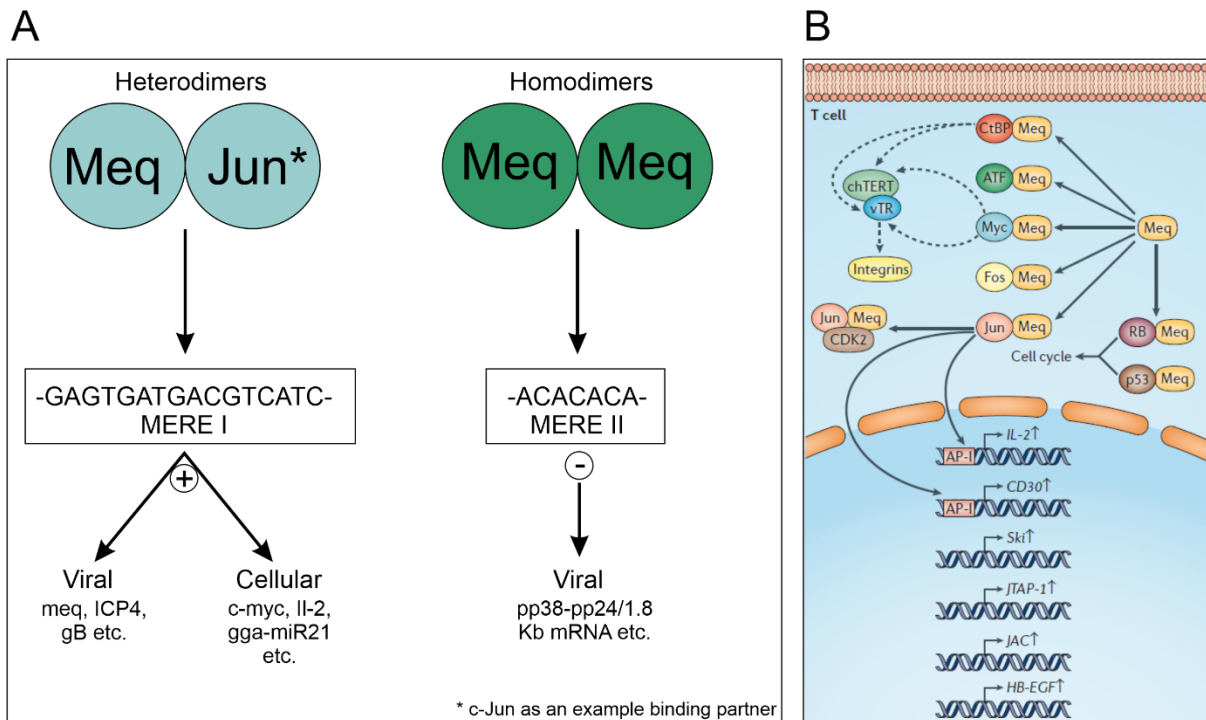
Figure 7: Summary of Meq as a transcription factor. (A) Meq forms homodimers (Meq/Meq) or heterodimers with c-Myc, c-Fos, ATF and c-Jun, the latter being the strongest transactivator. They bind to Meq response element I (MERE I) and MERE II, respectively. Binding of Meq/c-Jun to viral and cellular promoters promote gene transcription, while the binding of Meq/Meq represses gene expression. (B) These dimers function to up- or downregulate particular cellular or viral target genes, for example here a T cell. Proven interactions are indicated by solid lines, putative interactions by dashed lines. (B) was obtained from [12] with permission from Springer Nature (licence number 4878661017149).

5.3.2 Meq binding proteins and functions

It has been shown that the heterodimers, Meq/Jun, activate the heparin-binding epidermal-growth-factor-like growth factor (HB EGF), the cathepsin-like protein JTAP-1, JAC and all proteins that are capable of independently transforming chicken cells [12]. The upregulation of the tumor-associated genes by the Meq/Jun heterodimer, and the upregulation of anti-apoptotic factors such as Bcl-2 and c-Ski, the cellular homologue of retroviral v-Ski, suggests convergent evolution of the transforming pathways of oncogenic avian retroviruses and herpesviruses (Fig. 7B) [108]. In addition to homo- and heterodimerization with proto-oncoproteins, Meq can bind to several other cellular proteins including, CDK2, p53, Retinoblastoma (Rb), C-Terminal Binding Protein 1 (CtBP-1), p27^{Kip1}, S-Phase Kinase-Associated Protein 2 (Skp2) and HSP-70 (Fig. 7) [12, 113-115]. Meq contains several PXXP motifs in its proline-rich regions in the C-terminus domain which act as binding modules for

SH3 (SRC Homology 3) domain-containing proteins. Meq interacts with p53 through the ZIP domain of Meq and C-terminal tetramerization domain of p53 [74]. Interaction of Meq with p53 leads to a decrease in the transcriptional and apoptotic activities of p53 [74]. Interaction of Meq with Rb protein is probably through the LXCXE motif located at the end of the ZIP domain [114]. Meq localizes to cajal bodies and nucleolar periphery where, through the interactions with p53, Rb, CDK2, p27[Kip1] and Skp2, likely deregulates the cell-cycle checkpoints leading to transformation [12, 116].

Meq interacts with the transcriptional repressor protein (CtBP1) through PLDLS motif present at the amino acid terminus. Mutations introduced in the PLDLS motif of the *meq* gene completely abolished the interaction of Meq with CtBP1 and resulted in a complete loss in its oncogenic potential [113]. CtBP1 is recruited to the genetic loci by the DNA-binding proteins that have PLDLS motifs. Here the CtBP1 functions as a dimer and seemingly recruits a large number of proteins including Histone Deacetylases (HDAC), small Ubiquitin-like Modifier (SUMO) and H3-K9 Histone Methyltransferases that ultimately leads to repression [117, 118]. CtBP1 functions in the regulation of the development, differentiation, proliferation, apoptosis and cellular adhesion [118, 119]. Interaction of Meq with CtBP1 is supposed to be involved in the regulation of metastasis and apoptosis of transformed T cells. When chicken embryo fibroblasts (CEF) are infected with MDV, an increased accumulation of the nuclear accumulation of HSP-70 protein is observed. In addition, it is also observed that HSP-70 co-immunoprecipitated with Meq in transformed tumor cells [115]. HSP-70 plays roles in diverse functions including, inhibition of apoptosis and promoting cell survival [120, 121]. Meq has the ability to also regulate its expression, by binding or transactivation of its promoter.

5.3.3   Meq spliced products

Despite the full-length unspliced form of *meq*, at least two other splice variants were detected [122]. A 700 bp form of an alternative *meq* spliced transcript was named as Meq-sp or MeqΔC-BamL (and later described as Meq/vIL-8) [123]. The Meq-sp isoform encodes a 28kDa nuclear protein, and has been proposed to be a potential negative regulator for Meq oncogenic properties and it has been found to be expressed in MDV transformed lymphoid cell line, MKT-1 and a non-pathogenic vaccine strain, CVI-988 [124].

The dominant isoform, Meq/vIL-8 connects the bZIP domain of Meq to exons 2 and 3 of vIL8, an MDV encoded chemokine [125]. Peng and Shirazi reported that Meq/vIL-8 could bind to the AP-1 site with the consensus sequence of TGAGTCA when it dimerizes with cJun, but not with full length Meq [126]. They also reported that C-terminal of Meq/vIL8 has little transactivation activity in a CAT reporter assay and concluded it as a negative regulator of Meq. Subsequent

analysis via fluorescence resonance energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP) indicated that Meq does not interact with Meq/vIL-8 *in vivo* and they showed different nuclear mobilities [127]. In MDV-transformed cells as well as Meq expression cells, Meq localizes to the nucleus, nucleolus and at Cajal bodies. Within the nucleus, Meq migrates very quickly with mobility consistent with its function as a transcription factor. The spliced form of Meq, however, shows very different migration rates and appears to localize primarily to structural sites within the nucleolus, Cajal bodies, and nucleoplasm[127].

5.3.4   The major oncogene - *meq*

Meq, described as the major oncogene of MDV, is consistently expressed in MDV induced lymphomas. The oncogenic potential of Meq has been demonstrated with the overexpression in Rat-2 (rodent fibroblast) and DF-1 (immortalized chicken embryo fibroblast) cell lines. The overexpression resulted in morphological transformation, serum-independent growth [110], anchorage-independent growth and inhibition of apoptosis [74]. When the *meq* gene was deleted from the virus, no tumors were observed in chickens, indicating, the significance of Meq in transformation and lymphomagenesis [64, 102]. Nonetheless, given that the entire *meq* gene was deleted might also have an impact on other transcripts and proteins mentioned above that are regulated by Meq that could have caused this phenotype – absence of tumor formation. One example of this orchestrated effects of Meq that could have led to the demolishment of tumors in the *meq*-null experiment is the possibility of Meq to modulate vTR (Table 1). Meq can upregulate vTR by forming heterodimers with cellular protein c-Myc (Fig. 7B) [128, 129]. In my paper, [129], we show that Meq regulates vTR, and different point mutations in Meq impact vTR expression. Therefore, making it plausible that the *meq*-null virus had a substantial impact on vTR expression that led to this non-oncogenic phenotype.

5.4   MDV evolution

The continuous evolution of MDV remains a problem and a challenge for the poultry industries. When MD was first described, only mild inflammation was reported and, today, MD includes several other symptoms. The severity of the disease symptoms increased dramatically despite medical intervention strategies such as the introduction of vaccines. In this section, together with evolution, pathotypes and vaccines introduced will be discussed and is summarized in figure 8.
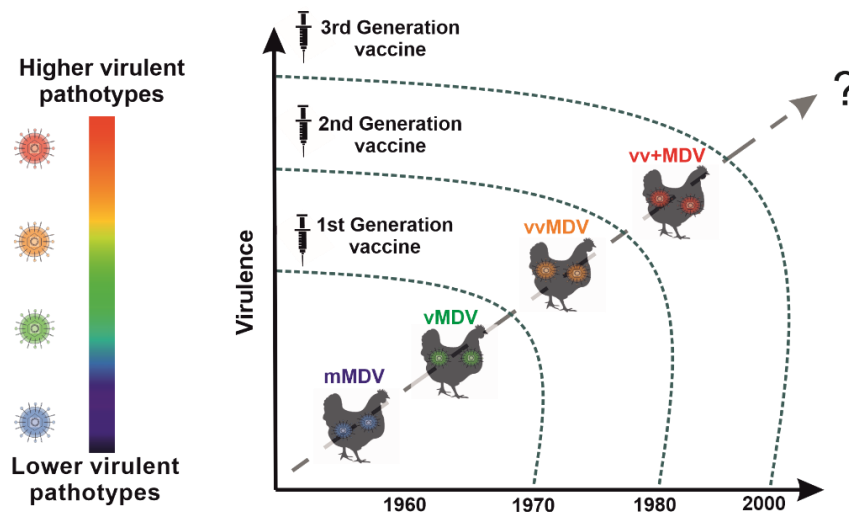
Figure 8: Schematic illustration of the stepwise evolution of MDV. MDV has increased in virulence over the past decades ranging from low to high virulent pathotypes. Despite the introduction of vaccines, strains continue to evolve towards higher virulence. Obtained from [130]

Despite the control through vaccination, MDV field strains continue to emerge that have an increased virulence. Virulence here is defined as the ability of the virus to replicate, cause disease, affect the host defence, and increased spread [131]. The clinical picture changed dramatically since almost 100 years ago and therefore also the need for introducing pathotype nomenclature. The MDV strains are currently classified into four pathotypes based on their pathogenicity in experimental infections in vaccinated and unvaccinated chickens [132, 133]. These pathotypes range from mild (m), virulent (v), and very virulent (vv), to the most virulent, very virulent plus (vv+) (Fig. 8). The initial mMDV strains, which were mostly assumed to have occurred before the 1950s, only cause a rather mild neurological disease. The vMDV emerged in the 1950s and has increased in virulence. These vMDV strains, such as JM102, induced tumors in various organs and led to an approximate 40% mortality rate [133]. The reason for the jump in virulence from mMDV to vMDV is unclear but it has been hypothesized that the increase in the density of chickens in the broiler industry and intensifying farming techniques [134]. To control and reduce disease symptoms, the first vaccine against MDV was introduced in the 1970s - the HVT vaccine [12, 46]. Soon after the introduction of the HVT vaccine, the jump in virulence again is observed surfacing another pathotype, vvMDV. The vvMDV strains, such as RB-1B, causes a transient paralysis in most chicken lines and high tumor incidence with high mortalities in unvaccinated flocks [48]. To control chickens against the vvMDV pathotypes, the bivalent vaccine, composed of HVT and SB-1, was introduced in the early 1990s [43]. The vaccine barrier did not protect long, and the fatal vv+MDV pathotypes emerged and circulated the population [43]. The vv+MDV pathotype, for example, the N-strain frequently

causes severe brain oedema, stunting and death within a few days in unvaccinated animals, and tumor lesions, even in vaccinated chickens [135-137]. The (CVI) vaccine was introduced in the mid-1990s to protect chickens up to vv+MDV pathotypes [138] and is today still used as the golden standard vaccine in chickens against MDV (Fig. 8). How long the CVI vaccine will still protect and remains a concern and a future challenge.

5.4.1 Determinants for MDV evolution

The mechanism facilitating the evolution of MDV strains towards higher virulence remains mostly unknown. Various factors, or pressures, could be associated with this evolution and increase in virulence. Some of these potential factors will be discussed individually.

5.4.1.1 Farming practices

Farming practices and techniques have changed dramatically over the last decades and could also be a potential driver for the increase in virulence [139]. The poultry industries up to the mid-1900s comprised of backyard farming. The population densities in these farming styles were very low, with low growth rate and egg production [139]. During this time, MDV was not considered as a threat, even though outbreaks were reported in different parts of the world. During this time, the mild pathotype resided, mMDV, and of not that concern. Since the 1960s, the poultry industry had significant changes and the poultry production was elevated aimed at higher productivity – meaning increased population density of chickens [134]. A large number of animals (>20,000) in a confined space and the dramatically shortened average lifespan of chickens with industrialization allow efficient virus spread between individuals [140]. In a cohort study by Rozins et al., it has been demonstrated that evolution towards higher virulence can be plausible with (i) longer cohort durations, (ii) larger flock sizes, and (iii) less intensive cleaning of the barn [134]. These intensive farming techniques are the most plausible factors for the observed increase in virulence of circulating field strains [134]. This could be at least true for the first observed jump in virulence from mMDV to vMDV (Fig. 8), as no vaccines were introduced yet and the only pressure was increased farming.

5.4.1.2 MDV vaccination

Examination of virus evolution revealed, apart from the first jump in virulence in the 1960s due to intensive farming. The occurrence of all subsequent waves of the evolution of virulence had been linked to the introduction of different generations of vaccines (Fig. 8)[141].

The live-attenuated vaccines that were developed against MD (Table 2) prevent clinical symptoms but have many inherent drawbacks, and little was known about how these vaccines would impact evolution. One major downside is that all the vaccines that were introduced are 'leaky vaccines' – vaccines that keep the host alive do not prevent infection or replication and still allow transmission. This phenomenon is also referred to as imperfect vaccination [142]. If all the vaccines introduced were sterilizing, and transmission is blocked, the evolution of MDV might not even be a subject now. Now we keep the host alive with leaky vaccines and therefore allowing virulent strains to circulate into the population. If vaccines were not introduced in the first place, natural selection would have taken its natural course, and the virulent pathogens would not reside and instead would have been removed from the population [143] (Fig. 9).

A great article demonstrating imperfect vaccination is a study from Read *et al*. They showed that when birds are infected with high virulent strains (strains from the vv+MDV pathotype), they all died within ten days before substantial shedding has begun. No sentinel birds (birds hosted with the infected birds) were infected or died. In contrast, when the birds were vaccinated and then infected with these higher virulent strains, they survived for much longer and therefore allowing substantial shedding – putting unvaccinated individuals at higher risk [144].
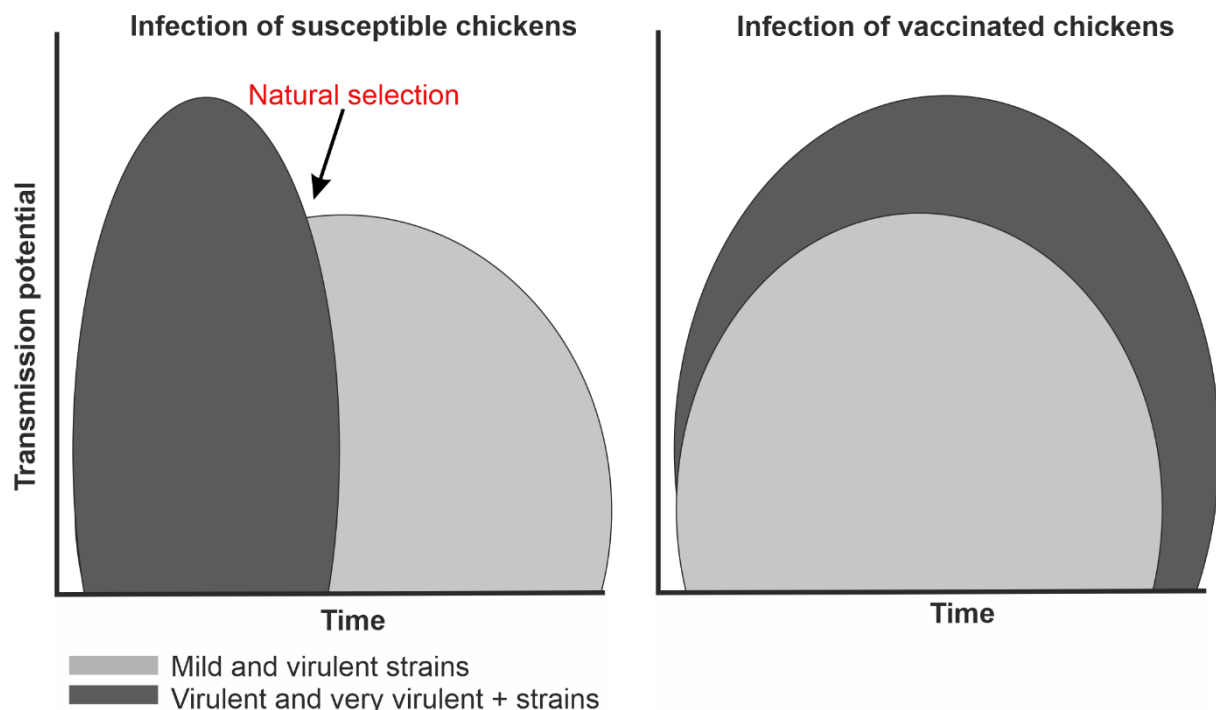
Figure 9: Illustration of the imperfect vaccination theory. Infection of susceptible chickens with higher virulent MDV would have killed the host, through natural selection, and further spread of these strains would have been prevented. In contrast, infection of vaccinated hosts prolongs survival and allows circulation of the higher virulent strains, and evolution continues.

It was recently demonstrated with transmission experiments that vaccination with a leaky vaccine substantially reduces viral load in both vaccinated individuals and unvaccinated contact individuals they infect [145]. While this article shows some positive consequences, these 'imperfect' vaccines still allow virus spread and evolution in the field and are associated with the emergence of field strains with increased virulence. The efficiency of virus shedding is also influenced by chicken breeds, farm hygiene, and biosecurity [146]. As far it goes, vaccines have a stimulating effect for today's observed diversity and evolution towards higher virulence. If the viral evolution is allowed to continue at the present rate with the current type of vaccines and the vaccination strategies, MD could become a significant economic problem for the poultry industry again.

5.4.1.3 Changes in the MDV genome

One of the major goals pertaining to the MDV evolution is to identify the genes that changed and to improve vaccines and control measures. MDV strains are currently classified into four pathotypes based on their pathogenicity in vaccinated and unvaccinated chickens, as mentioned previously. During evolution, there are changes within these pathotypes, in the MDV genome, documented that could contribute to the increase in virulence and allow the virus to overcome vaccine protection [130, 147-149].

Comparative bioinformatic studies have been performed to identify genes that could contribute to the increase in MDV virulence. In a study by Trimpert et al. [148], their temporal phylogeny of MDV yielded an evolutionary rate of approximately 1.6×10^{-5}, which is in line with rates that have been recorded for similarly sized dsDNA viruses such as variola ($\sim 9 \times 10^{-6}$) [150] and myxoma virus (MYXV) ($\sim 1 \times 10^{-5}$) [151]. These rates are surprisingly higher than what is typically expected for dsDNA viruses. Nonetheless, candidates, or hotspots in open reading frames (ORFs), have been identified that appeared to be explicitly associated with higher virulent strains and include: *meq* (MDV076), ICP4 (MDV084), and ICP27 (MDV068). The *meq* gene was also identified as 'greater-than-average' amount of specific mutations [148].

The number of candidates genes were expanded with MDV isolates from recent disease outbreaks [149]. Additional candidate genes are UL6 (MDV018), UL15 (MDV027), UL36 (MDV049), UL37 (MDV050), UL41 (MDV054), and R-LORF8. In this study by Dunn et al., they also find a clear separation of low virulent strains from higher virulent strains, in their phylogenetic tree [149]. These changes identified in the genome could be used as pathotype markers in the future.

5.4.2 The contribution of *meq* in MDV evolution

The strongest association with the observed increased virulence is the polymorphisms identified in the *meq* gene (Fig. 10). Despite the rather low evolutionary rate of double-stranded DNA viruses [152, 153], it has been reported that the *meq* gene is evolving at a much faster rate than most genes in double-stranded DNA viruses [154]. Computerized models show that point mutations in the *meq* gene correlate with the increase in virulence and have evolved under positive selection [154]. Point mutations in the N-terminus (position 71 and 77; Fig. 10) are present in *meq* from the lower virulent strains, such as JM102. The lower virulent *meq* genes also have a differing number of proline-rich repeats in their transactivation domain. The lower virulent *meq* gene, contains five PRR in their C-terminus, whereas vvMDV (e.g. RB-1B) and vv+MDV strains (e.g. MK) possess only three PRR [147]. This phenomenon is not entirely true as we observe in contrast that a *meq* gene expressed from the CVI vaccine that composes of five PRR is still virulent and oncogenic when expressed from the RB-1B strain [129]. The mutations that accumulated in the C-terminus of *meq*, as seen for the vv+MDV strains, appear to be unique and correlate with virulence and the putative role of *meq* in evolution (Fig. 10) [154].

Specific mutations in:
Lower virulent strains and higher virulent strains
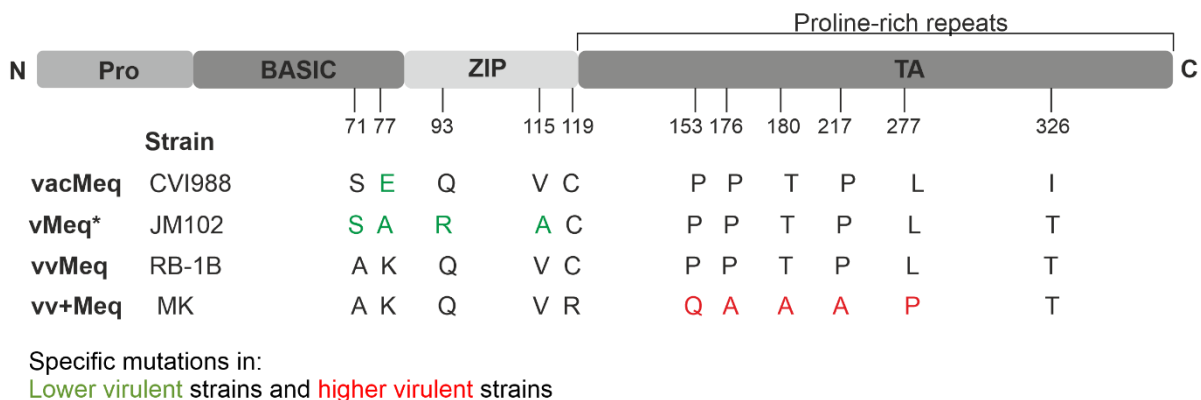
Figure 10: A schematic representation of the evolutionary acquired point mutations in the *meq gene*. The point mutations in *meq* for each pathotype, and an example of an MDV strain of each pathotype is shown.

The *meq* isoforms of different pathotypes (vMDV, vvMDV, vv+MDV and the CVI988 vaccine) were inserted individually into the very virulent RB-1B strain and replaced the original *meq* gene. Virus replication was not significantly affected *in vitro* and *in vivo*. However, insertion of less virulent *meq* isoforms (vacMeq and vMeq) either abrogated or severely impaired MDV pathogenesis while higher virulent *meq* isoforms (vvMeq and vv+Meq) readily caused disease and tumors. Even in vaccinated chickens, viruses harbouring the higher virulent *meq*s caused disease and efficiently shed into the environment. Strikingly, only viruses harbouring the

vv+Meq were able to overcome vaccine protection and cause tumors in vaccinated animals. Furthermore, we show that the point mutations in *meq* isoforms of higher virulent MDV strains help the virus to overcome innate cellular responses, potentially contributing to vaccine failure. Overall, our data show that the evolutionary adaptations in *meq* alone substantially contribute to the increased virulence, vaccine resistance, and enhanced transmission – therefore, together with vaccination, playing a central role in the evolution of MDV.

5.5   Project aims

In this thesis I address two specific aims. Work from both aims are published and the results are summarized:

1. **Investigate the contribution of the two isoforms of the major oncogene encoded by the golden standard vaccine in its attenuation.**

   Interestingly, commercial vaccine stocks, CVI988, express two predominant isoforms of the major MDV oncogene *meq*. The vaccine expresses these oncogenes but is not oncogenic. The longer forms (Lmeq) are identical to the short *meq* (Smeq) form, but with an in-frame insertion of 180 bp (60 amino acids) in the transactivation domain. To determine the role of the *meq* isoforms expressed in the CVI vaccine, we replaced the *meq* gene in the very virulent MDV strain RB-1B with either the Smeq (vSmeq) or Lmeq (vLmeq) isoform. Intriguingly, we found that viruses with these vaccine-derived *meq* isoforms strikingly differ in pathogenesis and oncogenesis in infected chickens.

   The paper was published in mSphere, 'A Common Live-Attenuated Avian Herpesvirus Vaccine Expresses a Very Potent Oncogene' and is in Section 7 of this thesis.

2. **To determine the contribution of the major oncogene *meq* in MDV virulence and vaccine resistance.**

   Recombinant viruses were generated based on a well-characterized MDV strain, RB-1B. The recombinant viruses express *meq* isoforms from different pathotypes to investigate Meq-specific contributions directly. I analysed the replication and the measure of the spread of the recombinant viruses *in vitro*. Also, I determined the contribution of the *meq* isoforms in MDV pathogenicity and their role in the resistance to vaccines *in vivo*. The paper was published in PLoS Pathogens, 'Distinct polymorphisms in a single herpesvirus gene are capable of enhancing virulence and mediating vaccinal resistance.' and is in Section 8 of this thesis.

6   References

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7   A Common Live-Attenuated Avian Herpesvirus Vaccine Expresses a Very Potent Oncogene

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Vaccines play a crucial role in the protection of animals and humans from deadly pathogens. The first vaccine that also protected against cancer was developed against the highly oncogenic herpesvirus Marek's disease virus (MDV). MDV infects chickens and causes severe immunosuppression, neurological signs and fatal lymphomas, a process that requires the virus-encoded oncogene, *meq*. The most frequently used Marek's disease vaccine is the live-attenuated CVI988/Rispens (CVI) strain, which efficiently protects chickens and prevents tumorigenesis. Intriguingly, CVI expresses at least two isoforms of *meq*; however, it remains unknown to what extent these isoforms contribute to virus attenuation. In this study, we individually examined the contribution of the two CVI-*meq* isoforms to the attenuation of the vaccine. We inserted the respective isoforms into a very virulent MDV (strain RB-1B), thereby replacing its original *meq* gene. Surprisingly, we could demonstrate that the longer isoform of *meq* strongly enhanced virus-induced pathogenesis and tumorigenesis, indicating that other mutations in the CVI genome contribute to virus attenuation. On the contrary, the shorter isoform completely abrogated pathogenesis, demonstrating that changes in the *meq* gene can indeed play a key role in virus attenuation. Taken together, our study provides important evidence on attenuation of one of the most frequently used veterinary vaccines worldwide.

7.2 Importance

Marek's disease virus (MDV) is one of several oncogenic herpesviruses and causes fatal lymphomas in chickens. The current "gold standard" vaccine is the live-attenuated MDV strain CVI988/Rispens (CVI) that is widely used and efficiently prevents tumor formation. Intriguingly, CVI encodes two predominant isoforms of the major MDV oncogene *meq*, one variant with a regular size of *meq* (*Smeq*) and one long isoform (*Lmeq*) harboring an insertion of 180 base pairs in the transactivation domain. In our study, we could break the long-standing assumption that the *Lmeq* isoform is an indicator for virus attenuation. Using recombinant viruses that express the different CVI-*meq* isoforms, we could demonstrate that both isoforms drastically differ in their ability to promote pathogenesis and tumor formation in infected chickens.

7.3 Introduction

Marek's disease virus (MDV) is a lymphotropic alphaherpesvirus that infects chickens and causes 1 to 2 billion-dollar losses worldwide annually (1). MDV causes a variety of clinical symptoms including immunosuppression, ataxia, chronic wasting, and formation of T cell lymphoma in various visceral organs (2). MDV vaccines are widely used to protect chickens from this deadly disease and were the first vaccines that prevented cancer, long before this

approach was applied to human medicine (3, 4). The current "gold standard" vaccine is the live-attenuated MDV strain, CVI988/Rispens (CVI) that efficiently protects chickens against very virulent field strains (5, 6). Intriguingly, commercial vaccine stocks express two predominant isoforms of the major MDV oncogene meq (7). The Meq protein is a basic leucine zipper (bZIP) protein that is essential for tumorigenesis, represses apoptosis, dysregulates the cell cycle and modulates cellular and viral gene expression (8-10). One of the cellular targets is c-myc that influences the expression of MDV-encoded viral telomerase RNA (vTR) (11), a non-coding RNA that plays an important role in tumorigenesis (12). One of the two CVI-encoded meqs has the same size as its counterparts in virulent MDV strains, but harbors several point mutations (Smeq; Fig. 1A) (13). The other isoform is identical to Smeq except for an in-frame insertion (Lmeq) of 180 base pairs (60 amino acids) in the carboxy-terminal transactivation domain (14, 15). The insertion consists of proline-rich repeats that likely arose from a domain duplication (16). It has been shown that these two CVI-meqs are weak transactivators of viral gene expression, which could contribute to the non-oncogenic phenotype of the CVI virus in chickens (7). To determine the role of the *meq* isoforms encoded in the CVI vaccine, we replaced the meq gene in the very virulent MDV strain (RB-1B) with either the Smeq (vSmeq) or Lmeq (vLmeq) isoform. Intriguingly, we found that viruses with these vaccine-derived meq isoforms strikingly differ in pathogenesis and oncogenesis in infected chickens.

7.4   Results

7.4.1   Generation of recombinant viruses

To examine the contribution of the two meq isoforms in the attenuation of the CVI vaccine, we generated recombinant viruses that harbor either the Smeq or Lmeq isoform. CVI-meq isoforms were individually inserted into the very virulent RB-1B MDV strain instead of its native *meq* gene (Fig. 11A). We confirmed the resulting clones with PCR, RFLP, Sanger sequencing and Illumina MiSeq whole genome sequencing with a ~1000-fold coverage to confirm the integrity and the sequence of the entire virus genome.
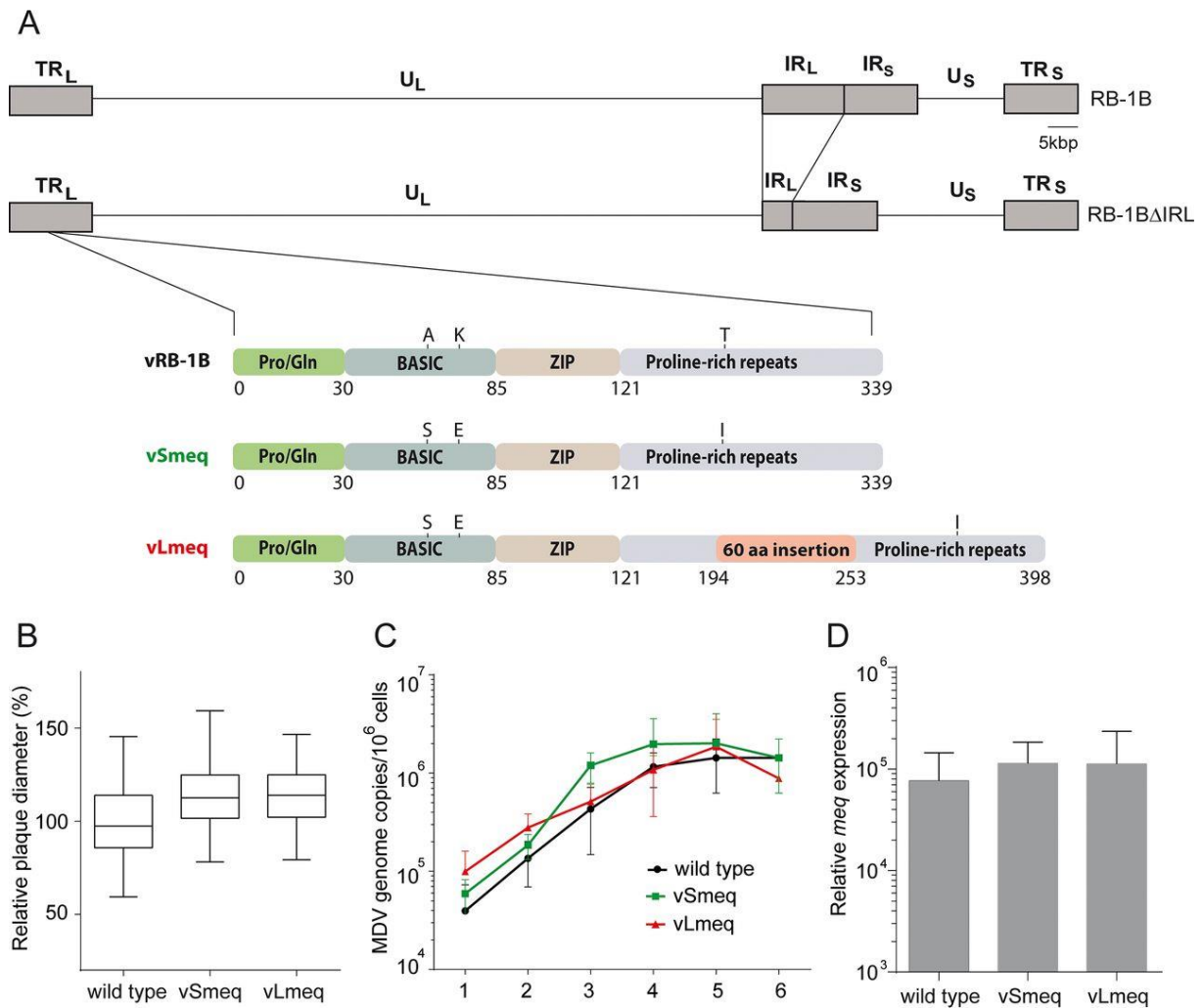
Figure 11: Construction and in vitro characterization of recombinant viruses. (A) Schematic representation of the MDV RB-1B genome with a focus on the different meq genes including mutations in the basic domain and proline-rich repeats. (B) Virus spread was assessed by plaque sizes assays (n = 150) and replication by (C) multi-step growth kinetics 1 to 6 days post infection. Spread and replication of indicated recombinant viruses were not statistically different (p > 0.05, one-way ANOVA). (D) meq expression levels in infected chicken embryo cells relative to GAPDH were not statistically different (p > 0.05, Kruskal-Wallis). Data are shown as the means of a minimum of three independent experiments with standard deviation (SD; error bars).

7.4.2 *In vitro* characterization of recombinant viruses

To determine if insertion of the *meq* isoforms affects virus replication, we performed plaque size assays and multi-step growth kinetics. We could demonstrate that the recombinant viruses efficiently replicate similar to the parental (wild type) virus (Fig. 11B and 11C), indicating that the insertion of the CVI-*meq* isoforms in a very virulent RB-1B does not affect virus replication

in vitro. To ensure that both CVI-*meq* isoforms are efficiently expressed, we quantified the expression levels of *meq* in virus-infected cells by RT-qPCR. We could demonstrate that the *meq* gene expression of vSmeq or vLmeq was comparable to the *meq* expression in the wild type virus (Fig 11D).

7.4.3 Replication of recombinant viruses *in vivo*

To assess if the CVI-meq isoforms affect virus replication, pathogenesis and/or tumor formation *in vivo*, we infected one-day old Valo SPF chickens subcutaneously with 4000 plaque forming units of wild type virus, vSmeq or vLmeq. Viral load in the blood was assessed by qPCR and revealed that the recombinant viruses replicated efficiently in infected animals (Fig. 12A), indicating that the CVI-meq isoforms do not affect virus replication *in vivo*. Moreover, all viruses efficiently spread to co-housed contact chickens, confirming that the insertion of Smeq and Lmeq did not significantly influence virus transmission to naïve contact chickens (Fig. 12B).
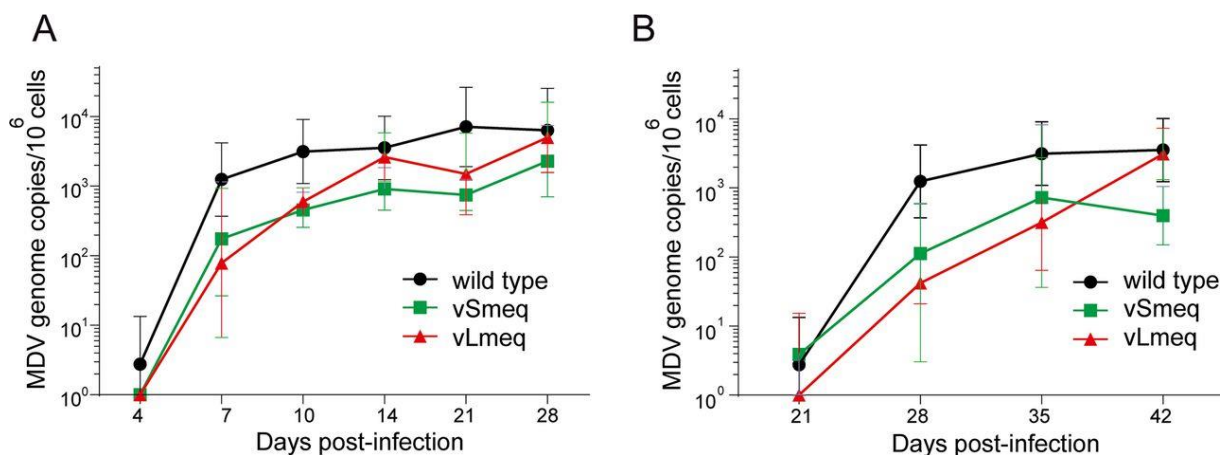
Figure 12: Replication of recombinant viruses *in vivo*. MDV genome copies were detected in blood samples of (A) chickens infected with indicated viruses as well as in (B) contact chickens infected via the natural route by qPCR. Genome copy numbers were not statistically different (p > 0.05, Kruskal-Wallis test).

7.4.4 Pathogenesis and tumorigenesis of recombinant viruses *in vivo*

We monitored the animals for clinical symptoms and tumor development over the course of the experiment. The recombinant virus harboring the *Smeq* isoform did not cause any disease (Figure 13A); indicating that the small number of amino acid changes in *meq* can indeed attenuate the virus. Surprisingly, an increase in disease was observed in animals infected with vLmeq (Fig. 13A), revealing that the 180 bp insertion in *Lmeq* enhances the potency of the

meq oncogene. Almost all the chickens infected with vLmeq succumbed to disease (96%), while a lower incidence was observed for wild type virus (84%). Consistently, contact animals infected via the natural route with vLmeq had a significantly higher disease incidence (45%) compared to the wild type control (9%). No pathogenicity was observed in the vSmeq contact animals (Fig 13C). We confirmed the increased disease incidence caused by vLmeq in a second independent animal experiment (Fig. 13E).

In addition, we quantified the number of animals that developed macroscopic tumors. Remarkably, the virus harboring the *Lmeq* showed the highest tumor incidence (88%) when compared to wild type virus (76 %; Fig. 3B), while no tumors were present in the vSmeq group (Fig. 13B). In line with this, an increase in the tumor incidence was also observed in vLmeq contact chickens (64%) compared to the wild type virus group (45%; Fig. 13D). In the case of vSmeq, none of the contact animals developed tumors, confirming the data of the experimentally infected animals (Fig. 13D). This increased tumor incidence was confirmed by an independent animal experiment (Fig. 13F). Intriguingly, tumor dissemination was also enhanced in vLmeq-infected chickens as more organs harbored tumor lesions per animal (Fig. 14A), highlighting the high oncogenic potential of the *Lmeq* isoform.
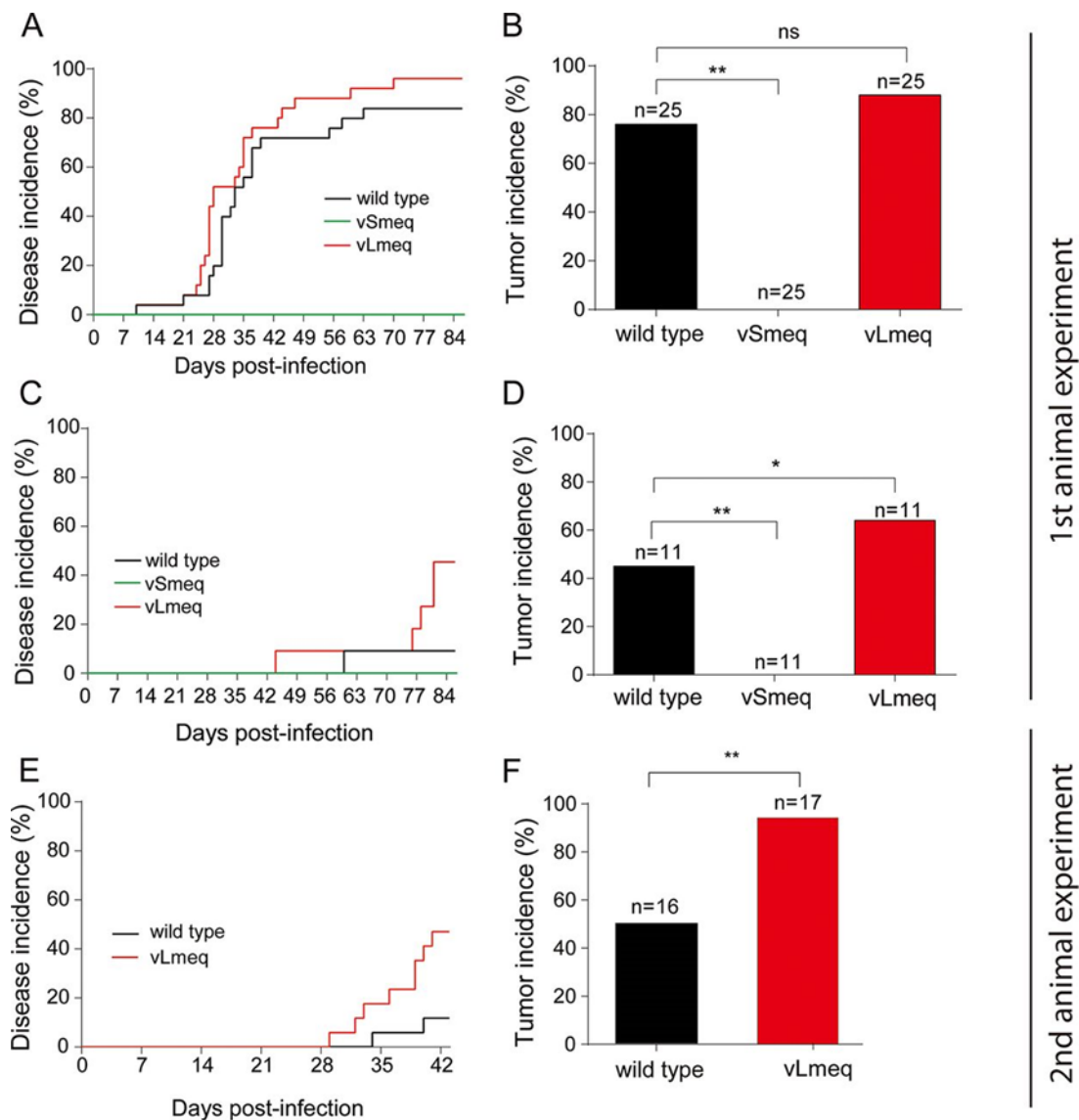
Figure 13: *In vivo* characterization of recombinant viruses. Kaplan–Meier analyses of Marek's disease incidence in chickens infected with indicated recombinant viruses (A and E) and naïve chickens infected via the respiratory route (C) of two independent animal experiments. Statistical analyses using the log-rank test revealed a significant difference between vLmeq and vSmeq in A (p = 0.0001) and in C (p = 0.0142). As significant difference between vLmeq and wild type was observed in C (p = 0.0142) and E (p = 0.02). Tumor incidences are shown as percentage per group in infected chickens (B and F) and in naïve contact chickens (D). Asterisks indicate significant differences (*p<0.05 and ** p<0.0125; Fisher's exact test). ns = not significant.

7.4.5 Role of CVI-*meq* isoforms in vTR expression

To provide a possible mechanistic explanation for the increased oncogenic potential of the *Lmeq* isoform, we examined the expression of vTR in cells infected with wild type, vSmeq and

vLmeq using RT-qPCR. Remarkably, the *Lmeq* isoform significantly upregulated vTR by 12-fold compared to the already highly expressed RB-1B-encoded *meq* and *Smeq,* suggesting that the 180 bp insertion in the transactivation domain strongly influences vTR expression and in turn transformation efficiency in chickens (Fig. 14B). The increase in vTR copies thereby provide a reasonable explanation for the increased tumor promoting activity of the *Lmeq* isoform in chickens.
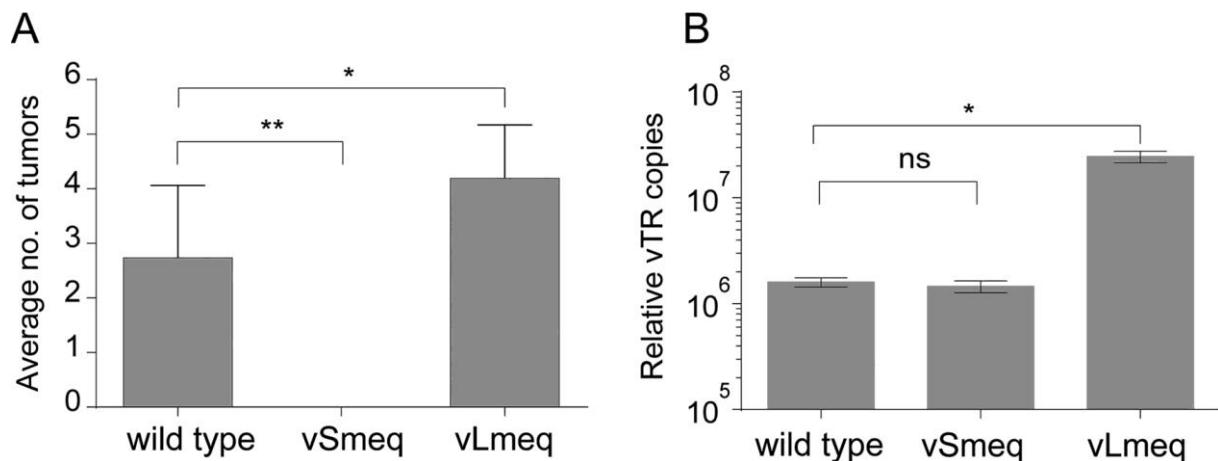
Figure 14: Analysis of tumor dissemination and vTR expression of recombinant viruses. (A) Mean number of organs with gross tumors per animal in the indicated groups (1st animal experiment). Significant differences are indicated by asterisks (*p < 0.05 and ** p < 0.0125; Fisher's exact test). (B) Mean genome copies of vTR for the indicated viruses are shown relative to the cellular GAPDH (*p < 0.05, Kruskal-Wallis test, n = 3). ns = not significant.

7.5   Discussion

The CVI vaccine is the current "gold standard" vaccine against MDV and efficiently protects against very virulent MDV strains. In previous studies, we and others observed that at least two meq isoforms are expressed in vaccine stocks (7, 17, 18). The two predominant meq isoforms expressed from the vaccine are Smeq and Lmeq and were considered weak oncogenes due to that fact that the CVI vaccine does not induce tumors. These two meq isoforms differ by an insertion of 180 bp in the transactivation domain (Lmeq) compared to the Smeq. Both isoforms are only encoded in the CVI vaccine and have not been detected in other MDV strains, such as RB-1B and MD5 [17]. In this study, we individually delineated the contribution to attenuation and the oncogenic potential of Smeq and Lmeq.

First, we performed growth kinetics and plaque assays to determine if the insertion of Smeq and Lmeq affects growth properties, as the oncogene is also expressed during lytic replication.

Our results show that replacing the original meq of the very virulent RB-1B strain with the CVI-meq isoforms does not significantly influence its growth properties in vitro (Fig. 11B and C). The recombinant viruses expressing CVI-meq isoforms even replicated slightly better compared to the wild type. This is an intriguing observation, as CVI replicates more efficiently compared to virulent MDV strains (4), suggesting that meq isoforms partly contribute to this growth advantage (Fig. 11B). However, more work is needed to fully understand why the CVI replicates better compared to other virulent MDV strains. We could also demonstrate that the meq gene expression of vSmeq or vLmeq was comparable to its counterpart in the wild type virus (Fig. 11D), confirming that the observed effects in this study are not due to differences in the meq expression levels.

Next, we characterized the recombinant viruses *in vivo*. One-day old chickens were infected with wild type and recombinant viruses to determine the role of Smeq and Lmeq in pathogenesis and assess their oncogenic potential. Insertion of the Smeq completely abrogated MDV pathogenesis and oncogenesis in the chickens. The inserted Smeq only differs by three amino acid changes compared to the wild type meq from the very virulent RB-1B MDV strain. It is intriguing that this small number of amino acid changes in meq could completely attenuate the very virulent RB-1B strain. In contrast, the pathogenesis and oncogenesis were severely enhanced upon insertion of the Lmeq. Moreover, it is remarkable that only an insertion of 180 bp in the proline-rich region into Smeq drastically enhanced disease incidence (Fig. 13A) and tumorigenesis (Fig. 13B and 14A) in infected animals. The same trend was observed in contact chickens that were infected via natural route. All recombinant viruses were able to spread efficiently to contact chickens (Fig. 12B). We observed a slight delay with vLmeq, however this was not statistically significant. Only wild type and vLmeq viruses were able to cause disease (Fig. 13C) and tumors (Fig. 13D) in the contact chickens. To confirm these exciting results, we performed a second animal experiment using a different chicken line. This independent animal confirmed the disease incidence (Fig. 13E) and the high oncogenic potential of vLmeq (Fig. 13F).

To explain the *in vivo* data, we focused on a viral gene that i) plays a role in transformation and ii) is regulated by the Meq protein. It has previously been shown that the Meq protein modulates the expression levels of vTR, which plays a crucial role in MDV-induced lymphomagenesis and tumor dissemination via cellular c-myc (11, 12, 19). We quantified the expression levels of vTR in cells infected with the respective viruses and could show that expression of the Lmeq isoform significantly upregulates vTR compared to the wild type meq and Smeq. This suggest that the 180 bp insertion in the transactivation domain of Lmeq strongly influences vTR expression via c-myc (Fig. 14B), and could therefore explain the

increased tumorigenesis observed in chickens infected with vLmeq in both animal experiments.

Here we demonstrate that Lmeq alone increases tumorigenesis *in vivo* and that this is due to the 180 bp insertion in the transactivation domain. This insertion extends the six proline-rich regions (PRR) to nine PRR and therefore changes its transactivation potential of genes involved in pathogenesis and oncogenesis, as shown for vTR in this study.

Moreover, our data on Lmeq suggests that other mutations in the CVI genome contribute to attenuation of the vaccine, resulting in a fully attenuated virus despite the presence of this potent oncogene. Strikingly, CVI harbors a number of mutations/indels and amino acid changes compared to virulent strains as published previously (16); however, whether these changes have an effect on oncogenesis remains elusive. An alternative explanation for the apathogenic nature of CVI would be that the oncogenic potential of Lmeq is masked by heterodimerization with the Smeq isoform. It remains unknown if Smeq and Lmeq can repress each other, an aspect that will be addressed in future studies. In previous studies, co-expression of CVI Smeq or Lmeq with the oncogene of the MD5 strain resulted in a suppression of the meq promoter (7, 20). However, this suppressive effect was not observed in cells infected with our recombinant viruses expressing Smeq and Lmeq individually (Fig. 1D).

In summary, we assessed the contribution of the CVI-meq isoforms to the attenuation of the vaccine strain. Our study revealed that the two CVI-meq isoforms allow efficient virus replication; however, they vastly differ in their tumor promoting properties. Strikingly, the Lmeq isoform enhances MDV pathogenesis and oncogenesis of a very virulent MDV strain, while insertion of the Smeq isoform completely abrogated MDV pathogenesis. Our results on the Lmeq isoform breaks with the long-standing assumption that it is a marker for attenuation (21-23) and demonstrates that other mutations in the CVI genome contribute to its attenuation.

7.6   Materials and methods

7.6.1   Cells

Primary chicken embryo cells (CEC) were prepared from 11-day old specific-pathogen-free (SPF) chicken embryos (Valo BioMedia, Germany) as described previously (24). Cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% bovine serum and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) at 37°C in a humidified atmosphere containing 5% CO_2.

7.6.2 Generation of recombinant viruses

We generated recombinant viruses each harboring either Smeq or Lmeq (GenBank accession no. 8AY243333 and 8AY243338) derived from the commercial CVI988/Rispens vaccine strain (5). Smeq and Lmeq were inserted into a bacterial artificial chromosome (BAC) of the very virulent MDV strain RB-1B that lacks most of the internal repeat long region (IRL; pRB-1BΔIRL), which is rapidly restored upon virus reconstitution. (25). Therefore, only one copy of the meq region had to be manipulated by two-step Red-mediated mutagenesis as described previously (26, 27), while the resulting recombinant virus contained the meq substitution in both loci as confirmed by PCR (25). First, we deleted the meq and then introduced either Smeq (vSmeq) or Lmeq (vLmeq). We confirmed the BAC clones by RFLP, PCR, Sanger and Illumina MiSeq sequencing to verify the integrity and the sequence of the entire virus genome. The primers used for mutagenesis and sequencing are listed in Table 1. All viruses were reconstituted and propagated on CEC and stocks were prepared as described previously (25, 28)

7.6.3 Plaque size assays and multi-step growth kinetics

The spread and replication of the recombinant viruses were first analyzed by plaque size assays as described previously (29). Briefly, one million CEC were infected with 100 plaque-forming units (pfu) of the recombinant viruses and cells were fixed at 6 days post infection (dpi). Images of randomly selected plaques (n=50) were taken and plaque areas were determined using Image J software (NIH).

Plaque size data was confirmed by qPCR-based multi-step growth kinetics as described previously (29). Briefly, one million CEC were infected with 100 plaque-forming units (pfu) of the recombinant viruses and virus replication assessed by qPCR over 6 days of infection. Primers and probes specific for the MDV infected cell protein 4 (ICP4) and chicken inducible nitric oxide synthase (iNOS) are shown in Table 1. Virus genome copies were normalized against the chicken iNOS gene as published previously (30).

7.6.4 Quantitative reverse transcription PCR (RT-qPCR)

To ensure that the CVI-meq isoforms are expressed comparable to its counterpart in wild type RB-1B, we quantified the expression levels of the different meqs using RT-qPCR as previously described (31). Briefly, total RNA was extracted from virus-infected CEC using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. The samples were treated with DNase I (Promega) and cDNA was generated using the High-Capacity cDNA Reverse

Transcription Kit (Applied Biosystems). meq expression levels were normalized to the expression levels of cellular GAPDH. We also used this approach to examine the expression of vTR in cells infected with wild type, vSmeq and vLmeq by RT-qPCR. The vTR expression levels were normalized to the expression levels of cellular GAPDH (32). Primers and probes used for qRT-PCR are shown in Table 1.

Table 4: Primers and probes used for construction of recombinant viruses, DNA sequencing and qPCR

Construct		Sequence 5' – 3'
meq kana_in (transfer construct)	for	AATTCGAGATCTAAGGACTGAGTGCACGTCCCTGTAGGGATAACAGGGTAATCGATTT
	rev	GTCCTTAGATCTCGAATTTCCTTACGTAGGGCCAGTGTTACAACCAATTAACC
Δ*meq* (deletion of RB-1B *meq*)	for	CAGGGTCTCCCGTCACCTGGAAACCACCAGACCGTAGACTGGGGGGACGGATCGTCAGCGGTAGGGATAACAGGGTAATCGATTT
	rev	GGGCGCTATGCCCTACAGTCCCGCTGACGATCCGTCCCCCCAGTCTACGGTCTGGTGGGCCAGTGTTACAACCAATTAACC
IRL restoration (sequencing)	for	CGAACGGAATGTACAACAGCTTGC
	rev	GATAAGACACTTTCCCACTCATAC
MDV_*meq* (insertion of CVI-*meqs*)	for	ATGTCTCAGGAGCCAGAGCC
	rev	GGGTCTCCCGTCACCTGG
ICP4 (qPCR)	for	CGTGTTTTCCGGCATGTG
	rev	TCCCATACCAATCCTCATCCA
	probe	FAM- CCCCCACCAGGTGCAGGCA-TAM
iNOS (qPCR)	for	GAGTGGTTTAAGGAGTTGGATCTGA
	rev	TTCCAGACCTCCCACCTCAA
	probe	FAM- CTCTGCCTGCTGTTGCCAACATGC-TAM
meq (RT-qPCR	for	TTGTCATGAGCCAGTTTGCCCTAT
	rev	AGGGAGGTGGAGGAGTGCAAAT
	probe	GGTGACCCTTGGACTGCTTACCATGC
	for	CCTAATCGGAGGTATT GATGGTACTG

vTR (RT-qPCR)	rev	CCCTAGCCCGCTGAAAGTC
	probe	FAM-CCCTCCGCCCGCTGTTTACTCG-TAM
GAPDH (RT-qPCR)	for	GAAGCTTACTGGAATGGCTTTCC
	rev	GGCAGGTCAGGTGAACAACA
	probe	FAM-TGTGCCAACCCCCAAT-TAM

a for, forward primer; rev, reverse primer.

b FAM, 6-carboxyfluorescein; TAM, TAMRA.

7.6.5 *In vivo* characterization of recombinant viruses

The replication properties, pathogenesis and tumorigenesis of the recombinant viruses was assessed in specific pathogen free (SPF) chickens as described previously (31). In the first animal experiment, one-day old Valo SPF chickens (Valo BioMedia) were randomly distributed into three groups. The chickens were infected subcutaneously with 4000 pfu of the wild type (n=25), vSmeq (n=25) and vLmeq (n=25). Each group was co-housed with 11 non-infected contact animals to assess the natural transmission of the respective virus from experimentally infected birds. The animal experiment was approved by the Landesamt für Gesundheit und Soziales in Berlin, Germany (LAGeSo; approval number G0294-17) and was conducted according to relevant national and international guidelines for humane use of animals. Animals were monitored daily for clinical symptoms throughout the 86-day experiment.

The phenotype of the vLmeq was confirmed in a second, independent animal experiment. White leghorn chickens (Sunrise Farms, Inc., Catskill, NY) were inoculated with a 1000 pfu of either the wild type (n=16) or vLmeq (n=17). This animal experiment was approved by the agricultural animal care and use committee (AACUC; approval number (22) 05-23-13b-R). Animals were monitored for clinical symptoms throughout the 43-day experiment. To eliminate bias, the examining veterinarian had no knowledge of the viruses in the different groups. All chickens were humanely euthanized and examined for gross tumor lesions if symptoms appeared or upon termination of the experiment. DNA was isolated from spleens and tumors to confirm the sequence of the respective meq gene.

7.6.6 Quantification of MDV genome copies in blood samples

The virus load in the blood of infected animals was analyzed at 4, 7, 10, 14, 21 and 28 dpi and for contact animals at day 21, 28, 35 and 42 by qPCR as described previously (33). DNA was isolated from whole blood samples of infected and contact chickens using the E-Z96 blood DNA kit (OMEGA Biotek, USA) according to the manufacturer's instructions. We determined

MDV genome copy numbers by qPCR using primers and probes specific for the MDV ICP4 as described above.

7.6.7   Statistical analysis

Statistical analysis was performed using Graph-Pad Prism v7 and the SPSS software (SPSS Inc.). Analysis for plaque size assays and growth kinetics included one-way analysis of variance (ANOVA). Fisher's exact test and Kaplan-Meier survival analysis were analyzed using log-rank test (Mantel-Cox test) was used for analyses of the animal experiment data with Bonferroni correction on multiple comparisons and were considered significant if p < 0.0125.

7.7   Acknowledgements

7.8   Author contributions

A.C., L.D.B. and N.B. conducted the experiments; A.C., M.P. and B.B.K. designed the experiments; A.C. and L.B.D. drafted the manuscript and all four authors edited the final version.

7.9   Declaration of interests

The authors declare no competing interests.

7.10   References

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Article 2

8   **Distinct polymorphisms in a single herpesvirus gene are capable of enhancing virulence and mediating vaccinal resistance.**

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8.1 Abstract

Modified-live herpesvirus vaccines are widely used in humans and animals, but field strains can emerge that have a higher virulence and break vaccinal protection. Since the introduction of the first vaccine in the 1970s, Marek's disease virus overcame the vaccine barrier by the acquisition of numerous genomic mutations. However, the evolutionary adaptations in the herpesvirus genome responsible for the vaccine breaks have remained elusive. Here, we demonstrate that point mutations in the multifunctional *meq* gene acquired during evolution can significantly alter virulence. Defined mutations found in highly virulent strains also allowed the virus to overcome innate cellular responses and vaccinal protection. Concomitantly, the adaptations in *meq* enhanced virus shedding into the environment, likely providing a selective advantage for the virus. Our study provides the first experimental evidence that few point mutations in a single herpesviral gene result in drastically increased virulence, enhanced shedding, and escape from vaccinal protection.

8.2 Author summary

Viruses can acquire mutations during evolution that alter their virulence. An example of a virus that has shown repeated shifts to higher virulence in response to more efficacious vaccines is the oncogenic Marek's disease virus (MDV) that infects chickens. Until now, it remained unknown which mutations in the large virus genome are responsible for this increase in virulence. We could demonstrate that very few amino acid changes in the *meq* oncogene of MDV can significantly alter the virulence of the virus. In addition, these changes also allow the virus to overcome vaccinal protection and enhance the shedding into the environment. Taken together, our data provide fundamental insights into evolutionary changes that allow this deadly veterinary pathogen to evolve towards greater virulence.

8.3 Introduction

Vaccines have revolutionized modern medicine and industrial animal farming by dramatically lowering disease incidence and mortality [1, 2]. While vaccines are ideal interventions for eradication, some viruses can evolve to overcome vaccinal protection [3]. Therefore, it is crucial to understand the evolutionary changes that facilitate vaccine resistance in order to develop more effective vaccines. [4]. A well-documented example of virus evolution towards a greater virulence is the highly oncogenic Marek's disease virus (MDV) [5, 6]. MDV is an alphaherpesvirus that infects chickens and is controlled by the wide application of modified live virus vaccines. In the absence of vaccination, infected chickens typically develop an acute

rash, and edematous neuronal and brain damage, severe lymphomas, paralysis, and death at a very young age [7, 8]. The tumors induced by MDV are considered to be one of the most frequent cancers in the animal kingdom [9].

MDV has undergone three major shifts in virulence over the past decades (Fig. 15A). This evolution resulted in ever more virulent field strains that cause increased severe clinical symptoms and vaccine evasion [8, 10, 11]. MDV strains are currently classified into four pathotypes based on their pathogenicity in vaccinated and unvaccinated chickens [8, 12, 13]. First-generation MDV vaccines, such as the related herpesvirus of turkey (HVT), were introduced in the 1970s to prevent chickens from emerging virulent MDV (vMDV) strains [14]. Soon after the introduction of the HVT vaccine, very virulent (vvMDV) strains emerged that were more pathogenic, immunosuppressive, and were able to overcome this vaccinal protection [15]. Protection against vvMDV was achieved using a second-generation bivalent vaccine, composed of a combination of a non-oncogenic, related herpesvirus of chickens (MDV-2, strain SB1) in combination with HVT that protected chickens from clinical disease [14]. Subsequently, very virulent plus (vv+MDV) strains emerged that are controlled by the third-generation vaccine (CVI988/Rispens); however, it remains unknown if more virulent strains will arise in the future (Fig. 1A) [14, 16]. This stepwise evolution of MDV directly correlates with the introduction of MD vaccines [17], suggesting that the 'leaky' MDV vaccines that protect from disease but are unable to provide sterilizing immunity may have directly contributed to the increase in virulence [18].

A large number of MDV field strains from all pathotypes have been sequenced over the years to identify mutations that could be responsible for changes in virulence [19, 20]. A few defined point mutations in the coding sequence of the major MDV oncogene meq have been identified that coincide with increased virulence (Fig. 1A) [10, 20]; however, their contribution in the evolution of MDV towards a greater virulence has never been proven.

Meq is a 339 amino acid basic leucine zipper protein (bZIP) that is expressed in lytically and latently infected cells, and is encoded in the internal and terminal repeat regions of the MDV genome [21]. Meq regulates viral and cellular genes by forming heterodimers with other bZIP proteins such as c-Jun to promote transcription [22]. In addition, Meq can form homodimers that repress the expression of numerous genes [22-25]. The C-terminus of *meq* encodes a transactivation domain characterized by proline-rich repeats (PRR) [26]. Low virulent vMDV strains (e.g. JM/102W) contain five PRR in their C-terminus, whereas vvMDV (e.g. RB-1B) and vv+MDV strains (e.g. N-strain) possess only three PRR (Fig. 15A) [27].

In this study, we set out to determine if these point mutations acquired in *meq* through the years contribute to the increase in MDV virulence, vaccine resistance and virus transmission.

The *meq* isoforms of different pathotypes (vMDV, vvMDV, vv+MDV and the CVI988/Rispens vaccine strain) were individually inserted into the very virulent RB-1B strain, thereby replacing its original *meq* gene. Virus replication was not significantly affected in vitro and *in vivo*. However, insertion of less virulent *meq* isoforms (vacMeq and vMeq) either abrogated or severely impaired MDV pathogenesis while higher virulent *meq* isoforms (vvMeq and vv+Meq) readily caused disease and tumors. Even in vaccinated chickens, viruses harboring the higher virulent meqs caused disease and efficiently shed into the environment. Strikingly, only viruses harboring the vv+Meq were able to overcome vaccinal protection and cause tumors in vaccinated animals. Furthermore, we show that the point mutations in *meq* isoforms of higher virulent MDV strains help the virus to overcome innate cellular responses, potentially contributing to vaccine failure. Overall, our data show that the evolutionary adaptations in *meq* substantially contribute to the increased virulence, vaccine resistance, and enhanced transmission – therefore playing a central role in the evolution of this highly oncogenic alphaherpesvirus.

8.4   Results

8.4.1   Generation of recombinant viruses

To determine if the point mutations in the *meq* isoforms contribute to MDV evolution towards a greater virulence, we replaced the *meq* gene in the very virulent RB-1B MDV strain with the *meq*s from different pathotypes as described previously [28]. Briefly, the *meq* gene from the CVI988/Rispens vaccine strain, JM/102W (vMDV), RB-1B (vvMDV) or N-strain (vv+MDV) were inserted into a virus lacking the *meq* gene (Δmeq) [28] by two-step Red-mediated mutagenesis [29, 30]. The insertion of *meq* isoforms were confirmed by next-generation sequencing (Fig. S21A). The recovered recombinant viruses were termed vacMeq, vMeq, vvMeq and vv+Meq. Sequencing of the recombinant viruses, passage level 4, confirmed the presence of the respective *meq* isoforms in the TRL and IRL without any secondary mutations in the genome (Fig. S21B).

8.4.2   Characterization of recombinant viruses *in vitro*

To determine if the *meq* isoforms of different pathotypes affect virus replication, we performed plaque size assays and demonstrated that all viruses efficiently replicated in vitro, while minor changes were observed that were not statistically significant. The *meq* genes from less virulent strains slightly enhanced replication in vitro (Fig. 15C), a phenotype also observed with the corresponding parental strains [31]. We confirmed this phenotype by plaque size assays (Fig. 15D), underlining that the insertion of *meq* isoforms only mildly affects MDV replication. We

verified that all *meq* isoforms are expressed at comparable levels by performing RT-qPCR on samples from infected chicken embryo cells (CEC) (Fig. 15E). Furthermore, ae analyzed whether the splice variant of *meq* to exons II and exons III of vIL8 (meq/vIL8) is affected through the differences in *meq*. Our data revealed the meq/vIL8 splicing is not affected in CECs and CU91 T cells (Fig. 15F), which is consistent with the absence of changes in the splice sites.
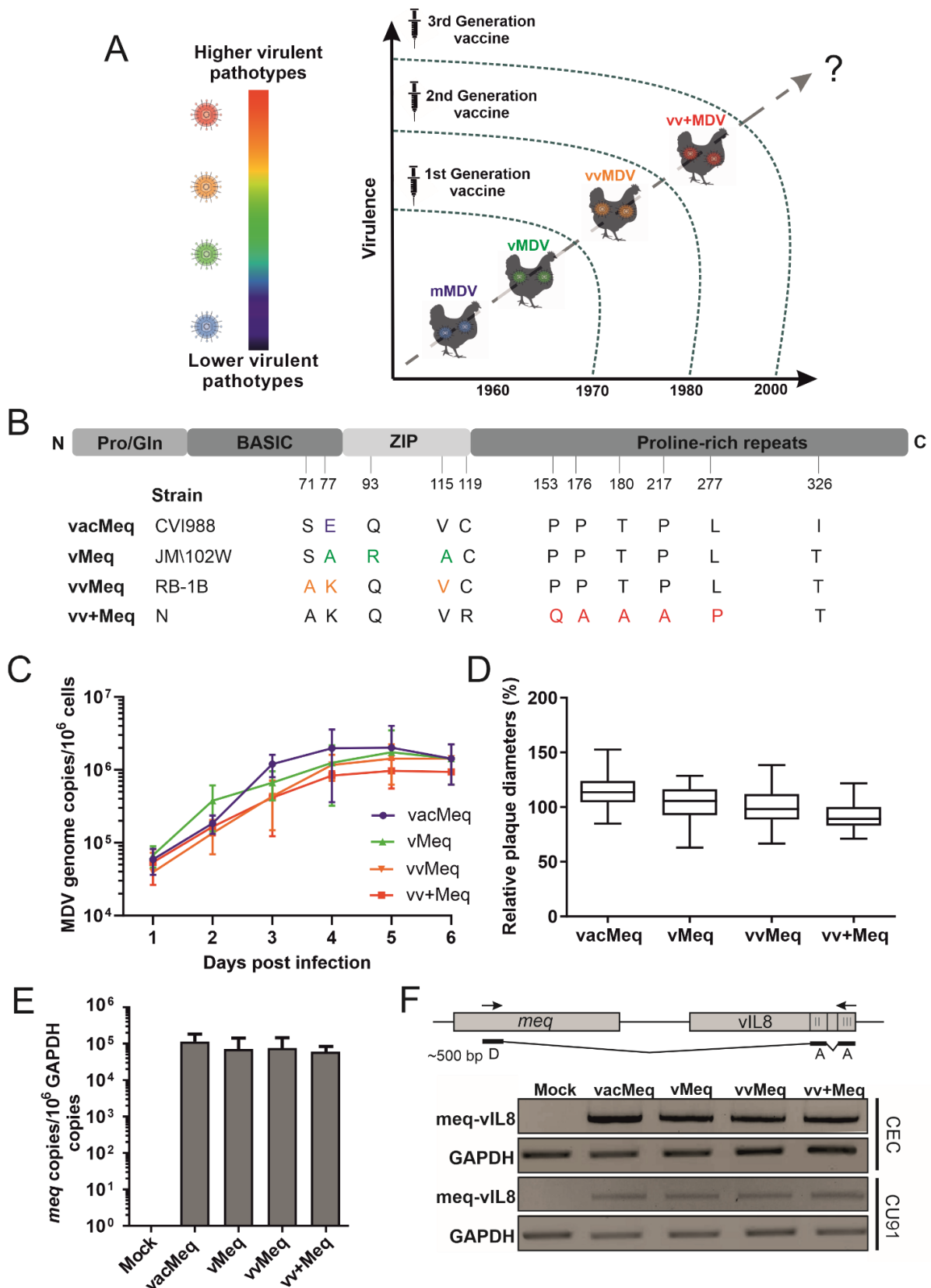
Figure 15: Characterization of the recombinant viruses *in vitro*. (A) A schematic illustration of the evolution of MDV towards increased virulence in the context of the indicated vaccine generations (B) The representation of the Meq protein with its domains. The N-terminal region

comprises of a proline/glutamine (Pro/Gln) rich domain followed the basic region and leucine zipper (ZIP). (C) Virus replication was assessed by multi-step growth kinetics. Mean viral genome copies per one million cells are shown for the indicated viruses and time points (p>0.05, Kruskal–Wallis test, n=3). (D) Plaque size assays of indicated recombinant viruses. The mean plaque diameters of three independent experiments are shown as box plots with minimum and maximums (p>0.05, one-way ANOVA, n=150). (E) The *meq* expression levels in infected CEC were assessed by RT-qPCR. Meq expresion is shown relative to one million copies of the cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and were not statistically different (Kruskal-Wallis test). (F) RT-PCR analysis of the meq/vIL8 splice variant using primers specific for the donor site "D" in *meq* and the acceptor sites "A" in vIL8. GAPDH was used as a control.

8.4.3 Role of the *meq* isoforms in MDV pathogenesis

To investigate if the evolutionary acquired point mutations in the *meq* gene contribute to MDV-induced pathogenesis and tumor formation, one-day old unvaccinated chickens were infected subcutaneously with 4,000 pfu of the respective recombinant viruses. To determine the effect of the *meq* isoforms on MDV replication, we quantified viral genome copies in the blood of infected animals by qPCR. All viruses efficiently replicated in infected animals (Fig. 16A), indicating that the changes in the *meq* isoforms only have a minor contribution to lytic replication *in vivo*. We monitored the animals for clinical disease symptoms and tumors during the experiment. Replacement with the MDV vaccine *meq* isoform completely abrogated virus-induced pathogenesis and tumor formation (Fig. 16B and C). Viruses harboring the vMDV *meq* isoform only induced clinical disease in 20% of the animals, while only 10% developed gross tumors (Fig. 16B and C). Viruses expressing vvMeq and vv+Meq efficiently induced disease and tumors, while the native vvMeq resulted in the highest virulence (Fig. 16B and C).

To assess the effect of the *meq* isoforms on tumor dissemination, the number of visceral organs with macroscopic tumors were quantified during necropsy throughout the course of the experiment and at the day of final necropsy (91 dpi). Replacement with the vMDV *meq* severely impaired tumor dissemination (Fig. 16D), as only a single organ (spleen) was affected in each tumor-bearing animal. vvMeq and vv+Meq induced efficient tumor dissemination in contrast to the the lower virulent *meq* isoforms (Fig. 16D). The data of this *in vivo* experiment was validated in an independent animal experiment using a different chicken line. In this second animal experiment, we observed a comparable MD incidence and tumor incidence (Fig S20). To ensure that the viruses did not develop compensatory mutations in the animals, we performed next-generation sequencing on viruses derived from organs and tumors (n=12).

Most viruses did not acquire any mutations in the animals, while three viruses had a single mutation that was either silent or in a non-coding region (Fig. S21C). In addition, we confirm that the *meq* was not altered in the host (Fig. S21C). These experiments revealed that the mutations in the *meq* isoforms affect virus-induced pathogenesis, tumor formation, and dissemination.
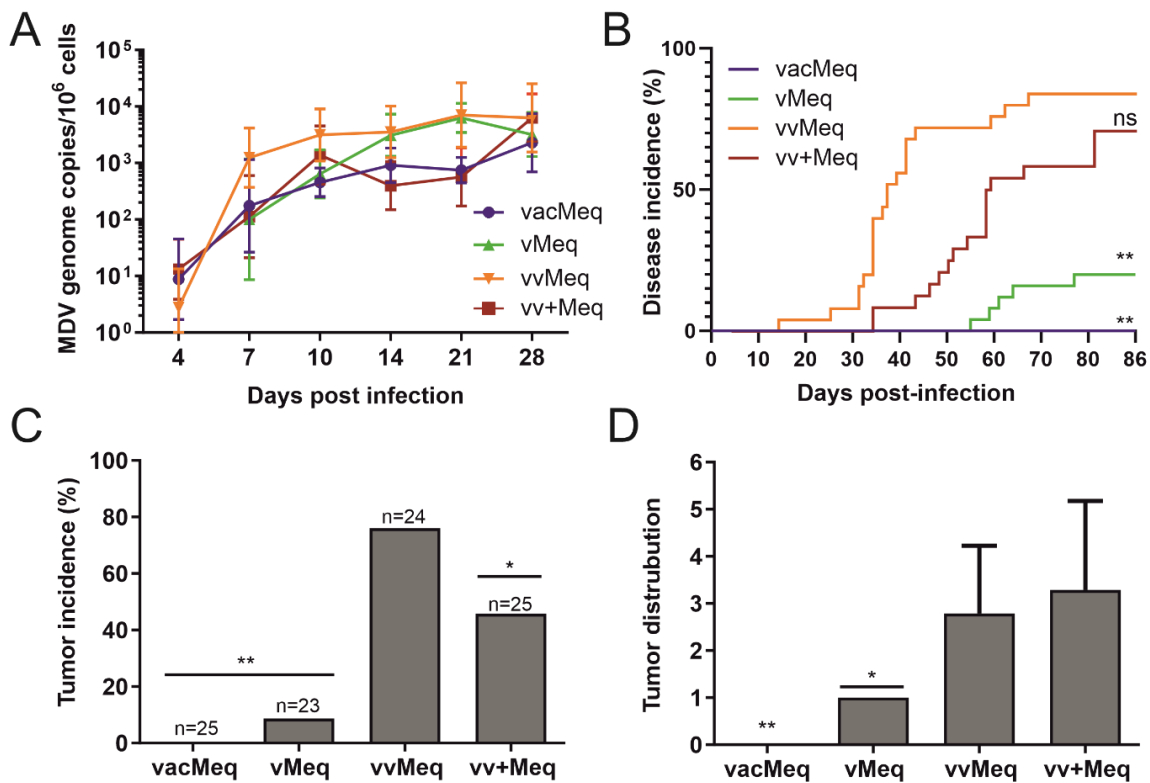
Figure 16: Influence of *meq* isoforms from various pathotypes on MDV pathogenesis. (A) MDV genome copies were detected in the blood samples of chickens infected with indicated viruses by qPCR. Mean MDV genome copies per one million cells are shown for the indicated time points ($p > 0.05$, Kruskal-Wallis test). (B) Disease incidence in chickens infected with indicated recombinant viruses and significant differences in comparison to vvMeq (** $p < 0.0125$, Log-rank (Mantel-Cox) test). (C) Tumor incidence as percentage of animals that developed tumors during the experiment. Asterisks indicate significant differences compared to vvMeq (* $p < 0.05$ and ** $p < 0.0125$; Fisher's exact test). (D) Tumor distribution is shown as the number of tumorous organs in tumor-bearing animal with standard deviations (* $p < 0.05$ and ** $p < 0.0125$; Fisher's exact test).

8.4.4 Natural spread and pathogenesis of recombinant viruses in contact animals

To confirm that these effects are also observed upon the natural spread of the virus via the respiratory tract, we co-housed naïve chickens with the subcutaneously infected animals. All *meq* isoform viruses were readily transmitted to the contact chickens as viral copies were detected in the blood (Fig. 17A), but only viruses harboring the vv and vv+ meq isoforms caused disease (Fig. 17B). Insertion of *meq* isoforms from the CVI988/Rispens vaccine and vMDV pathotypes completely abrogated tumor formation (Fig. 17C). Viruses harboring the

vvMDV and vv+MDV *meq* isoforms both efficiently induced tumors in the contact animals. As observed in the subcutaneously infected animals, tumor dissemination of the vv+Meq was slightly enhanced, although not statistically different, compared to the very efficient vvMeq (Fig. 17D).

Our data demonstrate that the few point mutations in the *meq* gene directly contribute to MDV virulence in experimentally and naturally infected animals.
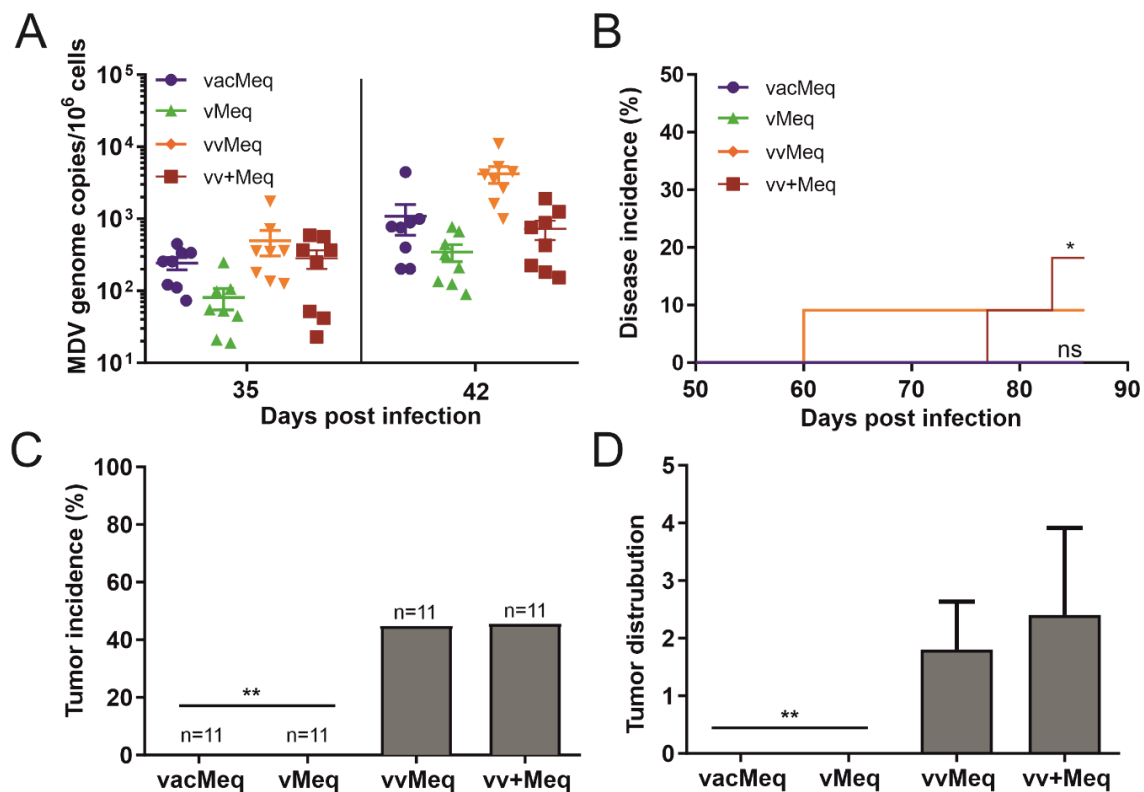
Figure 17: Pathogenesis and tumor incidence in naïve contact animals. (A) qPCR analysis of blood samples from naive chickens where MDV genome copies were determined (p>0.05, Kruskal-Wallis test). (B) Disease incidence in naïve chickens infected via the natural route and tumor incidence (C) and tumor distribution (D) are shown for co-housed contact animals. Asterisks (** p<0.0125; Fisher's exact test) indicate the significant differences in (C).

8.4.5   Pathogenesis of *meq* isoforms in vaccinated animals

Next, we determined if the different *meq* isoforms contribute to vaccine resistance and affect virus shedding in vaccinated animals. One-day old chickens were vaccinated subcutaneously with 4,000 pfu of the commonly used HVT vaccine. At seven days post-vaccination, we infected all vaccinated chickens with 5,000 pfu of the respective recombinant viruses to determine if *meq* contributes to vaccine breaks. Replication of the recombinant viruses (Fig. 18A) and HVT vaccine (Fig. 18B) was not statistically different between the groups. Vaccination completely

protected chickens from the less virulent *meq* isoform viruses (vacMeq and vMeq; Fig. 18C). On the other hand, the higher virulent *meq* isoform viruses were able to overcome the vaccinal protection and caused disease (vvMeq and vv+Meq; Fig. 18C). Strikingly, insertion of the vv+Meq isoform strongly enhanced virulence in vaccinated animals (Fig. 18C). Only chickens infected with vv+Meq developed tumors (Fig. 18D), indicating that the few point mutations in *meq* allow the virus to overcome the vaccinal protection and cause tumors in vaccinated animals.
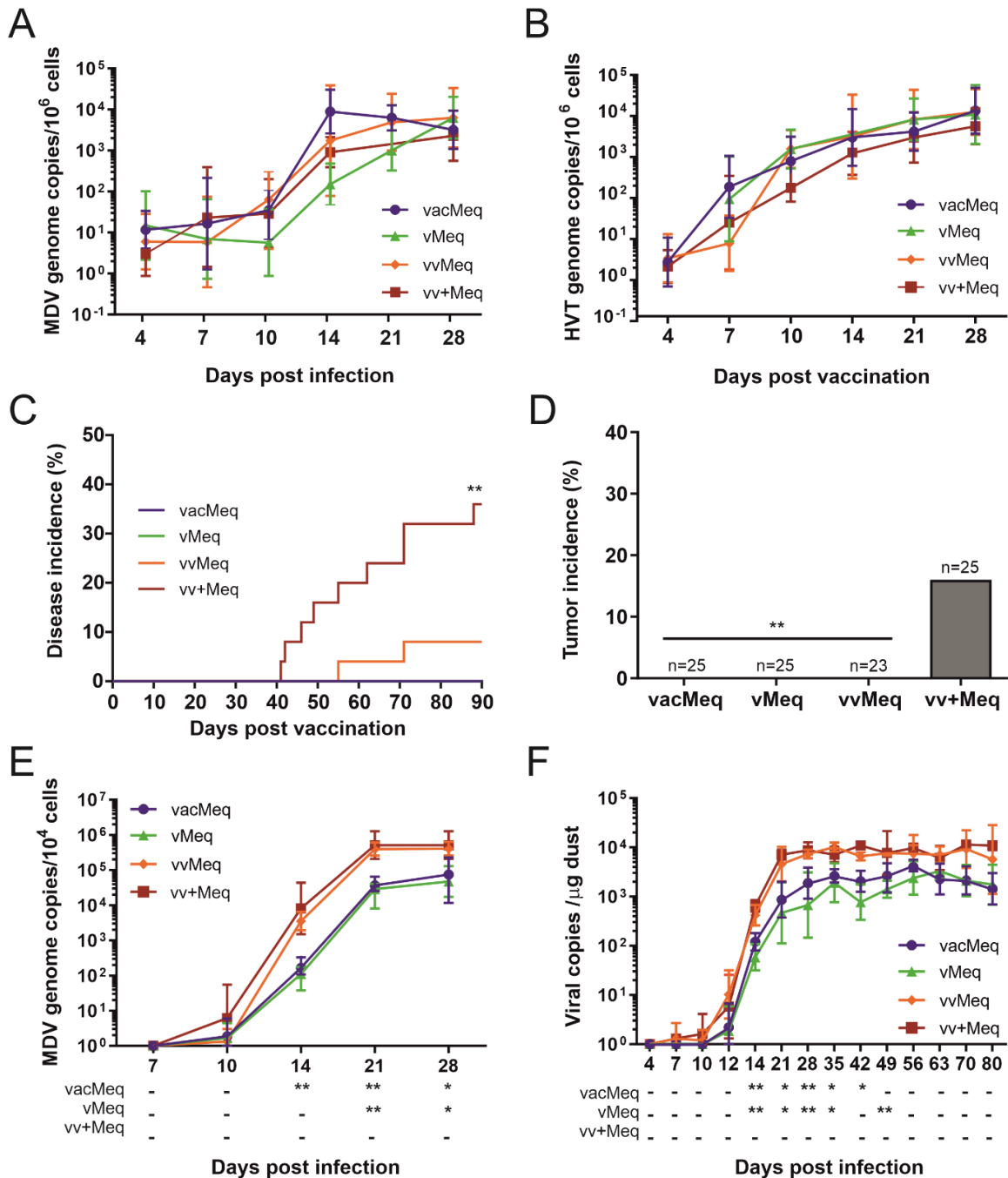
Figure 18: Pathogenesis and shedding of different *meq* isoform viruses in vaccinated chickens. Viral genome copy numbers of (A) the *meq* isoform viruses and (B) the HVT vaccine detected in blood of vaccinated chickens infected with the *meq* isoform viruses (p>0.05, Kruskal-Wallis test). (C) Disease incidence and (D) tumor incidence in vaccinated chickens infected with indicated recombinant viruses. Asterisks (** p<0.0125, Fisher's exact test) indicate statistical differences to vv+Meq in (D). (E) Viral copies from feathers of the *meq* recombinant viruses. (A), (B) and (E): mean MDV genome copies per one million cells are shown for the indicated time points. (F) Viral copies per µg of dust are shown for each group as validated previously [32]. Statistical differences in the feathers and dust samples are displayed as a comparison to vvMeq. Asterisks indicate significant differences (* p<0.05 and ** p<0.0125; Tukey's multiple comparisons test).

### 8.4.6   Role of *meq* isoforms in virus shedding from vaccinated animals

Efficient virus shedding plays an essential role in virus evolution. During infection, MDV is transported to the feather follicle epithelia in the skin, where it is shed with the feathers into the environment [33]. To assess if the *meq* isoforms also affect virus shedding, we collected feathers and dust during the experiment and measured MDV copy numbers by qPCR (Fig. 18E and F). Even though all viruses reached the feather follicles at approximately ten days post-infection (dpi), virus load was significantly increased in viruses harboring vvMeq and vv+Meq (Fig. 18). In addition, shedding was significantly higher upon infection with the vvMeq and vv+Meq viruses (Fig. 18F), indicating that these mutations provide an evolutionary advantage due to the higher virus levels in the environment. Taken together, we could demonstrate that few mutations in *meq* contribute to a higher virulence, allow the virus to overcome vaccinal protection and enhance virus shedding.

8.4.7   Mutations in *meq* allow the virus to overcome cellular innate responses

To determine if the specific mutations in *meq* affect innate immune responses, we stimulated primary chicken T cells with innate immune agonists (Poly I:C, LPS and cGAMP) and infected these cells with the different recombinant viruses. Upon infection, we measured the effect of these innate immune agonists on virus spread to CEC and subsequent virus replication (Fig. 19). Poly I:C, LPS, and cGAMP treatments in general significantly decreased the number of plaques (Fig. 19A), and the plaque sizes (Fig. 19B) compared to the media control. Strikingly, viruses harboring the higher virulent *meq* isoforms (vv and vv+Meq) formed significantly more plaques than the one with lower virulent isoforms (Fig. 19A). Consistently, CEC infections with higher virulent *meq* isoform viruses led to increased plaque sizes compared to vacMeq and

vMeq (Fig. 19B). These results indicate that the mutations in the higher virulent *meq*s allow the virus to overcome innate cellular responses induced by these agonists and provide a potential explanation for the vaccine breaks mediated by *meq* [34].
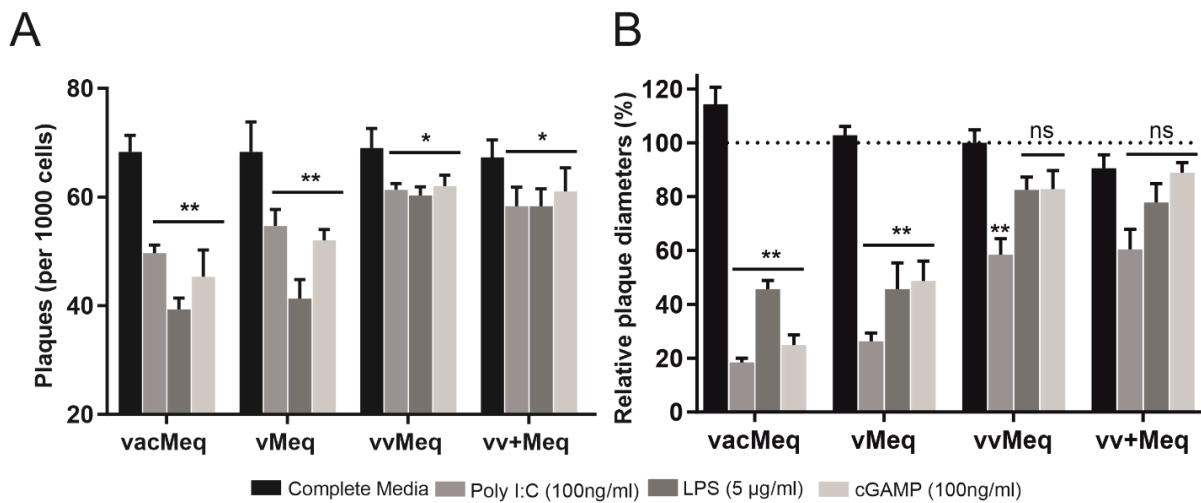
Figure 19: Efficiency of *meq* isoform viruses to overcome innate immune response. Primary T cells were activated by innate immunity agonists (Poly I:C, LPS, or cGAMP). Activated T cells were infected with the different meq isoform viruses to determine the effects on virus shedding and replication. (A) Plaque counts were performed on CECs overlaid with 1,000 activated infected primary T cells. (B) Corresponding changes in plaque sizes on infected CEC (normalized to vvMeq). Asterisks indicate significant differences (* $p<0.05$ and ** $p<0.0125$; Tukey's multiple comparisons test).

8.5 Discussion

MDV strains have repeatedly increased in virulence and overcame vaccinal protection [35, 36]. Virulence is a complex trait and several virulence factors act alone or orchestrated with other to drive pathogenesis and tumor formation. These factors include the oncoprotein Meq, the viral telomerase RNA (vTR), the virus-encoded chemokine vIL-8/vCXCL13, RLORF4, RLORF5a, pp14, pp38 and telomere arrays present at the ends of the virus genome [6, 37]. In this study, we determined the contribution of *meq* isoforms alone in MDV pathogenicity, oncogenicity, and shedding in unvaccinated and vaccinated animals. We provide the first experimental evidence that distinct polymorphisms in the *meq* have a substantial impact on the evolution of MDV towards greater virulence. Our data revealed that only four amino acid changes (AKQV) are involved in an increase in tumor incidence by more than 50% in our experiments.

We first evaluated the growth properties of the *meq* isoforms in vitro and *in vivo* to determine if *meq* isoforms from different pathotypes affect virus replication. The *meq* isoforms did not differ in their replication properties in tissue culture and in the host. Even though Meq is expressed during lytic infection, these few mutations in *meq* do not provide an advantage for

its replication properties. Consistently, Lupiani and colleagues previously demonstrated that *meq* is dispensable for virus replication [21]. We demonstrate that the minor mutations residing in the *meq* isoforms did not affect *meq* expression in primary CEC (Fig. 15E). In addition to the Meq protein, alternative splicing gives rise to a splice form with exon 2 and 3 of vIL-8, designated as meq/vIL8 [38]. We assessed the expression of this splice variant by qRT-PCR in both CEC and CD4 T cells, revealing that these minor changes in *meq* do not affect meq/vIL8 splicing (Fig. 15F). This is consistent with a previous study that showed that splice variants did not differ between different pathotypes in infected primary chicken B cells [39]. The comparable expression of meq/vIL8 likely due to the absence of mutation in the splice donor site encoded in the leucine zipper domain in the *meq* isoforms, while the branch point and acceptors sites are outside of *meq* and were not altered in our study.

Deletion of *meq* led to an abrogation of tumor formation, indicating that *meq* has essential transforming properties [40]. The observed increase in virulence of strains over the years has been characterized by the ability to induce lymphoproliferative lesions [13] and an increase in shedding [5], thereby shifting our focus towards these aspects and the contribution of *meq*.

In the first animal experiment, we infected one-day-old chickens with viruses harboring *meq* isoforms from different pathotypes to determine their individual contribution to virus-induced pathogenesis and oncogenesis *in vivo*. In this experiment, we also co-housed the infected with naïve contact chickens to measure the horizontal spread via the natural route of infection. The *meq* gene from the lowest virulence class vacMeq, completely abrogated MDV pathogenicity and tumor formation. It has been previously shown that the meq isoform of the CVI988/Rispens vaccine, is a weaker transactivator, decreasing the expression of cellular and viral genes due to mutations in the DNA binding domain at positions 71 and 77 (Fig. 15A) [41]. Meq binds to its own promoter and through its weak transactivation properties on its own promoter it could alter the development of T cell tumors. However, we did not observe a reduction in vacMeq expression on our experiments. The two point mutation differences in vacMeq ultimately rendered the very virulent RB-1B strain apathogenic (Fig. 16). Insertion of the vMDV meq into RB-1B reduced disease incidence and tumor incidence in infected chickens. The vMDV meq (JM/102W) harbors a 177 bp insertion or duplication of a proline-rich (PRR) domain [42] located in the transactivation domain (Fig. 15A). This insertion increased the copy number of the PRR, which exerts a transrepression effect [42, 43]. The higher virulent forms vvMeq and vv+Meq showed higher disease incidence rates and enhanced oncogenesis compared to the less virulent pathotypes (Fig. 16B-D). An independent animal experiment using a different chicken line confirmed the markedly elevated disease incidence (Fig. S20A) and the higher oncogenic potential for the higher virulent *meq* isoform viruses (Fig. S20B). The vv+Meq had a slightly lower disease incidence than vvMeq (Fig. 16B and Fig. S20A). This could be due to

epistatic effects, where the fitness of the virus is impacted not by *meq* alone, but by its interaction with the rest of the viral genome. Interestingly, this effect was not detected upon natural infection in contact animals (Fig. 17B). Kumar and colleagues previously inserted *meq* of RB-1B into rMd5 (both vv strains), in which the *meq* only differs in three amino acid positions. This exchange altered the phenotype of the resulting virus in the subtle way and allowed the establishment of tumors cell lines (UD36-38) which could not be achieved with the parental rMd5 [42]. Tumors induced by the recombinant showed similar cellular expression profiles to rMd5 tumors, suggesting that the context of the strain encoding the Meq protein plays an important role in pathogenesis. Potential epistatic effects are a limitation in our study and it remains to be addressed whether different backbones expressing the *meq* isoforms might behave differently.

All recombinant viruses were successfully transmitted to contact chickens (Fig. 17A), but only contact chickens in the higher virulent *meq* isoform groups showed clinical signs and tumors (Fig. 17B and C). The tumor dissemination was also altered upon insertion of the different *meq* isoforms. While the vMeq tumors were only localized in one organ (spleen), multiple organs were affected with the higher virulent *meq* viruses (Fig. 16D). We found the highest number of tumors in the vv+Meq group (Fig. 16D) and observed the same trend of tumor dissemination in the contact chickens (Fig. 17D). Importantly, experimentally and contact birds were hatched on the same day and housed together for the duration of the experiment. Therefore, the contact animals were infected much later (~ day 14) when they were already more resistant to MDV. However, our results clearly show that the higher virulent meq isoforms allow tumor formation in more organs in unvaccinated hosts.

In the next animal experiment, we aimed to assess the ability of the different recombinant viruses to break the vaccinal protection and promote efficient horizontal spread. We vaccinated chickens with the HVT vaccine that protects chickens from vMDV (Fig. 15A). We then challenged the chickens at day seven post-vaccination using the viruses that harbor the different *meq* isoforms. All viruses replicated efficiently in the vaccinated chicken (Fig. 18A and B). We observed no mortalities in groups infected with the less virulent *meq* viruses, as observed with the parental strains that cannot overcome the HVT protection (Fig. 18C).

The only birds that succumbed to disease despite vaccination were the birds challenged with the higher virulent *meq* isoforms (Fig. 18C). However, only the virus harboring the vv+Meq was able to induce tumors in the vaccinated animals. It is remarkable that the virus only required five distinct point mutations in the vv+Meq, allowing the vv+Meq to overcome vaccinal protection and cause malignant tumors (Fig. 18D). All of these mutations found in vv+Meq resides in the transactivation domain and affect the number of PRRs. Since the PRRs exhibit a transrepression effect, the mutations interrupt the number of PRRs and thereby influence the

transactivation activity of Meq [44]. Moreover, Meq functions in target cellular and viral gene transactivation and the higher transactivation properties of vv+Meq could alter and increase proliferation, mobility and apoptosis resistance of cells that develop tumors, perhaps through the upregulation of adhesion molecules via vTR [45, 46]. In addition, the chicken CD30, which is discussed to be involved in MDV lymphomagenesis, has 15 potential binding sites for Meq [47]. Thus, the enhanced transactivation of vv+Meq could also lead to CD30 overexpression, favoring neoplastic transformation. The latter hypothesis is consistent with observation on other oncogenic viruses such as Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus [48]. However, CD30 overexpression in MDV-induced tumors could not be confirmed in follow-up studies [49].

Efficient virus transmission provides strong evolutionary advantages [50]. Here we found that the mutations in *meq* had a strong influence on the amount of virus presence in the feather follicles and on viral shedding into the environment. The higher virulent *meq* isoform viruses were detected at higher levels in feather follicles compared to the less virulent *meq* isoforms (Fig. 18E). Consequently, the levels of virus shedding of the higher virulent *meq* isoforms were increased (Fig. 18F), likely providing an evolutionary advantage for the virus. There are two potential reasons for increased virus shedding: i) that the viruses harboring the higher virulent *meq* isoforms replicate better in the feather follicles or ii) that the increased number of transformed cells that can travel to the skin facilitate a more efficient delivery to the feather follicles, enhancing virus production and shedding [51]. Read et al. recently demonstrated that vaccination with leaky vaccines prolongs viral shedding and onward transmission of vv+MDV strains as the host is kept alive for extended periods [5]. Also, they showed that the cumulative shedding of less virulent strains is reduced by vaccination, but increased by several orders of magnitude with highly virulent strains [5].

It would be interesting to evaluate virus competition between the *meq* isoforms to determine which virus sheds at higher rates as performed previously by Dunn et al [52]. They show for pathogenically similar (rMd5 and rMd5/pp38CVI) or dissimilar (JM/102W and rMd5/pp38CVI) virus pairs that the higher virulent strains had a competitive advantage over the less virulent strains [52].

The *meq* isoforms we chose are representative of a broad range of viruses and pathotypes [53]. We did test two *meq* isoforms from the vMDV pathotype, JM102 (Fig 15 to 19) and 617A (Fig. S1) that behaved similar, resulting in lower disease and tumor incidence compared to viruses harboring a vv and vv+ meq. However, it would be interesting to test additional *meq* isoforms from the respective pathotypes in future studies.

Nonetheless, our data indicate that the minor mutations in *meq* contribute to this enhanced shedding that increases the level of infectious virus in the environment and provides a selective advantage for more virulent strains.

Next, we turned to the first line of defense against MDV, the innate immunity. It has been previously shown that Meq blocks apoptosis and interferes with antiviral activity [54, 55]. As Meq regulates viral and host genes, we evaluated whether the individual *meq* isoforms affect cellular innate immune responses. The lower virulent *meq* isoforms showed a significant reduction in growth and plaque sizes in cells treated with the agonists (Fig. 19). In contrast, the higher virulent *meq* isoforms allow the virus to overcome the antiviral response activated in primary T cells stimulated by Poly I:C-, LPS- and cGAMP (Fig 5). It has been previously shown that MDV has the ability to evade the cGAS-STING DNA sensing pathway (stimulated by cGAMP) as Meq delayed the recruitment of TANK-binding kinase one and (interferon) IFN regulatory factor 7 (IRF7) to the STING complex, thereby inhibiting IRF7 activation and IFN-β induction [34]. Especially the vv and vv+Meq isoforms were able to block the cGAS-STING DNA sensing pathway, as compared to the lower virulent *meq* isoforms (Fig. 19). It remains unclear how Meq mechanistically modulates the signaling pathway and should be investigated to understand the role of Meq in the innate immunity in the future. Overall, our findings suggest that the mutations in the higher virulent *meq* isoforms provide an advantage in the vaccinated animals by allowing the virus to overcome these innate responses early upon infection.

In summary, our data demonstrate that minor polymorphisms in *meq* drastically alter disease outcomes in naïve and vaccinated chickens. The *meq* isoforms from highly virulent MDV strains are required for efficient disease and tumor formation, while those from less virulent strains severely impair or abrogate disease and tumor incidence. Also, we show that the mutations that arose in the *meq* from higher virulent strains permitted vaccine resistance and the ability to shed at higher rates in the environment; all factors promoting the evolution of this pathogen.

8.6   Materials and methods

8.6.1   Ethics statement

All animal work was conducted in compliance with relevant national and international guidelines for care and humane use of animals. Animal experimentation was approved by the Landesamt für Gesundheit und Soziales in Berlin, Germany (approval numbers G0294-17 and T0245-14) and the Agricultural Animal Care and Use Committee protocol (64R-2019-0, UBC protocol 16-023).

8.6.2 Cells and viruses

CEC were prepared from 11-day old specific-pathogen-free (SPF) chicken embryos (VALO BioMedia, Germany) as described previously [56]. CEC were cultured in Eagle's minimal essential medium (MEM; PAN Biotech, Germany) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). Reticuloendotheliosis virus-transformed T cells (CU91) were propagated in RPMI 1640 media (PAN Biotech, Germany) supplemented with 1% sodium pyruvate, 1% nonessential amino acids, 10% FBS, and penicillin–streptomycin, and maintained at 41°C in a 5% CO_2 atmosphere. Viruses were reconstituted by transfecting bacterial artificial chromosome (BAC) DNA into CEC as described previously [56]. Viruses were propagated on CEC for four passages thereafter virus stocks were frozen in liquid nitrogen and titrated on CEC as described previously [57, 58].

8.6.3 Generation of recombinant viruses

To generate recombinant viruses that harbor meq isoforms from the different pathotypes, we inserted the meq isoforms into the very virulent RB-1B strain (GenBank accession no. MT797629) instead of the native meq gene as described previously[28]. This resulted in the viruses containing the meq isoforms from CVI988/Rispens vaccine (vacMeq), vMDV strain JM/102W (vMeq), vvMDV strain RB-1B (vvMeq) and vv+MDV N-strain (vv+Meq). Primers used for mutagenesis are listed in Table 5. Insertions of the meq genes were confirmed by PCR, restriction fragment length polymorphism (RFLP), Sanger- and Illumina MiSeq sequencing with a ~1000-fold coverage to ensure that the entire virus genome is correct. The GenBank accession numbers for each *meq* isoform and resultant recombinant viruses can be found in Table 6.

Table 5: Primers and probes used for construction of recombinant viruses, DNA sequencing and qPCR

Construct/target	Primer or probe[a]	Sequence (5' – 3')[b]
meq kana_in (transfer construct)	for	AATTCGAGATCTAAGGACTGAGTGCACGTCCCTGTAGGGATAACAGGGTAATCGATTT
	rev	GTCCTTAGATCTCGAATTTCCTTACGTAGGGCCAGTGTTACAACCAATTAACC
Δ*meq* (deleting RB-1B *meq*)	for	CAGGGTCTCCCGTCACCTGGAAACCACCAGACCGTAGACTGGGGGGACGGATCGTCAGCGGTAGGGATAACAGGGTAATCGATTT

		GGGCGCTATGCCCTACAGTCCCGCTGACGATCCGTC
	rev	CCCCCAGTCTACGGTCTGGTGGGCCAGTGTTACAAC CAATTAACC
MDV_*meq* (insertion of *meq*s)	for	ATGTCTCAGGAGCCAGAGCC
	rev	GGGTCTCCCGTCACCTGG
	for	CGTGTTTTCCGGCATGTG
meq/vIL8 (RT-PCR)	for	GCAGGGCGCAGACGGACTA
	rev	TCAAAGACAGATATGGGAACC
	for	CGTGTTTTCCGGCATGTG
ICP4 (qPCR)	rev	TCCCATACCAATCCTCATCCA
	probe	FAM-CCCCCACCAGGTGCAGGCA-TAM
	for	TTGTCATGAGCCAGTTTGCCCTAT
meq (qPCR)	rev	AGGGAGGTGGAGGAGTGCAAAT
	probe	FAM-GGTGACCCTTGGACTGCTTACCATGC-TAM
	for	GGCAGACACCGCGTTGTAT
HVT-SORF1 (qPCR)	rev	TGTCCACGCTCGAGACTATCC
	probe	FAM-AACCCGGGCTTGTGGACGTCTTC-TAM
	for	GAGTGGTTTAAGGAGTTGGATCTGA
iNOS (qPCR)	rev	TTCCAGACCTCCCACCTCAA
	probe	FAM-CTCTGCCTGCTGTTGCCAACATGC-TAM
GAPDH (RT-PCR and qPCR)	for	GAAGCTTACTGGAATGGCTTTCC
	rev	GGCAGGTCAGGTGAACAACA
	probe	FAM-CTCTGCCTGCTGTTGCCAACATGC-TAM

[a]for, forward primer; rev, reverse primer.

[b]FAM, 6-carboxyfluorescein; TAM, TAMRA.

8.6.4 Plaque size assays

Replication properties of the recombinant viruses were analyzed by plaque size assays as previously described [59]. Briefly, one million CEC were infected with 100 plaque-forming units (pfu) of the recombinant viruses and cells were fixed at five dpi. Images of randomly selected plaques (n=50) were captured and plaque areas were determined using Image J software (NIH, USA). Plaque diameters were calculated and compared to the respective control.

8.6.5 *In vitro* replication

In vitro replication of recombinant viruses was measured over six days by qPCR as previously described [60, 61]. Briefly, primers and probes specific for MDV-infected cell protein 4 (ICP4) and chicken inducible nitric oxide synthase (iNOS) genes were used (Table 1). The qPCR

analysis was performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems Inc., USA) and the results were analyzed using the Sequence Detection System v.1.9.1 software. Virus genome copies were normalized against the chicken iNOS gene as published previously [51].

8.6.6   Quantitative reverse transcription PCR (RT-qPCR) and RT-PCR

To assess the expression levels of the meq isoforms we performed RT-qPCR as previously described [62]. Briefly, total RNA was extracted from virus-infected CEC and CU91 using the RNeasy Plus minikit (Qiagen) according to the manufacturer's instructions. The samples were treated with DNase I (Promega), and cDNA was generated using the High-Capacity cDNA reverse transcription kit (Applied Biosystems).

ICP4 and GAPDH were used to control for the infection levels and the number of cells (Fig. 22). *meq* expression levels were normalized to the expression levels cellular GAPDH (per million GAPH copies). The primers and probes used for RT-qPCR are shown in Table 1. To investigate the expression of the meq/vIL8 splice form in cells infected with the recombinant viruses, we performed RT-qPCR using primers specific for the meq/vIL8 splice variant as previously described [58].

8.6.7   *In vivo* characterization of recombinant viruses

Animal experiment 1 (pathogenesis of recombinant viruses). One-day old VALO SPF chickens (VALO BioMedia) were randomly distributed into four groups and housed separately. Chickens were infected subcutaneously with 4,000 pfu of vacMeq (n=25), vMeq (n=23), vvMeq (n=24) and vv+Meq (n=25). With each group, 11 non-infected contact animals, same age, were housed to assess the natural transmission of the respective viruses. The experiment was performed in a blinded manner to avoid bias. Animals were kept under a 12 h light regime in stainless steel cages with wood and straw litter. Enrichment was provided by perches, sand baths and picking stones. Rooms were air-conditioned and temperature was regulated starting from an air temperature of 28 °C on day 1 decreasing to 20 °C on day 21. In the first 10 days, heat lamps were provided. Food and water were provided ad libitum. Whole blood samples were collected for infected animals at 4, 7, 10, 14, 21 and 28 dpi and for contact animals at day 21, 28, 35 and 42 to measure virus load in the blood. The chickens were assessed every day to monitor for MDV-specific clinical symptoms that include severe ataxia, paralysis, torticollis and somnolence. If symptoms appeared, chickens were humanely euthanized and examined for gross tumor lesions. Tumors were also assessed in chickens that did not show Marek's disease signs upon termination of the experiment at 85 dpi. DNA was isolated from

spleens and tumors to confirm the sequence of the inserted meq gene and integrity of viral genome. The phenotype of the *meq* isoforms were confirmed in a second, independent animal experiment. White leghorn chickens (Sunrise Farms, Inc., Catskill, NY) were inoculated with 1,000 PFU of the respective recombinant viruses (n=18).

8.6.8 *In vivo* characterization of recombinant viruses

Animal experiment 2 (infection of vaccinated animals). One-day old VALO SPF chickens were randomly distributed into four groups as described for animal experiment 1. Chickens were subcutaneously vaccinated with 4,000 pfu of the HVT vaccine (strain FC 126; Poulvac; Zoetis Inc., USA) for each group of 25 chickens. At seven days post-vaccination, chickens were challenged with 5,000 pfu of vacMeq (n=25), vMeq (n=25), vvMeq (n=23) and vv+Meq (n=25) and similar experimental procedures were followed as in animal experiment 1. Whole blood samples were collected to measure virus load in the blood as described above. Feathers were collected at 7, 10, 14, 21 and 28 dpi to monitor the time and the concentration of the viruses that reached the feather follicles to be shed into the environment. Dust shed from the infected chickens was collected from filters of each room once a week to assess the shedding rates until termination of the experiment at 90 dpi. DNA was isolated from spleens and tumors to confirm the sequence of the inserted *meq* genes.

8.6.9 Extraction of DNA from blood, feathers and dust

DNA was isolated from blood samples of infected and contact chickens using the E-Z96 blood DNA kit (OMEGA Biotek, USA) according to the manufacturer's instructions. Feathers were collected from birds and the proximal ends of each feather containing the feather pulp (referred to as feather tip). In addition, dust samples (three 1-mg aliquots) were collected from the filters in each room at indicated time points. DNA was extracted from feathers and dust samples as previously described [63]. All samples were analyzed by qPCR. The primer and probes (Table 1) for the differential quantification between MDV and HVT were described previously [64, 65]. Briefly, the *meq* gene and SORF1 that are exclusively encoded in MDV and HVT respectively were used as targets in the qPCR.

8.6.10 DNA extraction from organs and tumor tissue

The innuSPEED tissue DNA Kit (Analytik Jena) was used to extract DNA from organs, according to the manufacturer's instructions. Briefly, 50 mg of tissue were homogenized. The homogenate was treated with RNase A and proteinase K digestion, with the exception to the

protocol, that proteinase K treatment was extended to 90 min to release viral DNA from the nucleocapsids. The lysate was cleared by addition of a protein-denaturing buffer following high speed centrifugation. The DNA in the supernatant was isolated on DNA binding columns. After subsequent washing steps, the DNA was eluted in 150 µl elution buffer and used for qPCR or next-generation sequencing analyses.

8.6.11 Next-generation sequencing of recombinant viruses

DNA sequencing of the recovered viruses and DNA from tumors and spleens were performed on an Illumina MiSeq platform as previously described [66]. Briefly, one to five micrograms of total DNA extracted were fragmented to a peak fragment size of 500–700 base pairs (bp). The fragmented DNA (100 ng to 1 µg) was subjected to next-generation sequencing library preparation using the NEBNext Ultra II DNA Library Prep Kit for Illumina platforms (New England Biolabs). The bead-based size selection step was performed with Agencourt AMPure XP magnetic beads (Beckman Coulter Life Sciences) selecting for inserts of 500–700 bp. To achieve a library yield >500 ng, five PCR cycles were performed.

We used a tiling array method to enrich the viral sequences from the DNA extracts that were harvested from organs or tumors that contained mainly sequences of chicken origin [66]. The array contained 6,597 biotinylated RNA 80-mers that were designed against the sequence of the RB-1B strain (MYcroarray; Arbor Biosciences). The enrichment was performed according to the manufacturer's instructions.

8.6.12 Next-generation sequence data analysis

All Illumina reads were processed with Trimmomatic v.0.36 [67] and mapped against the RB-1B strain using the Burrows-wheeler aligner v.0.7.12 [68]. The single nucleotide polymorphisms (SNPs) were assessed with FreeBayes v.1.1.0-3 [69]. The data were merged by position and mutation using R v.3.2.3. The SNPs were additionally assessed and generated using Geneious R11 software.

8.6.13 Quantification of virus genome copies

MDV genome copy numbers were determined by quantitative PCR (qPCR) with primers and probes specific for either the HVT vaccine or meq isoform recombinant viruses, to distinguish between the viruses from vaccination and infection (Table 1). Virus genome copies were normalized against the chicken iNOS gene as published previously [51]. The qPCR analysis on feathers and dust was performed as described previously [5, 32]. Briefly, for the feather tip

samples, viral DNA copies were quantified as genomes per 104 feather tips and for dust, genomes per microgram of dust (MDV genomes/mg dust; based on the mass of dust used to prepare DNA and the volume of dust DNA used per reaction).

8.6.14 Assessment of virus spread and replication upon treatment with innate immune agonists

Next, we determined if meq isoforms allow the virus to overcome cellular innate immune responses in primary T cells. Primary T cells were extracted from the thymus of 12-day old chickens as previously described [70]. T cells were stimulated with either LPS (5 µg/ml), Poly I:C (100 ng/ml), and cGAMP (100 ng/ml), and control (medium only) to induce innate immune responses. At six hours (h) post-activation, T cells were infected with the different meq isoform viruses harboring a GFP reporter by co-cultivation with infected CEC due to the strict cell-associated nature of MDV. At 24 h post-infection, viable infected GFP-expressing T cells were isolated by FACS, and 1,000 infected cells were seeded on a fresh CEC monolayer. The number of plaques and plaque sizes were determined at five dpi as described above.

8.6.15 Statistical analyses

Statistical analyses were performed using Graph-Pad Prism v7 (GraphPad Software, Inc., USA) and the SPSS software (SPSS Inc., USA). The multi-step growth kinetics were analyzed with the Kruskal–Wallis test. Analysis for plaque size assays included a one-way analysis of variance (ANOVA). Kaplan-Meier disease incidence curves were analyzed using the log-rank test (Mantel-Cox test), and Fisher's exact test was used for tumor incidences and distribution with Bonferroni corrections on multiple comparisons. Tukey's multiple comparisons test was used for the analysis of feather and dust samples and for the innate immunity experiments. Data were considered significant if $p < 0.05$.

8.7 Acknowledgements

8.8 Author contributions

A.M.C., L.D.B., J.T, J.N.P., and S.M. conducted the experiments; A.M.C., M.S.P. and B.B.K. designed the experiments; all authors wrote and edited the paper.

8.9 Declaration of interests

The authors declare that they have no conflict of interest.

8.10 Supporting Information
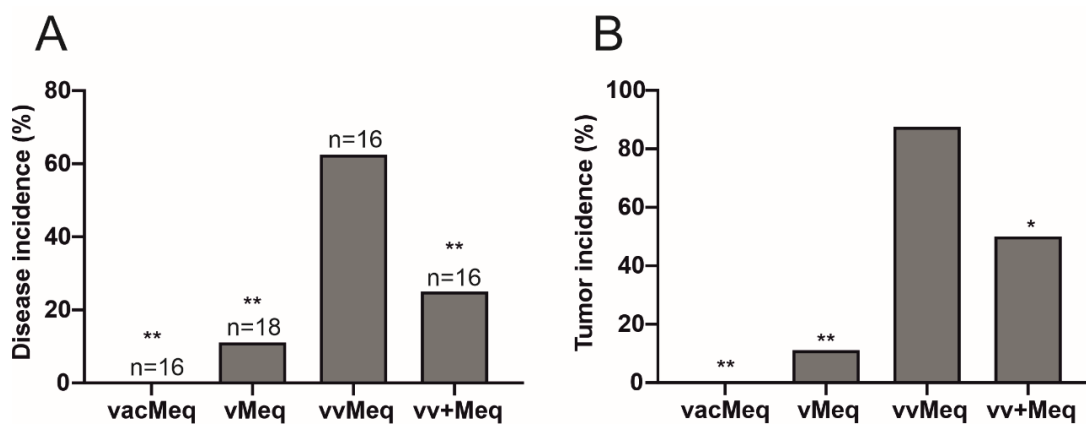
Figure 20: Pathogenesis in animals infected with *meq* recombinant viruses. (A) Disease incidence of chickens infected with the indicated recombinant viruses and (B) tumor incidence as percentage of animals that developed tumors during the experiment. Asterisks indicate significant differences compared to vvMeq (* $p<0.05$ and ** $p<0.0125$; Fisher's exact test).

Table 6: *meq* genes from different MDV pathotypes and genomic sequences from all viruses used in this study.

meq isolated	Genbank accession	Recombinant virus	Genbank accession
CVI988/Rispens	AY243335.1	vacMeq	MT797630
JM/102W	HM488348.1	vMeq	MT813453
617A*	AY362712.1		
RB-1B	AY243332.1	vvMeq	MT797629
N	AY362718.1	vv+Meq	MT797631

* *meq* gene and recombinant virus used in the biological replicate (Fig. S1)

Recombinant virus	Polymorphism type	Nucleotide change	Coding region affected	Protein effect
vacMeq	SNP transition	A -> G	None	No effect
vvMeq	SNP transition	A -> G	None	No effect
vv+Meq	SNP transition	T -> C	UL31	No effect (silent)
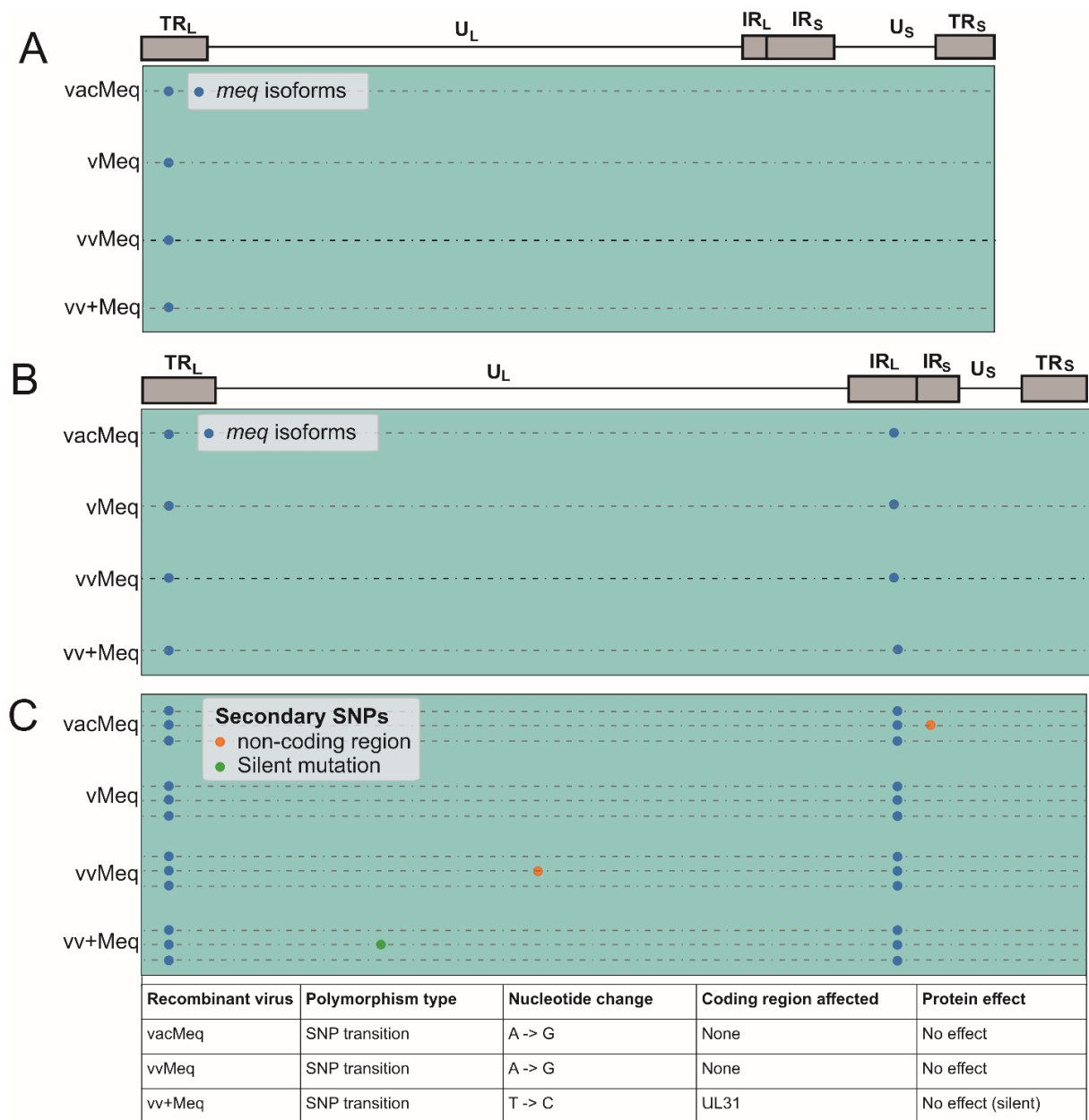
Figure 21: Next-generation sequencing of recombinant viruses. (A) The recombinant BACs generated only harbored the natural mutations in *meq* of the different *meq* isoforms inserted in the RB-1BΔIRL. (B) The recovered recombinant viruses in cell culture (passage 4) had no secondary mutations in the genome. Both copies of meq are present, as the IRL is restored. (C) Three representative samples from each recombinant virus from organs or tumor samples were extracted and sequenced. The sequences were aligned with the respective recombinant virus from passage 4. No mutations were detected in *meq*, and only minor point mutations in the minority of the viruses as summarized.
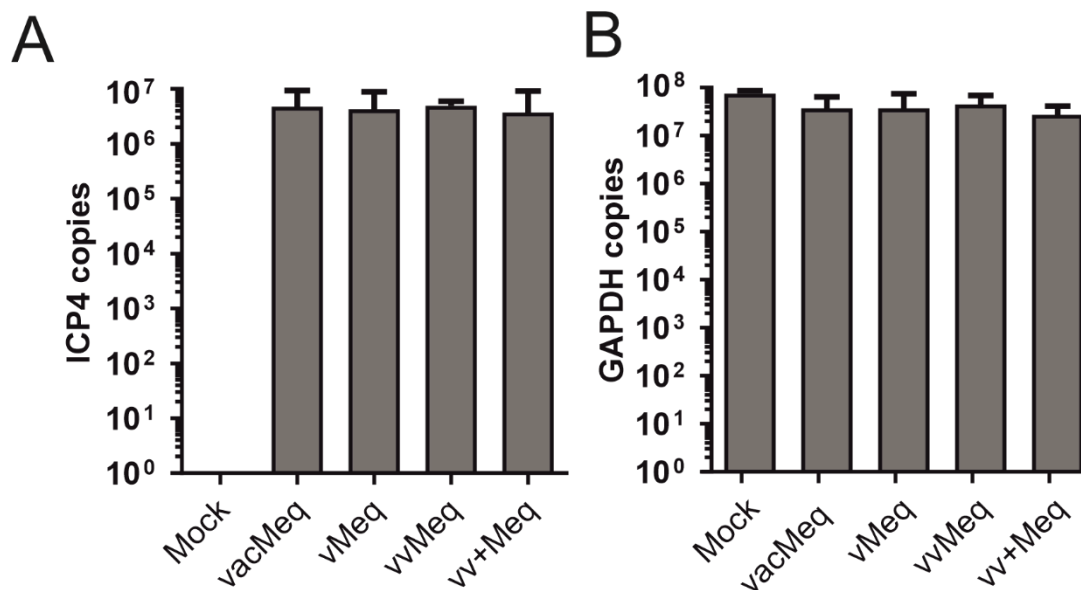
Figure 22: RT-qPCR analysis _in vitro_. The viral ICP4 (A) and cellular GAPDH (B) expression levels were used to control for the infections and the number of cells respectively. Viral ICP4 copies (A) and cellular GAPDH (B) were assessed by RT-qPCR and were not statistically different ($p > 0.05$, Kruskal-Wallis test).

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9 Discussion

9.1 General discussion

Marek's disease virus is an economically devastating diseases of poultry. It is also one of the most frequent virus-induced cancers in the animal kingdom and typically results in the death of infected birds [1]. To control the disease symptoms and to minimize financial costs, vaccines were developed that protected chickens [2]. Unfortunately, there are weaknesses to all the generations of vaccines that were introduced – they do not provide sterilizing immunity, or also known as 'leaky' vaccines. Although these vaccines are designed to prevent disease development, they had a severe impact downstream which allowed the viruses to become resistant and to break the vaccine barriers [3]. These leaky vaccines led to the emergence of vaccine-resistant MDV strains and the evolution toward higher virulence. Since the first introduction of vaccines against MD, MDV continued to evolve, and since today no new vaccines have been introduced to stop the spread of MDV. The mechanisms underlying MDV evolution are complex, and several factors have been discussed to delineate the emergence of higher virulent MDV strains. Some of these determinants for MDV evolution have been discussed, which includes farming practices and vaccination with leaky vaccines.

Several studies were able to pinpoint changes in the genome and identified genes that acquired polymorphisms throughout the decades that could correlate with increased virulence and the emergence of new viral strains with greater fitness [4-6]. In all these studies, one common discussed gene is the *meq* gene. The major oncogene *meq* is one of the major players in MDV pathogenesis and oncogenesis. Therefore, it is no surprise that this gene is involved in the evolutionary process of MDV. The *meq* gene acquired several mutations in the past decades, and these polymorphisms are specific for each pathotype. I took the opportunity to determine experimentally whether these specific point mutations in *meq* relate to an increase in virulence and whether they could potentially drive MDV evolution. To do this, I introduced the *meq* genes from various virulence classes into the common RB-1B strain by using two-step Red-mediated mutagenesis [7]. These recombinant viruses recovered were then used to study their *in vitro* replication and spread, and also to determine their role in pathogenesis *in vivo*. I could show that the *meq* isoforms did not differ in their replication properties *in vitro* and *in vivo*. The significant results were obtained in their natural hosts. In unvaccinated hosts, I show that the *meq* isoforms differed dramatically and the lower virulent *meq*, abrogated or severely impaired pathogenesis. In contrast, the higher virulent *meq* isoforms readily caused disease and tumors. This alone was already an astonishing finding, as the *meq* isoforms only differ by a few point mutations, they vary dramatically in pathogenesis and oncogenesis. Next we vaccinated chickens with the HVT-vaccine and then challenged them with the respective *meq* isoform viruses. The lower virulent *meq* isoforms did not cause disease in the vaccinated

chickens, revealing that the vaccine barrier was quite robust. The higher virulent *meq* isoforms were able to break the vaccine barrier, especially vv+Meq, and caused disease. Interestingly only the vv+Meq was able to cause tumors in vaccinated hosts. Taken together, our data demonstrate that very few point mutations in a single herpesvirus gene are critical for the increase in virulence, vaccine resistance, and transmission.

While dissecting the role of *meq* isoforms, I concurrently investigated why the CVI vaccine expresses multiple *meq* genes. Interestingly, commercial vaccine stocks, CVI988, express two predominant isoforms of the major MDV oncogene *meq* [8]. The vaccine expresses these oncogenes but is not oncogenic. The longer forms (L*meq*) are identical to the short *meq* (S*meq*) form, but with an in-frame insertion of 180 bp (60 amino acids) in the transactivation domain. The insertion consists of proline-rich repeats (PRR) that likely arose from a domain duplication [9]. I then similarly as above exchanged the RB-1B *meq* with these two forms respectively to study them individually. I found, although they only differed by 60 aa insertion, they vary dramatically in pathogenesis and oncogenesis *in vivo*. MDV pathogenesis in chickens infected with the S*meq* is abrogated while L*meq* induced significantly higher MD incidence and tumor incidence as compared to the wild type. I showed mechanistically that the L*meq* strongly influences the vTR (also involved in tumorigenesis) expression via a cellular protein c-*myc* which could explain the observed phenotype.

All together from both studies, I demonstrated the significance of *meq* on MDV pathogenesis and how a few point mutations in one gene, could influence the evolutionary processes of MDV in a similar step-wise manner as the famous evolutionary model (summarized in Fig. 16). Also, how *meq* is involved in vaccine resistance and unexpectedly increased virus shedding into the environment. All will be discussed in greater detail.

9.2   The golden-standard vaccine expresses a potent oncogene?

Vaccines play a crucial role in the protection of animals and humans from deadly pathogens. As already mentioned, the vaccines against MDV are not sterilizing and allow viruses to still infect, replicate and then to spread to non-vaccinated animals – putting them at risk. Even worse, the vaccines against MDV are thought to drive the evolution and promote the emergence of pathogen strains that cause more severe disease in unvaccinated hosts [10, 11]. One vaccine of focus here is the golden-standard and most efficacious commercial vaccine – CVI988 – and that is currently used to protect chickens up to vv+MDV pathotypes. Interestingly, we and others observed that at least two *meq* isoforms are expressed in the commercially available CVI vaccine stocks [4, 8, 12]. These two *meq* isoforms differ by an insertion of 180 bp in the transactivation domain (L*meq*) compared to the S*meq* (Fig. 11). Both

isoforms are only expressed in the CVI vaccine and have not been detected in other MDV strains, such as RB-1B and MD5.

The reason for the two predominant *meq*s expressed from the CVI vaccine remains unknown. One plausible hypothesis is, is that both the short and long-form are present in the genome simultaneously – one in the TR_L and one in the IR_L [13]. As this would be difficult to prove with next-generation sequencing, it is not entirely impossible. Similarly, it has been shown that two related phosphoproteins, pp38 and pp24, that are located in the TR_L and one in the IR_L, could have been once identical that characteristically split years back [14]. Then others contradict this theory by hypothesizing if this would have been true, the band intensities following PCR would have been the same [15]. I support the latter theory and add to that the CVI vaccine stocks consist instead as a population of viruses harbouring different *meq*s. The major population of the stock harbours the Lmeq, in both TR_L and IR_L, and the minor population has the Smeq. It would be interesting to know what the original isolated CVI988 virus looked like before being passaged. A troublesome finding shows that with prolonged passaging of the CVI vaccine stock, the subpopulation harbouring the L*meq* overtook the entire population at passage 35 with no S*meq* detected [12]. Why this is unwanted, and on top questions the continuance of the CVI vaccine, is explained in our study in which I examine the S*meq* and L*meq* individually [16].

We, therefore, inserted the respective isoforms into a very virulent RB-1B MDV, thereby replacing its original *meq* gene (vSmeq and vLmeq) (Fig. 11A). I characterized these viruses *in vitro* and *in vivo.* When I recovered the respective viruses and performed growth kinetics and plaque size assays *in vitro*, no significant differences were observed between them (Fig. 11B, and C). The 180 bp insertion into L*meq* did not alter or benefit the *in vitro* replication or spread. Also, by replacing the original RB-1B *meq* with the vaccine, *meq*'s respectively did not influence its growth properties, and they replicated similarly. Next, I characterized the expression levels of the *meq* from the viruses generated. All the viruses generated had similar protein expression level as compared to its counterpart in the wild type virus (Fig. 11D), allowing us to proceed towards *in vivo* experiments.

I infected chickens with the recombinant viruses and evaluated the (i) replication *in vivo* (ii) MD incidence and iii) tumor incidence. I additional monitored the spread of these recombinant viruses by hosting the infected chickens with naïve contact chickens. No differences were observed in *in vivo* replication of the recombinant viruses in infected chickens and contact chickens, as compared to the wild type (Fig. 12A and B). The vSmeq completely abrogated pathogenesis, and none of the infected or contact chickens succumbed to disease (Fig. 13A). This is very intriguing, as the difference between the S*meq* and the RB-1B *meq* is only three amino-acid change located in the N-terminus (Fig. 11A). This indicates that although the SMeq

is expressed from a vvMDV strain, these few mutations render it apathogenic while the RB-1B was able to cause disease in chickens. This vSmeq recombinant virus could be a possible vaccine candidate. A few studies propose a vaccine that lacks the *meq* as a possible vaccine [17-21]. In the studies, the results varied where the Δ*meq*-virus protected chickens, at comparable levels as to the CVI vaccine or even better [22]. Some studies show that Δ*meq*-virus induces thymus atrophy [23], and showing that it has weaker immunosuppression [20]. It has been shown that in chickens infected with MDV developed T cell immune responses against Meq [24], therefore making the S*meq* virus here a potential candidate. In contrast, the vLmeq drastically enhanced pathogenesis and oncogenesis in infected and contact chickens (Fig. 13A and C). The vLmeq remarkably had higher MD incidence as compared to the wild type by just a 180 bp insertion in the transactivation domain. The spread of the vLmeq from the infected to contact chickens infected via the natural route was also significantly higher as 50% of the contact animals succumbed to disease, whereas the wild type counterpart had only a mortality rate of 18% (Fig 13C). These results were unexpected and therefore, the animal experiment was repeated independently by our collaborator (Prof. Mark S. Parcells). The animals again were infected with the wild type and the vLmeq viruses and held for 42 days using a different chicken line. The same trend was observed and vLmeq outcompeted the wild type thereby confirming our results where vLmeq markedly elevated disease incidence (Fig. 13E).

I also quantified the number of animals that developed macroscopic tumors. The vLmeq showed the highest tumor incidence as compared to the wild type in infected and contact animals, while no tumors were present in the vSmeq group (Fig. 13B and D). The tumor development in the independent animal experiment showed the same trend for the vLmeq oncogenic potential as it also had elevated tumor incidence as compared to the wild type (Fig. 13F). I was also interested in the number of organs that had displayed tumors (tumor dissemination) per animal. Intriguingly, tumor dissemination was significantly enhanced in birds infected with the vLmeq as more organs harboured tumor lesions per animals (Fig. 14A) - again highlighting the high oncogenic potential of the vLmeq.

In an attempt to answer this increased observed MD- and tumor incidence of vLmeq, I focussed on the 180 bp insertion present in L*meq.* The insertion is in the transactivation domain of *meq* that could influence the transcriptional activity of Meq. Previously it has been shown that mutations in the transactivation domain of *meq*, affects transactivation activity of higher virulent strains [25]. Specifically, the point mutations that arose in the vv+MDV strains at amino acid positions (217, 283 and 320) elevated the transactivation activity as compared to lower virulent pathotypes. It has also been reported that the 180 bp insertion that, in turn, increase the copy number of the PRR exerts a somewhat transrepression effect [13]. Due to the vast spectrum

of genes that could be affected by *meq*, I focussed on a gene that plays a role in transformation – the vTR – that shows transformation potential similar as to Meq [26, 27]. The expression of vTR is not directly regulated by Meq, but rather via cellular c-*myc* (Fig. 7B) [26, 28]. Transactivation assays and chromatin immunoprecipitation assays demonstrated the involvement of the c-*Myc* oncoprotein in the transcriptional regulation of vTR [26]. I quantified the expression levels of vTR in cells infected with the respective viruses. I could show that the expression of the L*meq* isoform significantly upregulates vTR expression as compared to the wild type and S*meq*. This demonstrates that the insertion in the transactivation domain of L*meq* strongly influences its transactivation potential, thereby interacting with cellular c-*myc* and affecting downstream vTR expression. Upregulation of vTR expression coud result in an increased telomerase activity, that has also been observed in chickens infected with higher virulent MDV strains – therefore, providing one of the explanations for the increased oncogenesis. There could be several other downstream processes potentially changed with altered transactivation potential, and these pathways remain to be explored, especially with other interacting partners such as c-Jun.

Why the commercial vaccine is not oncogenic, even expressing the potent Lmeq remains elusive. One hypothesis could be that the presence of two Meq proteins in an infection could interfere with the dimerization functions Meq has. Meq has the ability to autoregulate its expression and affect the expression of other genes [29]. In a study, the transactivation functions of MD5 strain Meq was tested in the presence of CVI-Meq proteins [8]. They found that by co-transfecting both or individual CVI vaccine Meq proteins with MD5-Meq, decreased the MD5-Meq mediated activation of the *meq* promoter. The LMeq and SMeq could affect with the expression and thereby interfere with the binding of MD5 Meq with its cellular dimerization partners. This 'mixed infection theory' of two Meq's has been shown to modulate dimer formation (Meq/Meq) or (Meq/c-Jun for example) that affects downstream pathways for transcriptional regulation by Meq [30]. Another explanation for the non-existent oncogenic potential of the CVI vaccine could be that the CVI genome contains a number of mutations in other essential genes that could render it apathogenic. These all could at least in part contribute to the protection mechanisms of the CVI vaccine. Additional experiments are necessary to examine further all these hypotheses mentioned above as to why the CVI vaccine does not cause tumors. Also, the possibility of the vSmeq as vaccine candidate remains to be addressed.

To summarize, I could show that the L*meq* isoform enhances MDV pathogenesis and oncogenesis of a very virulent MDV strain, while insertion of the S*meq* isoform completely abrogated MDV pathogenesis. Our results from the L*meq* isoform break with the long-standing

assumption that it is a marker for attenuation and demonstrate that other mutations in the CVI genome could contribute to its attenuation.

9.3   The *meq* oncogene: The critical player in MDV evolution

In this study, I determined the contribution of *meq* isoforms in MDV pathogenicity and oncogenicity. I could provide the first experimental evidence that small evolutionary changes in this major virus-encoded oncogene alone have a substantial impact on the evolution of MDV towards a greater virulence. In addition, I demonstrated that these minor mutations residing in the *meq* oncogene allows the virus to overcome the vaccine barrier and also enhanced shedding of MDV. Here, with two main animal experiments, the findings will be discussed in more detail.

9.3.1   The *meq* oncogene enhances pathogenesis in chickens

To enable us to study the individual *meq* genes from different pathotypes, the *meq* isoforms of different pathotypes (vMDV, vvMDV, vv+MDV and the CVI988 vaccine) were inserted individually into the very virulent RB-1B strain instead of its original *meq* gene. To confirm the insertion of *meq* into the correct location (TR$_L$) I sequenced the BACs generated with next-generation sequencing (Fig. 21A). The full genome of the recombinant viruses recovered from cell culture were sequenced. Sequencing results confirmed that no secondary mutations are present in the genome and that the *meq* is present in both locations (TRL and IRL) (Fig. 21B). The recovered recombinant viruses, termed vacMeq, vMeq, vvMeq and vv+Meq were then characterized *in vitro* and *in vivo.* The point mutations *meq* acquired in field strains through decades did not significantly alter the replication properties *in vitro* (Fig. 15C and D) and *in vivo* (Fig. 16A). Meq is expressed in lytic and latent phase during MDV infection [31]. It has been demonstrated that Meq is dispensable for replication with an MDV lacking the *meq* gene, rMd5 virus [32] and RB-1B Δmeq [33]. Although, the insertion of the vv+ *meq* gene slightly reduced virus replication as compared to the parental strain (Fig. 15C and D). Interestingly, this trend is also observed in the vv+ parental strains, as attenuated MDV strains grow more efficiently *in vitro* compared to vv and vv+ strains [34]. Previous *in vitro* studies have established in which Meq is consistently expressed in lymphoblastoid cell lines derived from MD tumors, such as MSB-1 [35, 36]. In *in vivo* experiments performed indicate that the ΔMeq virus is fully attenuated and the chickens did not develop any lymphomas [17, 18, 32] – indicating the critical transforming properties of *meq.* We demonstrate that the minor mutations residing in the *meq* isoforms did not affect *meq* expression in primary CEC as all the *meq* isoforms were expressed in equal amounts with no statistical difference (Fig. 15E). It has been demonstrated that there

at least two other splice variants of *meq* that were detected [37]. The alternative splicing gives rise to a splice form with exon 2 and 3 of vIL-8, designated as meq/vIL8. We therefore finally concluded the *in vitro* characterization of the *meq* isoforms by assessing the expression of this splice variant by qRT-PCR in both CEC and CD4 T cells. We could show that these minor changes in *meq* do not affect meq/vIL8 splicing *in vitro* (Fig. 15F). This is consistent with a previous study that showed that splice variants did not differ between different pathotypes in infected primary chicken B cells [33]. The comparable expression of meq/vIL8 likely due to the absence of mutation in the splice donor site encoded in the leucine zipper domain in the *meq* isoforms, while the branch point and acceptors sites are outside of *meq* and were not altered in our study.

The observed increase in virulence of strains over the years has been characterized by the ability to induce lymphoproliferative lesions [38] and an increase in shedding [10], thereby shifting our focus towards these aspects and the contribution of *meq* in MDV-induce pathogenesis and most importantly tumor formation. I infected one-day-old chickens with viruses harbouring *meq* isoforms from different pathotypes to determine their contribution to virus-induced pathogenesis and oncogenesis *in vivo*. In this experiment, I also hosted the infected chickens with naïve contact chickens to measure the horizontal spread via the natural route of infection. All viruses replicated efficiently and, like *in vitro*, did not display any significant difference between them (Fig. 16A). The insertion of the lowest virulent form, *meq* from the CVI988 strain, completely abrogated MDV pathogenesis (also shown previously by us [16]). It is interesting that only a few mutations residing at the N-terminus (Fig. 15B) of the CVI *meq* rendered it apathogenic as compared to the parental strain (Fig. 16B). These mutations, especially at position 71 and 77, are dominating in the lower virulent *meq* isoforms and possibly attenuate the MD [4]. It has been previously shown that these specific mutations interfere with Meq's transactivation potential and thereby affecting downstream cellular or viral pathways [39]. The insertion of the vMDV *meq* into RB-1B showed reduced disease incidence and tumor incidence in infected chickens (Fig. 16B). As expected, the higher virulent isoforms, vvMeq and vv+Meq showed higher disease incidence (Fig. 16B) in infected animals. When I assessed the tumor incidence of all infected chickens the lower virulent isoforms, vacMeq and vMeq, had either no or very low tumor incidence (Fig. 16C). In contrast, the oncogenic potential was enhanced for the higher virulent *meq* isoforms, vvMeq and vv+Meq (Fig. 16C). Also, when I monitored the number of organs with gross lesions, the vv+Meq group, displayed the highest tumor dissemination compared to all the other groups (Fig. 16D). Although the vMeq isoform caused tumors in infected animals, tumor dissemination was severely impaired as only the spleen developed macroscopic tumors. To confirm our findings, the experiment was also performed independently by our collaborator, Prof. Mark Parcells, using a different chicken

line. The data obtained were comparable to ours, therefore confirming the observed phenotype (Fig 20).

I investigated the disease in animals infected with the the recombinant viruses via the natural route by co-housing naïve chickens with the subcutaneously infected animals. The lower virulent isoforms, vacMeq and vMeq, did not drastically cause disease in infected animals, as compared to the parental strain, but were able to spread to the naïve contact chickens as MDV genome copies were readily detected (Fig. 17A) but did not cause disease in any contact chicken (Fig. 17B). However, the horizontal spread for the higher virulent *meq* isoforms, vvMeq and vv+Meq, were more pronounced. The vv+Meq group had the highest number of natural infected birds that succumbed to disease (Fig. 17B). I observed a similar trend for tumor incidence, where the lower virulent *meq* isoforms that spread via the natural route were unable to cause tumors. The higher virulent *meq* isoforms, in contrast, caused tumors in the contact animals and allowed dissemination into more organs (Fig. 18C and D).

I assessed the mutations in vv+ *meq*, especially those residing in the transactivation domain (Fig. 16B). The mutations found in the C-terminal of vv+Meq gene have been shown to correlate with its transactivation potential and transactivation is enhanced for several cellular and viral genes [40]. The specific mutations in the vv+ *meq* at position 217, 283, and 320 were shown to be important for its enhanced transactivation activity [25]. I examined these mutations and the effect on the inherent transactivation (unpublished data). To study the transactivation activity, I constructed effector plasmids containing the Gal4-DNA binding domain fused with the transactivation domain of either the vacMeq, vMeq, vvMeq or vv+Meq (Fig. 23A). I studied the transactivation activity of these *meq* isoforms using these effector plasmids by co-transfecting them with the pG5TK-Luc reporter construct containing five tandem Gal4-binding sites (DBS) adjacent to a minimal adenovirus E1B promoter upstream of the firefly luciferase gene. The E1B promoter is transcriptionally silent and once activated by the respective Meq's, luciferase is expressed and measured. The vv+Meq showed the highest transactivation activity compared to the others. The mutations in the transactivation domain acquired by vv+Meq triggered a significant 40-fold increase in transactivation. The vvMeq also had a high transactivation activity (27-fold) comparable to the vacMeq (21-fold). The vMeq had the lowest transactivation potential, with only a 12-fold increase in transactivation (Fig. 23B).
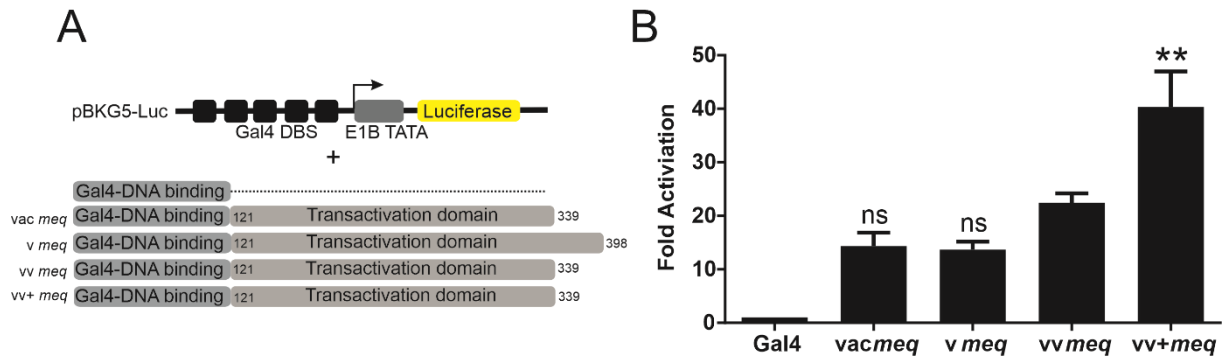
Figure 23: Transactivation of the different *meq* isoforms. (A) The pG5TK-Luc reporter plasmid, containing Gal4 binding sites, expresses luciferase when bound and transactivated by the transactivation domains of the different *meq* isoforms. (B) The Luciferase is measured and plotted as fold activation. Asterisks indicate significant differences to vv meq (** $p < 0.0125$; Tukey's multiple comparisons test).

The number of functions and pathways that could be affected by these mutations are vast and could benefit virus pathogenesis and increased virulence. One example is that Meq binds to and transactivate cellular miRNA gga-miR-21, which in turn targets chicken programmed death cell 4 (PDCD4) that promotes tumor cell growth and apoptosis [41]. This could be one of the possible explanations for the high tumor incidence observed for chickens infected with vv+Meq. Also, the mutations affecting a PLDLS motif in vv+Meq could affect the enhanced induction of lymphomas in chickens. The PLDLS motif binds and interact with cellular CtBP and regulate the development, proliferation, and apoptosis and have been previously reported to play a role in oncogenesis [42]. Also, the chicken CD30, which is discussed to be involved in MDV lymphomagenesis, has 15 potential binding sites for Meq [43]. Thus, the enhanced transactivation of vv+Meq could also lead to CD30 overexpression, favouring neoplastic transformation. The latter hypothesis is consistent with an observation on other oncogenic viruses such as Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus [44]. However, CD30 overexpression in MDV-induced tumors could not be confirmed in follow-up studies [45]. Another role for the enhanced transactivation of the higher virulent *meq* isoforms, is the potential to evade the innate immunity and is explained in the next section. The data from the first animal experiment revealed a trend that is consistent with the stepwise evolution of MDV and shows that the specific mutations that accumulated in the *meq* gene play a critical role in pathogenesis and oncogenesis of MDV.

9.3.2 Minor polymorphisms in *meq* drive vaccine resistance

In the second animal experiment, I aimed to assess the ability of the different recombinant viruses to break the vaccine barrier and whether the acquired polymorphisms influence

horizontal transfer. I used the HVT-vaccine that protects up to vMDV strains in order to measure whether the mutations in the *meq* isoforms facilitate vaccine resistance. I vaccinated chickens and seven days later I challenged them with the viruses harbouring the different *meq* isoforms. With primers differentially detecting all the recombinant viruses and the HVT-vaccine, I showed that all the viruses replicated efficiently in vaccinated animals (Fig. 18A an B). The disease incidence for all groups were delayed due to the vaccinal protection provided by HVT, when compared to the previous experiment with unvaccinated chickens. Nonetheless, the first chickens succumbed to disease were the birds challenged with the highest isoform, vv+Meq (Fig. 18C). Later the vvMeq virus also made its appearance with mortalities in chickens. In contrast, I observed no MD incidence in the groups that were infected with the lower virulent meq viruses (Fig. 18C). This observation was striking and demonstrated that the mutations aquired in *meq* allowed the virus to overcome the vaccine hurdle in the chickens. Even more impressive was when I quantified tumor incidence. Only the chickens infected with the vv+Meq were still able to cause tumors, despite the vaccination with HVT that was supposed to protects them from developing disease and tumors.

In contrast, none of the other groups displayed any macroscopic tumors in organs (Fig. 18D). In our previous experiment, I could show that the higher virulent *meq* viruses, vvMeq and vv+Meq, successfully transmitted to the naïve contact chickens and cause MD (Fig. 17B). Here I monitored spread by collecting feathers and dust from every group to assess the spread of the viruses. MDV is shed into the environment via scales and feather debris, which become the major source of contamination of other birds in the natural environment. Epithelial cells from the feather follicles are the only known cells that produce high levels of infectious mature virions as shown by transmission electron microscopy [46-48]. Finally, feathers harvested from animals and dust are today considered excellent materials to monitor spread of pathogenic viruses and transmission [49-51]. The viruses of the higher virulent *meq* isoform viruses, vvMeq and vv+Meq reached the feather follicle earlier (Figure 18E). They persisted at significantly higher concentrations in the feather follicle, as compared to the lower virulent meq isoforms (Fig. 18E). Consequently, the levels of virus shedding of the higher virulent *meq* isoforms were higher in dust throughout the whole duration of the animal experiment (Fig. 18F). The enhanced levels of shedding of virus into the environment likely provide an evolutionary advantage for the virus by extending the infectious period. The enhanced shedding could likely be due to the higher number of transformed T cells that transport the virus to the skin and efficiently reactivate it [52], resulting in the production and shedding of the virus. Read et al. recently demonstrated that vaccination with leaky vaccines enhances viral shedding and onward transmission of vv+MDV strains. Also, they showed that the cumulative shedding of less virulent strains is reduced by vaccination, but increased by several orders of magnitude

with highly virulent strains [10]. Our data indicates that the evolutionary acquired mutations in *meq* contribute to this enhanced shedding that increases the level of infectious virus in the environment and provides a selective advantage for more virulent strains.

To focus on the fact that only vv+Meq was able to cause tumors in vaccinated chickens, I looked at the transactivation domain, where most of the point mutations reside (Fig. 15B). I turned to the first line of defence against MDV – the innate immunity. We hypothesized that these mutations in *meq* are selected in such a manner that the virus can overcome vaccinal immune responses via interruption or deregulation of the innate signalling pathways. Meq can form homodimers and heterodimers with specific intracellular signalling proteins that modulate the innate immunity [40]. I stimulated primary chicken T cells with innate immune agonists (Poly I:C, LPS and cGAMP) and infected these cells with the different recombinant viruses. The agonists Poly I:C (triggering the TLR3 pathway), LPS (triggering the TLR4 pathway), and cGAMP (triggering the cGAS-STING pathway) all initiate innate responses critical for mounting the adaptive immune response [24, 53, 54]. All the treatments of the cells significantly decreased the number of plaques (Fig. 19A), and the plaque sizes (Fig. 19B) compared to the media control. Strikingly, viruses harbouring the higher virulent *meq* isoforms (vv and vv+Meq) formed significantly more plaques than the one with lower virulent isoforms (Fig. 19B), thereby possibly interfering with the signalling cascade and overcoming the innate immunity. Consistently, CEC infections with higher virulent *meq* isoform viruses led to increased plaque sizes compared to vacMeq and vMeq (Fig. 19). In previous work, it has been shown that MDV can evade the cGAS-STING DNA sensing pathway [53]. They show that Meq delayed the recruitment of TANK-binding kinase one and IFN regulatory factor 7 (IRF7) to the STING complex, thereby inhibiting IRF7 activation and IFN-β induction. The overexpression of Meq reduced antiviral responses and in contrast, a ΔMeq elevated MDV-triggered induction of IFN-β and downstream antiviral genes [53]. This type of evasion has also been demonstrated for other oncogenes such as, E7 from human papillomavirus (HPV) and E1A from adenovirus, as potent and specific inhibitors of the cGAS-STING pathway [55]. Also, it has been shown that the *meq* gene contributes to the enhanced NK-cell activity and IFN-γ production *in vitro* [56]. Also, p53 is a central mediator of the global innate immune response [54]. In a study by Deng et al., they found that Meq binds directly to p53 and that this interaction resulted in inhibition of the transcriptional and apoptotic activities of p53 [57], thereby explaining the dysregulation of the protective innate immunity pathways I observe. In our study, I could prove this is indeed true, and additionally, show that this was acquired through the evolution of MDV where the more virulent strains now cause vaccine breaks that are mediated by *meq.* It is unclear which step of the signalling pathway is modulated by Meq and this interplay should be investigated to understand the role of Meq in the innate immunity in future studies. Overall, our findings

suggest that the mutations in the higher virulent *meq* isoforms provide an advantage in the vaccinated animals by allowing the virus to overcome these innate responses early upon infection.

In summary, our data demonstrate that minor polymorphisms in *meq* drastically alter disease outcomes in naïve and vaccinated chickens. The *meq* isoforms from highly virulent MDV strains are required for efficient disease and tumor formation, while those from less virulent strains severely impair or abrogate disease and tumor incidence. Also, I show that the mutations that arose in the *meq* from higher virulent strains permitted vaccine resistance and the ability to shed at higher rates in the environment; all factors promoting the evolution of this deadly pathogen.

10 Concluding remarks and outlook

On a global scale, poultry is the most important source of animal protein for humans and secures the livelihood of millions of people. The spread of newly emerging MDV strains that overcome vaccine protection on a large scale would have catastrophic consequences for the global food supply and threaten the welfare and the lifes of billions of chickens. It is imperative, therefore, to obtain a better understanding of virus evolution and vaccine resistance in order to take measures against this imminent threat. My PhD project focused on the evolution of Marek's disease virus. Despite many years of research, the changes in the MDV genome that contribute to vaccine resistance remain poorly understood. Here I specifically addressed the mutations in *meq* from different pathotypes. I showed that these evolutionary point mutations that arose are key players in the observed increased MDV virulence. With the information obtained here, we understand the evolutionary processes better and will allow us to develop new vaccines that provide better protection and might even block virus evolution towards increased virulence.

Another aim I set to address is to determine if the selective pressure provided through vaccines results in the selection of more virulent viruses. I experimentally evolved MDV *in vitro* to determine 'hotspots' in the genome susceptible for mutations. With this information, it will be possible to examine genes that could be, like *meq*, be involved in an increase in virulence and vaccine resistance. The work for this is still ongoing. Briefly, experimental evolution is a method that is gaining popularity where the genetic basis of the evolutionary process can be revealed through high-throughput sequencing. Large DNA viruses like MDV is considered to be genetically stable, but with selective pressures, such as vaccines, allows for evolution and the emergence of more virulent strains. Because evolution relies on the random occurrence and subsequent spread of rare beneficial mutations, serial passaging varies considerably and could end up in attenuated virus stocks [58]. I used a simple *in vitro* based experimental setup where the rate of evolution is chemically accelerated using ethyl methanesulfonate (mutation rate of 5×10^{-4} to 5×10^{-2} mutations [59]). The fitness and the increase in virulence of the virus in cell culture was then measured in an animal model (vaccinated and unvaccinated animals). Our data obtained from next-generation sequencing of tumor samples will provide insight into how higher virulent strains could potentially emerge that are vaccine resistant and also which genes are essential in MDV evolution.

I covered the aspect that vaccines are leaky, and they circulate in the environment along with other environmental strains. Previous studies have demonstrated the presence of multiple strains of Marek's disease virus simultaneously circulating within poultry flocks, leading to the assumption that individual birds are repeatedly exposed to a variety of virus strains in their lifetime. Another experiment, 'a virus competition study' that could deliver exciting results is to

evaluate virus competition between the *meq* isoforms and between vaccines and *meq* isoforms to determine which virus sheds at higher rates. A similar study was performed by Dunn *et al.* [45]. They showed in different pathotypes, pathogenically similar (rMd5 and rMd5/pp38CVI) or dissimilar (JM/102W and rMd5/pp38CVI) virus pairs that the higher virulent strains had a competitive advantage over the less virulent strains [45]. This would be indeed interesting for the individual *meq* isoforms and to determine which will dominate following simultaneous infection. Virus competition within individual birds may be an essential factor that influences the outcome of co-infection under field conditions, including the potential outcome of emergence or evolution of more virulent strains

Although vaccines are considered as one of the most significant achievements of modern medicine to protect humans and animals, alternative interventions are possible for MDV. One solution will be, and features ongoing studies, in collaboration with the Technical University of Munich, to implement the CRISPR/Cas9 (clustered, regularly interspaced short palindromic repeats-CRISPR associated 9) system to target MDV and providing increased resistance to virus infection. This novel approach of bacterial CRISPR-Cas9 gene editing system can be used to engineer resistance to DNA viruses through direct cleavage of the virus genome. Our lab showed with several guide-RNAs (gRNAs) targeting essential MDV genes that virus replication can be abrogated [60]. An optimized combination of gRNAs and Cas9-expression cassettes will be tested in an *in vivo* setting. The MDV resistant chickens are currently being tested and will soon unveil whether the CRISPR/Cas9-based system can be used as an effective treatment strategy against MDV as opposed to vaccines.

On a different track, many aspects of MDV pathogenesis remain elusive, mostly due to the lack of technologies that allow tracking of the virus in infected animals. I recently developed recombinant MDVs that express luciferase either during lytic replication or in transformed cells, resulting to deadly lymphoma. With our collaborators at the INRA-ISP, Dr. Sascha Trapp and Dr. Caroline Denesvre, we want to use these viruses to determine i) how MDV really enters the chicken, ii) how the virus disseminates in the body of the host and iii) if we can visualize tumor development in animals over time. Animals will be infected at the INRA-PFIE facility which is specialized on *in vivo* imaging and have the complementary expertise to address these questions. These experiments would not only shed light on the MDV pathogenesis and provide strategies to block this deadly pathogen, but would also be a very valuable tool for the visualization of tumor development and drive our understanding of this process, leading to the most frequently diagnosed cancer in the animal kingdom.

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12 Summary

Vaccines are considered as one of the most significant achievements of modern medicine. The first vaccine that protected animals against cancer is a vaccine designed for Marek's disease virus. The most frequently used vaccine is the live-attenuated CVI988/Rispens (CVI) strain, which efficiently protects chickens against MD and prevents tumorigenesis. Interestingly, CVI expresses at least two isoforms of *meq,* the major oncogene of MDV. Meq is a basic leucine zipper (b-ZIP) protein consistently expressed in all MDV tumor and latently infected cells. We demonstrated that the longer isoform of *meq* strongly enhanced virus-induced pathogenesis and tumorigenesis, indicating that other mutations in the CVI genome contribute to virus attenuation. On the contrary, the shorter isoform completely abrogated pathogenesis, demonstrating that changes in the *meq* gene can indeed play a key role in virus attenuation.

Although vaccinated chickens are protected against developing MD symptoms, the development of vaccines has raised questions about the potential consequences of vaccine-driven evolution of viruses. MDV is continually evolving towards higher virulence despite generations of vaccination. Circulating field strains have acquired numerous genomic mutations in the last 60 years. However, the evolutionary adaptations responsible for the vaccine breaks remained elusive. Distinct mutations in the *meq* oncogene arose every time that new vaccines were introduced that ultimately provided an evolutionary advantage. We tested recombinant viruses harbouring *meq* isoforms from different field strains *in vivo*. Here, we demonstrate that a few distinct mutations in the virus-encoded oncogene *meq* are responsible for the increase in virulence and oncogenicity. The viruses expressing the lower virulent *meq* isoforms showed reduced pathogenicity while in contrast the higher virulent *meq* isoforms dramatically increased pathogenesis in unvaccinated hosts. Only viruses harbouring the highest virulent *meq* isoform were able to break the vaccine barrier and cause tumors in vaccinated hosts - likely by overcoming innate cellular responses. Concomitantly, the polymorphisms in *meq* enhanced virus shedding into the environment putting naïve animals at greater risk.

13 Zusammenfassung

Evolutionäre Polymorphismen im Onkogen meq erhöhen die Virulenz, die Virusausscheidung und die Impfstoffresistenz des Marek's Disease Virus

Impfstoffe gelten als eine der bedeutendsten Errungenschaften der modernen Medizin. Der erste Impfstoff, der Tiere vor Krebs schützte, war ein Impfstoff gegen das Marek's Disease Virus (MDV). Der am häufigsten verwendete Impfstoff ist ein abgeschwächte Lebendimpfstoff des CVI988/Rispens (CVI)-Stammes, der Hühner wirksam vor der Marekschen Krankheit schützt und die Tumorentstehung verhindert. Interessanterweise exprimiert CVI mindestens zwei Isoformen von *meq,* dem Haupt-Onkogen von MDV. Meq ist ein Leucin-Zipper-Protein (bZIP), das in allen MDV-Tumor- und latent infizierten Zellen konsistent exprimiert wird. Wir konnten zeigen, dass die längere Isoform des meq Gens die virusinduzierte Pathogenese und Tumorigenese verstärkt, was darauf hindeutet, dass andere Mutationen im CVI-Genom zur Virusabschwächung beitragen. Im Gegensatz dazu, verhinderte die kürzere Isoform die Pathogenese vollständig was zeigt, dass Veränderungen im *meq*-Gen tatsächlich eine Schlüsselrolle bei der Virusabschwächung spielen könnten.

Obwohl geimpfte Hühner vor der Entwicklung von Symptomen der Marekschen Krankheit geschützt sind, hat die Entwicklung von Impfstoffen Fragen über die möglichen Folgen einer durch Impfstoffe angetriebenen Evolution der Viren aufgeworfen. MDV entwickelt sich trotz generationenlanger Impfungen kontinuierlich in Richtung höherer Virulenz. Zirkulierende Feldstämme haben in den letzten 60 Jahren zahlreiche genomische Mutationen erworben. Die evolutionären Anpassungen, die für die Durchbrechung der Impfstoffbarriere verantwortlich sind, bleiben jedoch schwer zu ergründen. Spezifische Mutationen im *meq*-Onkogen traten jedes Mal auf, wenn neue Impfstoffe eingeführt wurden, die letztlich einen evolutionären Vorteil brachten. Wir testeten rekombinante Viren, die *meq*-Isoformen aus verschiedenen Feldstämmen enthielten, *in vivo.* Wir konnten zeigen, dass einige wenige bestimmte Mutationen im viruscodierten Onkogen *meq* für die Zunahme der Virulenz und der Onkogenität verantwortlich sind. Die Viren, die die weniger virulenten *meq*-Isoformen exprimieren, zeigten eine verminderte Pathogenität, während im Gegensatz dazu die stärker virulenten *meq*-Isoformen die Pathogenese in nicht geimpften Wirten dramatisch erhöhten. Nur Viren, die die stärkste virulente meq-Isoform enthielten, waren in der Lage, die Impfstoffbarriere zu durchbrechen und in den geimpften Hühnern Tumore zu verursachen - wahrscheinlich durch Überwindung der angeborenen zellulären Immunantwort. Gleichzeitig verstärkten die Polymorphismen im *meq* Gen die Ausscheidung von Viren in die Umwelt, wodurch naive Tiere einem größeren Risiko ausgesetzt waren.

14 List of publications

14.1 Scientific publications

Conradie, A., M., Bertzbach L.D, Trimpert J., Patria, J.N., Murata S., Parcells M.S., Kaufer B.B. 2020. Distinct polymorphisms in a single herpesvirus gene are capable of enhancing virulence and mediating vaccinal resistance. PLoS Pathogens. Accepted for publication

Vychodila, T., **Conradie, A. M**., T., Bertzbach, L. D., Kaufer., B. 2020.Marek's disease virus requires both copies of the inverted repeat regions for efficient *in vivo* replication and pathogenesis. Journal of Virology. Accepted for publication

Kooshkaki, O., Derakhshani, A., **Conradie, A. M**., Hemmat, N., Barreto, S.G., Baghbanzadeh, A., Singh, P.K., Safarpour, H., Asadzadeh, Z., Najafi, S., Brunetti, O., Racanelli, V., Silvestris, N., Baradaran, B. 2020. Coronavirus Disease 2019 (COVID-19): A brief review of the clinical manifestations and pathogenesis to the novel management approaches and treatments. Frontiers in Oncology.

Groenke, N., Trimpert, J., Merz, S., **Conradie, A.M.,** Wyler, E., Zhang, H., Hazapis, O., Rausch., Landthaler, M., Osterrieder, N., Kunec, D. 2020. Mechanism of Virus Attenuation by Codon Pair Deoptimization. Cell Reports, 31

Bertzbach, L. D., **Conradie, A. M.,** You, Yu., Kaufer, B. B. 2020. Latest Insights into Marek's Disease Virus Pathogenesis and Tumorigenesis. Cancers (Basel) 12 (2020)

Conradie, A. M., Bertzbach, L. D., Bhandari, N., Parcells, M. & Kaufer, B. B. 2019. A common live-attenuated avian herpesvirus vaccine expresses a very potent oncogene. mSphere, 4.

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Conradie, A. M., Stassen, L., Huismans, H., Potgieter, C. A. & Theron, J. 2016. Establishment of different plasmid only-based reverse genetics systems for the recovery of African Horse Sickness Virus. Virology, 499, 144-155.

Vermaak, E., **Conradie, A. M.,** Maree, F. F. & Theron, J. 2016. African Horse Sickness Virus infects BSR cells through macropinocytosis. Virology, 497, 217-232.

Vermaak, E., Paterson, D. J., **Conradie, A.,** Theron, J. 2015. Directed genetic modification of African horse sickness virus by reverse genetics. South African Journal of Science, 111, 7/8

Manuscript submitted or in preparation:

Wild, M., Kicuntod, J., Seyler, L., Wangen, C., Bertzbach, L.D., **Conradie, A.M**., Kaufer, B.B., Wagner, S., Michel, D., Eickhoff, J.,Tsogoeva, S. B., Bäuerle, T., Hahn, F., Marschall, M. 2020. Combinatorial drug treatments reveal promising anticytomegaloviral profiles for clinically relevant pharmaceutical kinase inhibitors (PKIs). Submitted to International Journal of Molecular Science.

Kunec, D., Dietert, K., Ebert, N., Thao, T. N, Vladimirova, D., Kaufer, A., Abdelgawad, A., **Conradie, A. M**., Höfler, T., Adler, J. M, Bertzbach, L. D., Gruber, A. D., Thiel, V., Osterrieder, N. and Trimpert, J. 2020. Development of safe, and highly protective live-attenuated SARS-CoV-2 vaccine candidates by synthetic genome recoding. Submitted to Nature Biotechnology

Conradie, A. M., Groenke, N., Kheimar, A., Kaufer., B. 2020. Vaccines drive selection of higher virulent viruses. Manuscript in preparation.

14.2 Talks and poster presentations

Conradie, A. M., Bertzbach, L. D., Parcells., M., Kaufer., B. (07.19). Evolutionary changes in the major oncogene directly determine the pathogenicity of a highly oncogenic alphaherpesvirus. 44th Annual international Herpesvirus workshop, Knoxville, USA

Conradie, A. M., Bertzbach, L. D., Bhandari, N., Parcells, M. & Kaufer, B. B. (04.19). A Common Live-Attenuated Avian Herpesvirus Vaccine Expresses a Very Potent Oncogene. ZIBI Graduate School Retreat, Akademie Schmöckwitz, Brandenburg

Conradie, A. M., Bertzbach, L. D., Parcells., M., Kaufer., B. (03.19). Evolutionary changes in the major oncogene directly determine the pathogenicity of a highly oncogenic alphaherpesvirus. 29th Annual Meeting of the Society for Virology, Düsseldorf

Conradie, A. M. and Kaufer., B. (04.18). A live-attenuated avian herpesvirus vaccine encodes a very potent oncogene. ZIBI Graduate School Retreat, Rheinsberg

Conradie, A. M., Bertzbach, L. D., Parcells., M., Kaufer., B. (09.17). Evolutionary changes in the major virus-encoded oncogene determine pathogenicity and shedding of Marek's disease virus. DRS Biomedical Sciences Doctoral Symposium, Berlin.

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17 Conflict of interests

There are no conflicts of interests

18 Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig und ausschließlich unter

Zuhilfenahme der genannten Quellen und Hilfen angefertigt habe.

Berlin, den 10.11.2020   Andelé Conradie