Marek's disease virus: from miasma to model

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Abstract | Marek's disease virus (MDV) is an oncogenic herpesvirus that causes various clinical syndromes in its natural host, the chicken. MDV has long been of interest as a model organism, particularly with respect to the pathogenesis and immune control of virus-induced lymphoma in an easily accessible small-animal system. Recent advances in MDV genetics and the determination of the chicken genome sequence, aided by functional genomics, have begun to dramatically increase our understanding not only of lytic MDV replication, but also of the factors and mechanisms leading to latency and tumour formation. This new information is helping to elucidate cellular signalling pathways that have undergone convergent evolution and are perturbed by different viruses, and emphasizes the value of MDV as a comparative biomedical model. Furthermore, the door is now open for rational and efficient engineering of new vaccines against one of the most important and widespread infectious diseases in chickens.

Polyneuritis
General inflammation of nerves.

Visceral lymphoma
Solid neoplasms in organs of the abdomen such as heart, liver, kidney or gonads that are characterized by an accumulation of transformed lymphocytes and immune cells.

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The first description of Marek's disease (MD) dates from 1907, when József Marek, the pre-eminent clinician of the Budapest veterinary school after whom the disease was named in 1960, reported a generalized polyneuritis in four chickens[1]. Histological examination revealed that both the sciatic nerve and areas of the spinal cord were infiltrated with mononuclear cells, an observation that is still made today after infection with most Marek's disease virus (MDV) strains[2,3]. In the late 1920s, Pappenheimer and colleagues proposed that polyneuritis and visceral lymphoma were symptoms of the same disease[4,5].

Marek's disease is similar to another neoplastic disease in chickens, retrovirus-induced lymphatic leukosis, and distinguishing between the two diseases was very difficult initially, although some clinical and pathological differences were apparent (reviewed in REF. 6). A clear separation between retrovirus- and MDV-induced neoplasia became possible in the late 1960s, when the herpesvirus aetiology of MD was established. The identification and cell-culture isolation of MDV quickly led to the development of vaccines that achieved unparalleled success in preventing the disease and provided a landmark: the first effective immune prophylaxis against a cancer[7–12]. However, during the past 25 years, the changing nature of the disease and the increased virulence of MDV (BOX 1) have led to concerns that new vaccines are now required (BOX 2).

In this review, we summarize our current understanding of how MDV interferes with host defence mechanisms to initiate and complete lytic replication, establishes latent infection in target cells and, ultimately, transforms T cells. In addition, the advantages of the MDV–chicken system as a versatile small-animal model for studying herpesvirus pathogenesis and oncogenesis will be presented. These include the ability to investigate tumour formation in a natural virus–host setting in which lymphoma and solid tumours form with remarkable kinetics and reliability. We will emphasize how recent advances in MDV genomics now allow for targeted disruption of MDV genes, which have a role in evolutionary conserved pathways of transformation as well as tumour formation and dissemination.

Classification of MDV in the family *Herpesviridae*

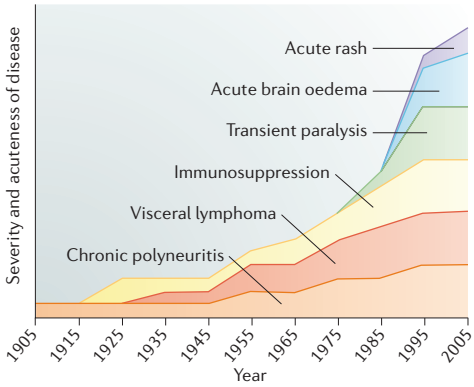
Owing to its biological properties, particularly its ability to induce T-cell lymphoma and its slow growth in cell culture, MDV was long thought to be closely related to Epstein–Barr virus (EBV), a member of the *Gammaherpesvirinae*. Electron-microscopy studies of the MDV genome provided the first evidence that this double-stranded DNA virus possesses repeat structures that are characteristic of the *Alphaherpesvirinae*[13], which was later confirmed by detailed restriction-enzyme mapping and sequencing, first of individual genes, and,

Box 1 | **Marek's disease virus — an agent undergoing fast-paced evolution**

The clinical picture of Marek's disease (MD) has changed dramatically since its initial recognition almost 100 years ago. Most strikingly, the sporadic and chronic nature of the disease has given way to an extremely aggressive, peracute or acute disease in which peripheral neuritis and lymphoma can be absent and protection with previously protective vaccines and immunization regimens can fail. The emergence of this new clinical picture has been paralleled by the isolation of strains with altered cell and tissue tropisms that can cause >90% morbidity and mortality in susceptible animal populations, although the relationship between genetic changes in the virus and the clinical presentation of MD remains largely enigmatic[100]. A scheme to classify the virulence of individual Marek's disease virus (MDV) strains has now been established, and the spectrum ranges from mildly virulent (m) to virulent (v), very virulent (vv) and very virulent plus (vv+)[109].

The most important criterion in this classification is the response of different strains to vaccination with various commercially available vaccines and combinations thereof, and it has been suggested that there is a close relationship between the evolution of strains towards increased virulence and their potential to overcome vaccine protection[109–111]. In the past 20 years, the character of MD has changed into a syndrome in which neurological signs dominate but differ from the polyneuritis originally observed in the early 1900s. Most v and vv MDV strains induce transient paralysis in most chicken lines, and the latest clade of MDV strains, vv+ strains, usually isolated from vaccine breaks, cause massive brain lesions that can be fatal[3,112,113]. Extensive damage to the brain is concomitant with, and probably caused by, an increase in nitric-oxide levels in both plasma and brain tissue, as well as a T_H2-biased proinflammatory response[3,114,115].

The figure below describes the increasing virulence of MDV over time. In the almost 100 years since the initial description of MD, the clinical picture of the disease has changed — the chronic polyneuritis that was prevalent until 1925 was joined by visceral lymphoma from 1925–1950, and from 1950 onwards more aggressive and faster-developing tumours were observed. During the past 25 years, MDV virulence has continued to increase and the clinical picture of the disease has changed again, so that along with the still prevalent visceral lymphoma and neurological symptoms, severe brain oedema and acute deaths, even in fully vaccinated animals, are observed.



later, entire genomes[14–16]. Therefore, MDV is genetically closely related to human herpesvirus 1 (herpes simplex virus type 1, HSV-1) and human herpesvirus 3 (varicella–zoster virus, VZV) (FIG. 1).

MDV belongs to the genus *Mardivirus*, into which three closely related but distinct species have been grouped: MDV (gallid herpesvirus type 2, GaHV-2), which is the type strain for the genus; GaHV-3 (previously referred to as MDV-2); and turkey herpesvirus 1 (HVT; meleagrid herpesvirus type 1, MeHV-1; previously MDV-3). The sequence similarity of virus proteins between the three viruses ranges from 50% to 80% (REFS 17–20). Only MDV causes clinical disease in chickens, the other two species are non-pathogenic. The genomes of MDV, GaHV-3 and HVT consist of a unique long (U_L) and a unique short (U_S) segment bracketed by inverted repeats known as terminal and internal repeats long (TR_L and IR_L) and terminal and internal repeats short (TR_S and IR_S), respectively. The genes located in the U_L and U_S segments are largely homologous to, and arranged collinearly with, those of HSV-1 and VZV, whereas genus- and virus-specific genes are located in the inverted repeat regions, predominantly TR_L and IR_L (FIG. 1) (REF. 13).

MDV as a model for human disease

MDV has proven to be a valuable model organism for understanding some of the principles of human disease. It provides a well defined small-animal model of general tumorigenesis, and virus-induced lymphomagenesis in particular. Infection of birds with MDV strains of varying virulence causes tumours to form within a few weeks in chicken lines from many different genetic backgrounds[21–24]. Depending on the virulence of the virus strain and the genotype of the chicken, T-cell lymphomas and solid visceral tumours that contain transformed CD4$^+$ T cells and immune effector cells such as macrophages, as well as MDV-specific B- and T cells, develop within two to six weeks of infection[25,26]. The reliable kinetics of disease induction and progression, together with the fact that lymphomagenesis can be studied in a natural virus–host system, make MDV unique and allow studies that are impossible to perform in other non-natural models of herpesvirus disease and oncogenesis, for example investigating the role of immune control and evasion in neoplasm formation.

Additionally, it has been recognized that the early stages of MDV infection of chickens closely mimic those of chickenpox in humans, which occurs following VZV infection of naive individuals. The similarities between the early pathogenesis of MD and chickenpox include the uptake of infectious virus by macrophages or dendritic cells (DCs) in the respiratory tract, the infection of activated CD4$^+$ T cells and the development of cell-associated viraemia mainly in CD4$^+$ T cells. Finally, free infectious virus, which is the means of virus transmission from one infected host to another, is produced only in terminally differentiated epithelial cells in feather (MDV) or hair (VZV) follicles[26–28]. It has also been shown that MDV and VZV require homologous viral gene products for efficient virus growth in cultured cells and *in vivo* (reviewed in REF. 18).

Although the promise of MD as a model for biomedical research had long been recognized and exploited, MDV fell from favour, mainly because of the limited availability of the molecular tools that are necessary for efficient and reliable virus manipulation. In addition, there was a shortage of genetic information about the virus host, the chicken, as well as a lack of well defined reagents that would have allowed in-depth elucidation of the functional processes leading to MDV uptake, dissemination, latency and tumour formation. In the past five years, however, some of these major impediments have been removed. Several MDV strains have been cloned, either as bacterial artificial chromosomes (BACs) or as a set of overlapping cosmids, so straightforward genetic manipulation is now not only feasible but has already brought rapid gains in our molecular understanding of MDV. Specifically, the genomes of four MDV strains — three avirulent vaccine strains (584Ap80C, CVI988 and an avirulent form of strain Md11) and one very virulent (vv) strain (RB-1B; see BOX 1) — are available as BACs, and the genome of

Neoplastic disease
A disease involving uncontrolled cell growth, a cancer.

Lymphomagenesis
Process involving the transformation and expansion of lymphocytes, resulting in a cancer of the affected cells.

CD4$^+$ T cells
A subpopulation of T cells that express the CD4 receptor. These cells aid in immune responses and are therefore referred to as T helper cells.

Macrophages
Cells of the mononuclear phagocyte lineage that are responsible for phagocytosis of foreign material.

Box 2 | **Marek's disease vaccines — an open-ended success story**

Immunization against Marek's disease (MD) was started in the late 1960s and first used avirulent Marek's disease virus (MDV) or a virus very closely related to MDV, turkey herpesvirus 1 (HVT), which does not cause disease. Vaccination reduced the incidence of MD by 99% and presents a unique example of the successful application of a modified-live virus (MLV) vaccine against an extremely aggressive agent that can routinely causes >90% morbidity and mortality in susceptible, unvaccinated hosts[7,116]. Because MDV strains are constantly evolving towards greater virulence in the face of vaccination[109] (BOX 1), combination vaccines consisting of HVT and gallid herpesvirus type 3 or an attenuated MDV strain, CVI988-Rispens, are currently used[117–119].

The principles underlying successful immunization remain unknown. MDV vaccination clearly stimulates the innate immune response, but this response alone is not protective[120]. The induction of an MDV-specific cytotoxic response appears to be important for protection, but cytotoxic T lymphocytes (CTLs) are detectable from day 8 post infection, when the initial phase of MDV lytic replication is virtually over and latency is established. In addition, downregulation of major histocompatibility complex (MHC) class I in lytically infected cells will largely preclude efficient lysis by CTLs, and cytotoxic responses are in fact weak compared to those induced by other virus infections[49,121]. These findings might indicate that MDV MLV vaccines have additional — or simply different — modes of action compared with ordinary herpesvirus vaccines. One of the possible scenarios that might aid in vaccine protection is 'substrate deprivation', so that the MLV vaccine infects target B and T cells, which are then no longer susceptible to infection with wild-type virus. In this regard, it is important to note that replication-incompetent vaccines as well as inactivated or subunit vaccines provide partial protection against MD at best[122,123].

There is justified concern that new strains are evolving and will circulate in chickens which have the ability to break vaccine protection, a property some recent strains already exhibit, even if the most efficacious vaccines or combinations of these vaccines are used. Because the development of novel vaccines by classical methods such as cell-culture adaptation of recently isolated strains has proven to be largely ineffective[108], efforts have been concentrated on the use of infectious clones to generate a new class of rationally designed and molecularly defined vaccines[30,93,124]. There could be light at the end of the tunnel, because some rationally engineered vaccines can induce protection in birds that is superior to the CVI988-Rispens strain, the current 'gold standard' of vaccination[125]. Also, the improvement and the use of so-called 'BAC-VACs' that combine the advantages of vaccination with DNA and an MLV are a prospect that will certainly be at the forefront of MD vaccine development in the near future[30,123,126].

Dendritic cells
(DCs). 'Professional' antigen-presenting cells that are found in the T-cell areas of lymphoid tissues and as minor cellular components in most tissues. They have a branched or dendritic morphology and are the most potent stimulators of T-cell responses.

Bacterial artifical chromosome
(BAC). A prokaryotic cloning vector derived from a single-copy or low-copy-number mini-F plasmid that can stably maintain a large DNA insert (average size 150–300 kb) and can be propagated in *Escherichia coli*.

Cosmid
A plasmid cloning vector containing two cohesive (cos) ends from phage λ and one or more selectable markers that allow efficient cloning and amplification of large DNA fragments (40–50 kb) in *Escherichia coli*.

Bursa of Fabricius
The primary lymphoid organ in which B-cell maturation occurs in the chicken.

$CD4^-CD8^-$ T cells
T cells that express neither CD4 nor CD8 on their surface. These cells represent approximately 1–5% of αβ T cells and have been associated with immunoregulatory and immunosuppressive functions.

$CD8^+$ T cells
A subpopulation of T cells that express the CD8 receptor. $CD8^+$ cells recognize antigens that are presented on the surface of host cells by MHC class I molecules, leading to their destruction, and are therefore also known as cytotoxic T cells.

vv strain Md5 has been established as a set of five overlapping cosmid clones[29–33]. With the cloned viral genomes and various *Escherichia coli*-based recombination systems at hand for manipulation, timely and efficient mutagenesis of MDV can be accomplished by both random and targeted approaches[29,34–37]. The ability to introduce markerless modifications, even in both copies of a given sequence in the repeat regions of the genome, has proven invaluable in the progress of MDV research (FIG. 2). Likewise, the completion of the chicken genome sequence and its ongoing annotation are major developments that will have a huge positive impact on MD research and the investigation of the molecular mechanisms of disease development and lymphomagenesis, especially if genetic polymorphisms of the host are considered[38–41].

The early cytolytic phase of MDV infection

Cellular tropism of MDV and early events after infection. MDV can persist for extended periods in the environment and is so ubiquitous that virtually every chicken worldwide faces MDV challenge from its first day of life. The first step of the MDV replication cycle in the definitive host is inhalation of the virus. The current model of MDV pathogenesis predicts that phagocytic cells in the respiratory tract — macrophages or DCs — become infected either directly or after an initial round of replication in epithelial cells[26]. Within 24 hours of uptake, virus is detectable in the spleen, thymus and the bursa of Fabricius[42]. Here, the virus meets its primary targets for the first phase of cytolytic replication: B cells and later activated $CD4^+$ and, rarely, $CD4^-CD8^-$ T cells or $CD8^+$ T cells. The peak of virus replication in these cells is observed between three and seven days post infection (p.i.)[43–45].

Infected $CD4^+$ T cells not only serve as a target for transformation and as the reservoir for latent MDV genomes, they are also the means of virus spread within an infected animal, and allow transport to the skin. Infected T cells appear to be the 'Trojan horse' by which MDV enters the feather-follicle epithelium, where free infectious virus assembles and is shed to infect chickens that come into contact with the infected animal[46–48].

A hallmark of lytic MDV infection is substantial and sustained downregulation of major histocompatibility complex (MHC) class I molecules on the surface of infected cells. The downregulation is encoded by an MDV early gene(s) and is maintained throughout the lytic cycle, resulting in virus-infected cells that are 'invisible' to the cytotoxic immune response carried out by $CD8^+$ T cells[49]. MDV seems to be a promising model to evaluate the *in vivo* effects of MHC class I downregulation. It has recently been shown that the product of the $U_L49.5$ gene, a protein that has previously been shown to be responsible for transporter associated with antigen processing (TAP) blockade and MHC class I downregulation in related varicelloviruses[50], is involved in this mechanism of immune evasion (FIG. 3; TABLE 1). It is currently surmised that escape from MHC class I restricted cytotoxicity facilitates the establishment of, and reactivation from, latency and is important for virus dissemination. This escape allows MDV to reach epithelia in the liver, lung, kidneys, oesophagus, proventriculus (glandular stomach), adrenal gland and skin, organs where productive infection can be established[26]. It is unclear, however, whether virus spread to inner organs and skin during the second cytolytic phase is initiated by latently or lytically infected T cells. In this

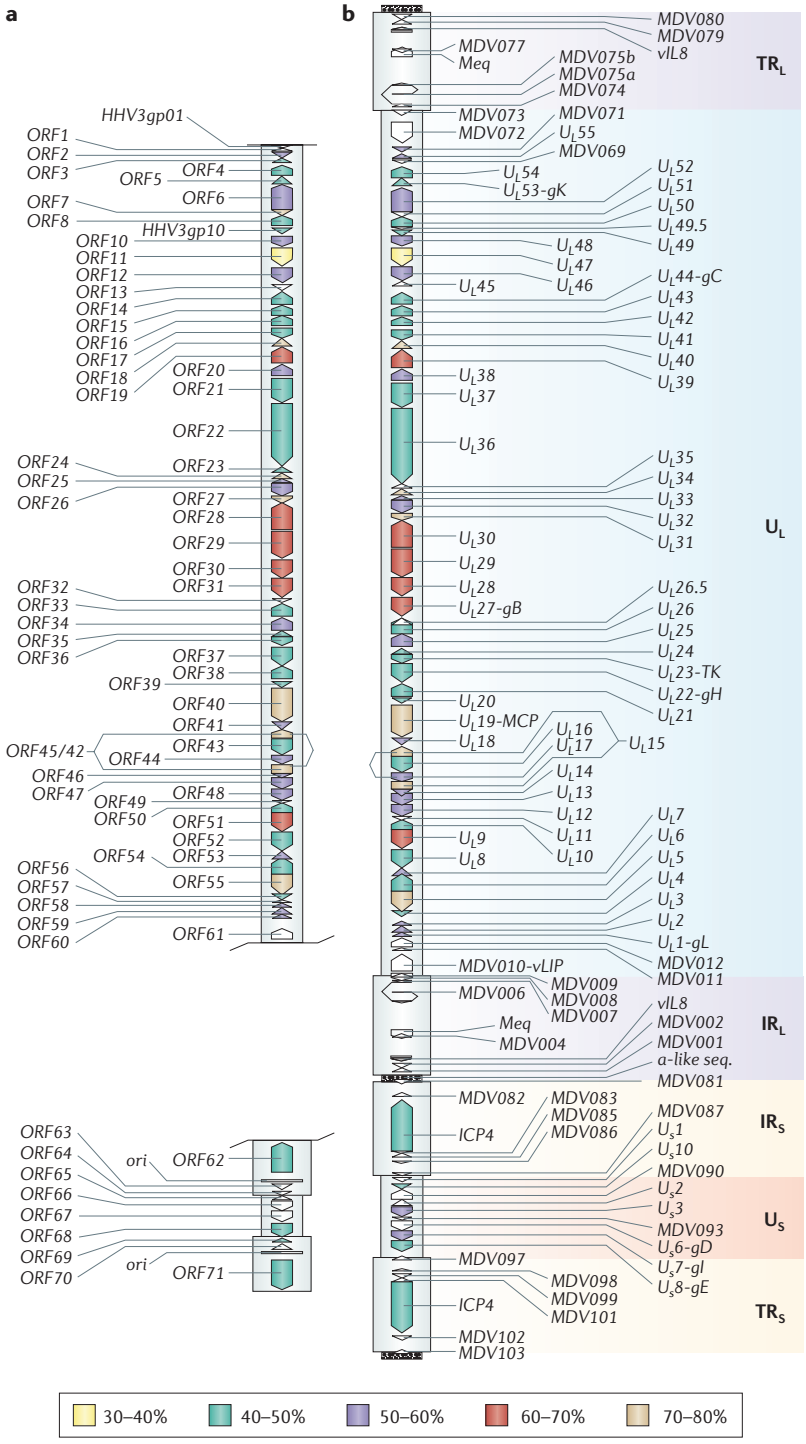


Figure 1 | **Organization of the Marek's disease virus (MDV) genome.** Comparison of the genomic organization of varicella–zoster virus (VZV) (**a**) with MDV (**b**). VZV is the closest relative of MDV affecting humans and the comparison shows significant homology, especially in unique regions of the genomes. The terminal and internal repeat long regions (TR_L, IR_L), the unique long region (U_L), the internal and terminal repeat short regions (IR_S, TR_S) and the unique short region (U_S) are shown. The sequence similarity between the proteins encoded by the individual genes is colour coded. Note that only five VZV genes (depicted in white in the VZV sequence) are not present in MDV. The largest variation between the MDV and VZV genomes is the presence of large repeats bracketing the unique long region (TR_L and IR_L) in MDV. The TR_L and IR_L regions contain genes that encode proteins or RNA structures that are important for cellular tropism (for example, viral interleukin 8, vIL-8) as well as tumorigenicity (for example, Meq and vTR)[15,16].

regard, the recent observation that VZV — at least in a SCIDhu model (a severe combined immunodeficient mouse reconstituted with human immune cells) — can reach the skin through infected memory $CD4^+$ T cells within 24 hours of entering the circulation could be of great interest to MDV pathogenesis[51]. The relatively long period of time between infection of skin (that is, the presence of VZV-infected cells) and rash formation and virus spread can be explained by a subtle, well balanced response of the innate immune system induced by the presence of virus-infected cells. Although the expression of interferon-α (IFN-α) is downregulated in VZV-infected epidermal cells, the cells surrounding the originally infected cell develop a strong and sustained IFN-α response, which is constantly stimulated by the presence of VZV antigens and keeps the infection in check[51]. By analogy, the presence of MDV-infected cells in the skin early in the lytic stage of the disease is conceivable and would offer a new perspective on virus dissemination within infected animals and the spread of the virus to uninfected birds.

Secreted MDV proteins and their role in lytic replication. During its long co-evolution with the chicken, MDV has developed strategies to efficiently recruit and infect target cells, some of which involve genes that were 'pirated' from the host and adapted for the benefit of the virus. In the TR_L and IR_L regions of the genome, MDV harbours a spliced gene (*MDV003*) that encodes a CXC chemokine of 18- to 20-kDa in size, referred to as viral interleukin-8 (vIL-8)[15,16,52,53]. MDV vIL-8, unlike its homologues in mice and humans, fails to attract chicken heterophils (corresponding to mammalian neutrophils), but is a potent chemoattractant for mononuclear cells[53]. Consistent with a role for MDV vIL-8 in attraction of target B and T cells as well as monocytes, deletion of the two vIL-8-encoding open reading frames resulted in mutant viruses that were severely impaired in their ability to cause efficient lytic replication, although two independently generated viruses devoid of *vIL-8* were able to enter latency and retained the ability for oncogenic transformation, albeit at reduced efficiency[53,54].

Another protein that has recently been shown to be important for efficient lytic replication of MDV *in vivo* is a large, 120-kDa *N*-glycosylated protein that is encoded by *MDV010*, a gene located at the extreme left terminus of the U_L genome segment[55]. The predicted amino-acid sequence of the protein exhibits high similarity to a fowl-adenovirus protein of unknown function and, in a stretch of ~200 amino acids, the α/β hydrolase fold of pancreatic lipases; the protein was therefore dubbed viral lipase (vLIP). The glycosylated protein is released into the supernatant of infected cells in apparently small quantities (FIG. 3), but is nonetheless required for efficient lytic virus replication in chickens[55]. The molecular mechanism of vLIP action is not known. Owing to a mutation of the critical acidic residue in the lipase catalytic triad, it is probable that the protein has lost enzymatic activity. It is tempting to speculate, however, that vLIP might still be able to

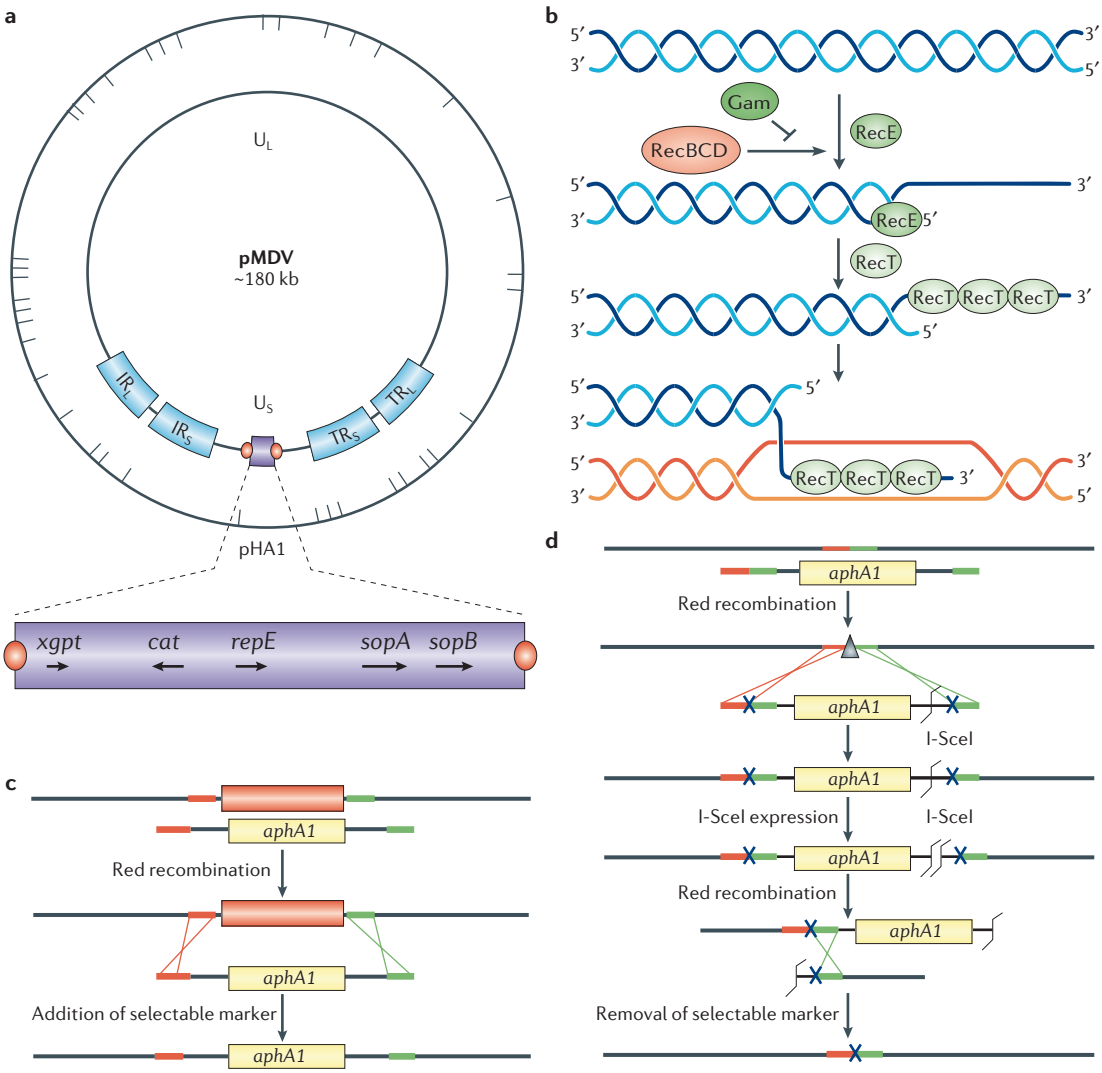


Figure 2 | **Manipulating infectious Marek's disease virus (MDV) bacterial artificial chromosome (BAC) clones.** **a** | Infectious MDV clones. Three of the four MDV BACs that have been generated and described so far contain mini-F sequences (pHA1) instead of *MDV094* (U_S2), which is dispensable for virus growth *in vivo* and tumorigenesis. The mini-F sequences in BAC20, pCVI988 and pRB-1B are flanked by *loxP* sites (red circles) that are amenable to Cre-mediated recombination, which allows vector sequences to be removed from the final virus construct[29,30,32]. **b**,**c** | The principle of Red (RecET) recombination, which exploits the DNA recombination machinery encoded by prophage Rac or phage λ. Red recombination requires double-strand breaks and short homology arms of only 30 to 50 base pairs. Linear DNA, usually a positive-selection marker amplified by PCR, is protected from degradation by the activity of Gam blocking RecBCD. The exonuclease RecE (Recα or Exo in phage λ) generates a 3′ overhang by processive degradation of the 5′ strand. The single-stranded-DNA-binding protein RecT (Recβ or Bet in phage λ) finally introduces the linear DNA fragment into a replication fork[127,128]. **d** | A two-step Red recombination that utilizes counter-selection of recombinants by utilizing homing endonucleases, such as I-SceI, allows markerless modifications such as point mutations and insertions of short or longer sequences. After a first Red recombination, which is selected for by screening for the presence of the positive selection marker, usually an antibiotic-resistance gene such as *aphAI*, which confers resistance to kanamycin, a second Red ('en passant') recombination then results in removal of the selection marker by utilizing a double-strand break generated by cleavage with I-SceI in close proximity to a sequence that had been duplicated[129]. $IR_{L/S}$, internal repeats long/short region; $TR_{L/S}$, terminal repeats long/short region; $U_{L/S}$, unique long/short region.

bind to lipids, because the serine residue responsible for the nucleophilic attack on lipid substrates is conserved in the context of the appropriate amino-acid residues for formation of the oxyanion hole that is required for stable covalent-bond formation[55]. Current research is focused on identifying whether vLIP is able to specifically bind to cell-surface molecules on subpopulations of chicken peripheral blood mononuclear cells (PBMCs), either to initiate or inhibit signalling cascades that would result in attraction of target cells, increasing their susceptibility for infection, or generating a cytokine environment that is favourable for efficient MDV replication and dissemination.

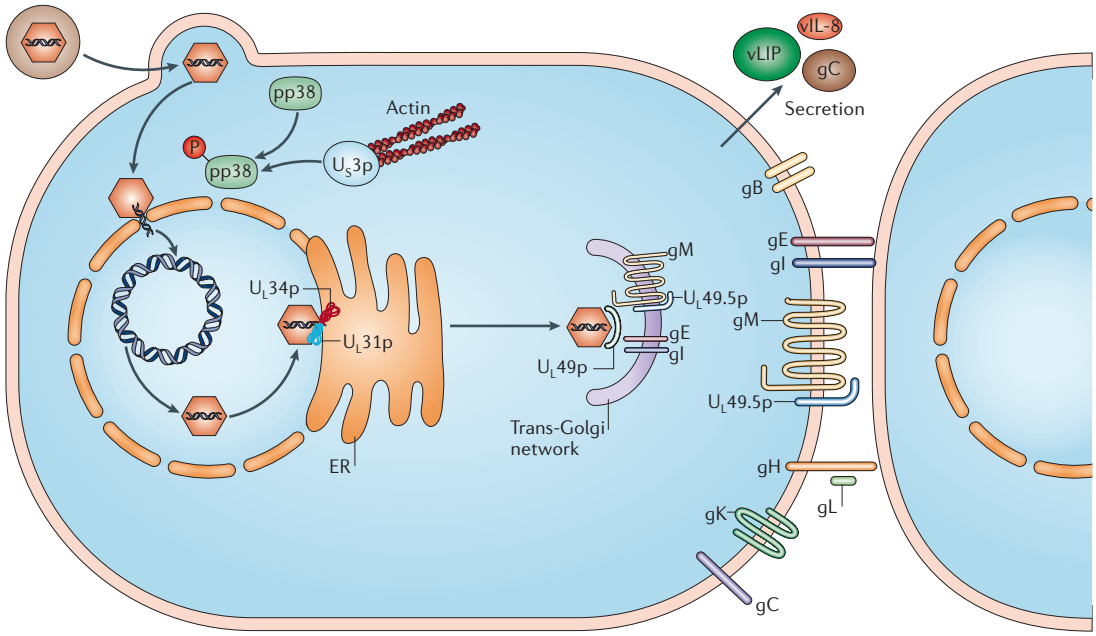


Figure 3 | **Genes and gene products essential for Marek's disease virus (MDV) replication *in vitro* and/or *in vivo*.** Shown are MDV gene products for which an essential function for growth *in vitro* or efficient growth *in vivo* has been shown. The membrane (glyco)proteins gB, gH/gL, gM/U_L49.5p (*MDV064*), gE/gI, gK, U_L31p/U_L34p (*MDV044*, *MDV047*), as well as the tegument protein VP22 encoded by the *U_L49* homologous gene (*MDV062*), were shown to be essential for virus growth *in vitro* and — consequently — *in vivo*[29,34,36,37,130,131]. The major phosphoprotein pp38 (*MDV073*), which is phosphorylated by the serine/threonine protein kinase U_S3p encoded by *MDV092*, was shown to be essential for growth of virulent MDV *in vitro* (TABLE 1; Schumacher *et al.*, unpublished data). The secreted factors viral interleukin-8 (vIL-8), viral lipase (vLIP) and glycoprotein C (gC) are required for full virulence of MDV *in vivo*[53,55] (TABLE 1; Tischer *et al.*, unpublished data). ER, endoplasmic reticulum.

MDV glycoprotein C (gC) — originally termed the A antigen[56] — is secreted (FIG. 3) and is one of the major antigens to which the chicken immune system mounts a substantial serological response. Intriguingly, gC expression levels are greatly reduced in MDV attenuated by serial passage in culture, again a striking similarity to VZV, in which the same phenomenon is observed[57]. Why this type I transmembrane protein with a long hydrophobic C-terminal α-helix is secreted into the supernatant of infected cells (FIG. 3), and whether there is a direct correlation between reduced expression levels and attenuation, has been the focus of research in many laboratories for the past 30 years.

With regard to gC secretion, it was speculated that the short cytoplasmic tail would cause instability of the molecule and therefore secretion[58], but recent data suggest that gC secretion is the result of the generation of a total of three splice variants, two of which encode glycoproteins devoid of a transmembrane domain (TABLE 1). The identification of alternative gC transcripts of different sizes is in agreement with the variability observed in the putative proteins encoded by *U_L44* (*MDV057*), which range in size from 44 kDa to 65 kDa[58,59]. Overexpression of gC in cultured cells led to a massive decrease in MDV replication, which was caused by a factor released into the supernatant of infected cells, presumably secreted forms of gC. By contrast, deletion of gC enhanced virus replication in cultured cells, but not *in vivo*[59] (Tischer *et al.*, unpublished data; TABLE 1).

The role of gC during replication of a virulent MDV *in vivo* was only recently addressed. A point mutation was introduced into the *U_L44* start codon in a BAC clone of the highly oncogenic vv strain RB-1B (pRB-1B)[32]. Growth of the resulting gC-negative virus in cultured cells was enhanced. Viral loads in peripheral blood after inoculation of birds were not significantly reduced compared with those detected for either the parental virus or a revertant virus in which the methionine start codon was restored (TABLE 1). The gC-negative mutant was severely impaired, however, in establishing latency and tumour formation, indicating that gC exerts its functions in MDV pathogenesis beyond lytic replication *per se*. These findings suggest a dual role for gC during MDV infection as both a membrane-bound protein facilitating virus spread and a secreted factor that might signal to target cells or interfere with the host's response to MDV infection. Point mutations in *U_L44* resulting in mutant viruses that can produce either membrane-bound gC or secreted gC exhibited different growth properties in cultured cells (Tischer *et al.*, unpublished data; TABLE 1), and animal studies using these mutant viruses should help to dissect the effects of the various forms of this glycoprotein[59].

The latent and tumour phase of MDV infection

Entry of MDV into latency. MDV enters the latent phase of infection from approximately 7 days p.i. Latency — defined as the presence and maintenance of viral

Table 1 | **MDV genes and gene products involved in pathogenesis and/or immune evasion**

Gene* (Protein)	Structure and function	Phase of infection	Refs
MDV001a (vTR)	RNA structure involved in transformation of infected cells and lymphoma dissemination	Latency/tumour formation	102;103; Trapp *et al.*, unpublished data
MDV003 (vIL-8)	Secreted CXC chemokine involved in early lytic replication and attraction of target cells	Lytic replication	53,54
MDV003a (R-LORF4)	Deletion results in large-plaque phenotype and complete attenuation *in vivo*; probably involved in the establishment of, or reactivation from, latency	Latency	135
MDV005 (Meq)	MDV oncoprotein involved in transformation *in vitro* and *in vivo* by the formation of homodimers and heterodimers, predominantly with c-Jun	Lytic replication/ latency/tumour formation	86,89,92,93
MDV010 (vLIP)	Forms a covalent bond with lipids; secreted protein with homology to a fowl adenovirus protein and pancreatic lipases; required for efficient lytic replication	Lytic replication	55
MDV057 (gC)	Three splice variants identified, two of which result in secretion of the glycoprotein that is necessary for full pathogenesis but not lytic replication	Lytic replication	59; Tischer *et al.*, unpublished data
MDV064 (U_L49.5p)	Essential, small non-glycosylated transmembrane protein required for processing of gM; involved in MHC class I downregulation	Lytic replication	37
MDV074 (pp38)	Abundant early protein phosphorylated by U_S3p ; involved in early lytic replication and implicated in reactivation from latency	Lytic replication/ latency	31,124
MDV092 (U_S3p)	Serine/threonine protein kinase involved in virus spread and rearrangement of the actin cytoskeleton; essential for growth of some virulent MDV strains; also shown to be involved in MHC class I downregulation	Lytic replication	131; Schumacher *et al.*, unpublished data

*Genes are designated according to the nomenclature by Tulman *et al.*[15] If the open reading frame was not listed in this paper, we referred to the designation proposed by Lee *et al.*[16] The vTR gene was not recognized in either of these reports and is tentatively designated *MDV001a*. gC, glycoprotein C; MDV, Marek's disease virus; MHC, major histocompatibility complex; vIL-8, viral interleukin-8; vLIP, viral lipase; vTR, viral homologue of telomerase RNA.

genomes without production of infectious progeny virus — is mainly restricted to $CD4^+$ T cells, although B cells, $CD4^-CD8^-$ T cells and $CD8^+$ T cells harbouring latent MDV have been isolated[60–65]. Relatively little is known about the sequence of events that leads to latent infection or determines the fate of a viral genome after infection of a susceptible cell. This 'black box' is not unique to MDV but is true for many herpesviruses, such as HSV-1, for which the viral proteins involved in establishment and reactivation from latency are known, but mechanistic details are sorely lacking[66]. In the case of MDV, we face another problem: discrimination of latently infected cells from transformed cells is nearly impossible, and the transitions and differences between the latent and transformed state are certainly not discrete — if there is coexistence of latency and transformation and the former does not by default result in the latter.

Estimates for the number of MDV genes that are transcribed in the latent (tumour) phase of infection are controversial and range from ~10–30 (REFS 67–69). Part of the problem with determining the exact number of latent transcripts and proteins is that these investigations were carried out using lymphoblastoid cell lines (LCL) transformed with MDV, some of which are prone to spontaneous reactivation[70]. Unlike the precise number of truly latent transcripts, it is clear that most latency-associated transcripts originate in both the long and short repeat regions[71].

Much of the work on MDV latency has concentrated on three regions: transcripts that are found in antisense orientation to *MDV084* (*ICP4*), the so-called 1.8-kb family of transcripts, and transcripts originating from the *meq* (*MDV004*) gene region in the MDV *Eco*RI Q fragment of the genome (see below). In the *MDV084* region, latency-associated transcripts (LATs) antisense to the MDV *ICP4* homologue are detectable in LCL but are also at least partially present in lytically infected cells[72–74]. A virus mutant from strain RB-1B unable to express LATs was capable of robust lytic replication but failed to produce tumours after infection of susceptible animals. The results indicated that MDV latency is at least partially controlled by modulation of expression of one of its immediate-early genes; this is identical to the situation in many related viruses, for example HSV-1 and VZV[73,75]. Transcripts originating from a bidirectional promoter located in the IR_L in close proximity to the IR_L/U_L junction are responsible for expression of one of the major phosphoproteins, pp38, expression of which is confined to the lytic cycle[31,76], and — on the opposite strand — the 1.8-kb family of transcripts[77,78]. The role, temporal organization and potential cooperation of the 1.8-kb family of transcripts remain elusive, although two proteins encoded by these transcripts, a 7-kDa protein and a 14-kDa protein that is also detected as an immediate-early protein in lytic infection, have been shown to be involved in the induction and maintenance of MDV latency by RNA-interference experiments[79–83].

MDV lymphomagenesis — the roles of Meq and vTR. The most important and obvious outcome of infection with MDV is transformation of infected cells followed by multifocal lymphoma formation[4,5]. The lymphocyte subpopulations transformed by the virus are identical to those in which latent infection is established, suggesting that latent infection is a prerequisite for oncogenic transformation and lymphomagenesis. Only a few members of

Immediate-early genes
Those herpesvirus genes encoding important transactivators that initiate and maintain the cascade-like expression of herpesvirus genes that also comprises early (mostly enzymes for DNA replication) and late (structural) genes.

the *Herpesviridae* are capable of inducing malignancies, and only EBV and human herpesvirus 6 (HHV-6) can integrate into the host genome[70,84]. Integration of MDV DNA into chicken chromosomes is common and seems to be random, as no recombination hot spots have been identified. It has been discovered, however, that the ends of integrated MDV genomes are elongated by the addition of telomeric repeats, suggesting a role for host telomeres, and possibly telomerase, in the process of integration (see below)[70].

Efficient MDV-induced transformation of T cells seems to require a robust cytolytic infection because a sufficient number of latently infected cells must be generated to reliably induce lymphoma. At present, it is thought that only a small subset of latently infected T cells will proliferate and disseminate to generate neoplasms[26]. In fact, it was reported that tumours in a given bird are derived from few originally transformed cells, and might even be monoclonal in origin in some animals[70,84]. Despite the oligo- or monoclonality of tumours in individual birds, whether only a limited number of latently infected cells are transformed and will develop into lymphoma or whether selection for ultimately tumorigenic, malignant and widely disseminating lymphoma cells occurs after the transforming event remains to be elucidated.

Malignant transformation by MDV and the basic leucine zipper protein (bZIP) Meq, which has similarity to the oncoproteins Fos and Jun, are inseparable. Since the detection of *meq*[85], researchers, mainly Kung and collaborators, have accumulated an impressive body of data dissecting the various functions of Meq, which include transactivation, DNA binding, chromatin remodelling and transcriptional regulation. Wild-type Meq is a 339-amino-acid protein that is expressed during both the lytic and the latent/tumour phase of infection[86] (FIG. 4). Probably dependent on the status of infection, Meq can — through its leucine zipper — dimerize with itself, c-Jun, JunB and Fos, with interaction with c-Jun being favoured[87]. It has been proposed that the Meq–c-Jun interaction stabilizes the cellular protein, thereby allowing c-Jun to act more like the retroviral v-Jun oncoprotein, which has been shown to activate the cathepsin-like protein JTAP-1, JAC and the heparin-binding epidermal-growth-factor-like growth factor (HB-EGF), all proteins that are capable of independently transforming chicken cells. The upregulation of identical transformation-associated genes by the Meq–c-Jun heterodimer, together with the upregulation of anti-apoptotic factors such as Bcl-2 and c-Ski, the cellular homologue of retroviral v-Ski, strongly suggests convergent evolution of the transforming pathways of oncogenic avian retroviruses and herpesviruses[88]. In addition to homo- and heterodimerization with proto-oncoproteins, Meq can bind to several factors that are involved in cell-cycle control, including RB, p53 and cyclin-dependent kinase 2 (CDK2)[86], which can also explain the role of Meq in oncogenic transformation of T cells (FIG. 4).

One important function of the Meq–c-Jun heterodimer is transactivation by binding to promoters containing AP-1 or so-called MERE sites (Meq responsive elements that harbour CRE/TRE cores), resulting in upregulation of *meq* transcription and the transformation-associated genes described earlier. Likewise, Meq-induced upregulation of interleukin 2 and CD30 (Hodgkin's disease antigen, a member of the tumour-necrosis-factor receptor

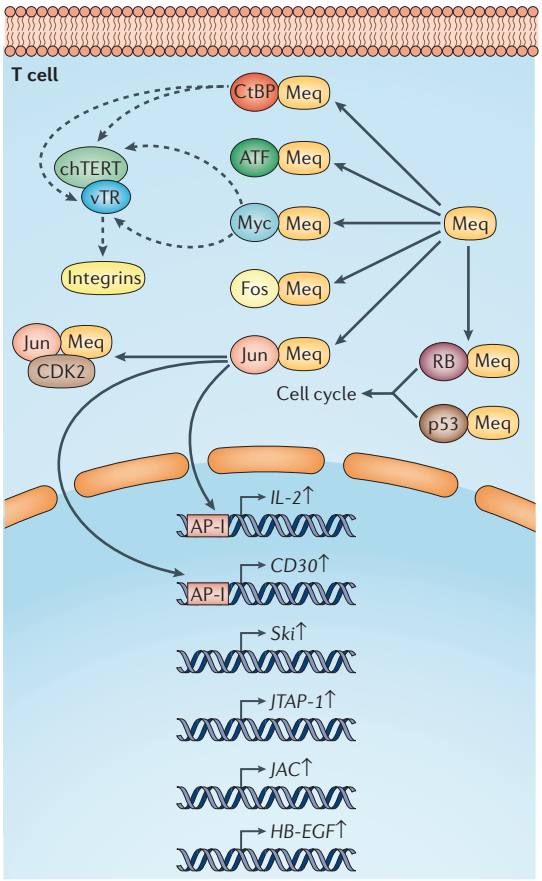


Figure 4 | **Transformation by Marek's disease virus (MDV) and the roles of Meq and vTR.** Two MDV gene products, Meq and the virus-encoded RNA subunit of telomerase, vTR, have been shown to be directly involved in MDV-induced lymphomagenesis. Meq is referred to as the 'MDV oncogene', and weak transformation of various cell types by Meq overexpression has been shown[88,89]. Meq forms homodimers, which bind to CRE/TRE elements and are *trans*-repressive, as are heterodimers between Meq and the C-terminal-binding protein (CtBP). In addition, Meq forms heterodimers with c-Myc, c-Fos, ATF and c-Jun, the latter being the strongest transactivator by binding to AP-1 sites, which results in upregulation of, for example, interleukin 2 (IL-2) and CD30. Meq was also shown to upregulate transcription of Ski, heparin-binding epidermal growth factor (HB-EGF), JAC and JTAP-1 (REFS 87,89,92, 132–134). The co-localization of Meq with cyclin-dependent kinase 2 (CDK2) in Cajal bodies as well as direct binding and sequestration of p53 and RB results in dysregulation of cell-cycle control[89,133]. The exact role of vTR in oncogenesis is unknown, but it was shown to be required for efficient tumour development and to impact on the aggressiveness of MDV-induced lymphomas (TABLE 1; Trapp *et al.*, unpublished data). Proven interactions are indicated by solid lines, putative interactions by dashed lines.

Transactivators
Proteins, such as the immediate-early genes or Meq, that function by enhancing the expression of other viral or cellular genes.

II family) expression through MERE sites was demonstrated[89,90]. The upregulation of CD30 in transformed cells is very interesting from a comparative viewpoint, because several human lymphomas, including Hodgkin's lymphoma, overexpress CD30. Furthermore, EBV latent membrane protein 1 (LMP-1) was shown to associate with TNF-receptor-associated factors TRAF-1, TRAF-2 and TRAF-3, which results in nuclear factor (NF)-κB activation, promotion of T_H2 cytokine production, cell proliferation and upregulation of co-stimulatory molecules such as CD30. Although overexpression of CD30 in MDV-transformed cells is not caused by NF-κB activation, interference with the CD30 pathway seems to be a common mechanism shared by these two oncogenic herpesviruses[89,90]. Based on these observations, it has been speculated that CD30 overexpression is evolutionarily conserved and defines a particular subset of neoplasms. Moreover, immunization against CD30 might be an effective anti-cancer therapy[91], and the MDV–chicken model provides an excellent experimental platform to further investigate such possible approaches to lymphoma control.

Does Meq have transforming capacity? And what is its exact role in lytic virus replication, latency, oncogenic transformation and virulence? The first question was addressed by attempts to transform or immortalize different cell types using Meq-expressing plasmids. Although Meq-induced transformation of chicken T cells has not yet been demonstrated, Meq clearly had transforming properties in continuous Rat-2 and chicken fibroblast DF-1 cell lines[88,92]. The second question was partially answered by recent studies using a *meq*-negative mutant derived from a vv strain, Md5; it was shown that the *meq*-negative mutant did not cause tumours in infected birds[93]. Given that an appreciable reduction in lytic virus replication was observed, and deletion of the entire *meq* gene might have an impact on other transcripts and proteins — such as a Meq–vIL-8 fusion protein[94] — originating from this transcriptionally highly active region of the genome, it could not be concluded that the inability of the mutant virus to cause tumours was solely dependent on the absence of Meq.

Very recently, however, further confirmation of an essential role for Meq in transformation and oncogenesis was provided[95]. Meq possesses a Pro-Leu-Asp-Leu-Ser (PLDLS) motif that is known to bind the C-terminal-binding protein (CtBP), a cellular transcriptional co-repressor with important roles in the regulation of development and oncogenesis[96]. Strikingly, Meq shares this CtBP-binding motif with adenoviral oncoprotein E1A and EBV nuclear antigens EBNA-3A and EBNA-3C (REF. 96). Previous reverse-genetics studies indicated that both EBNA3A and EBNA3C are crucial for efficient transformation of human B cells by EBV[97,98], and it has been shown that the CtBP-binding domain of EBNA-3A has a crucial, albeit non-essential, role in sustaining proliferation of EBV-transformed LCLs[99]. Meq can interact with CtBP physically and functionally through its PLDLS motif, and this interaction is crucial for MDV-induced lymphomagenesis, as mutations in the CtBP interaction domain completely abolish oncogenicity of an RB-1B-based PLDLS-negative mutant virus[95]. These results highlight the convergent evolution of molecular mechanisms of herpesvirus-induced tumorigenesis and show that MD is an excellent experimental model to study the role of CtBP in oncogenic transformation.

With respect to the increased virulence of recently isolated MDV strains (BOX 1), it is notable that Meq variants have been discovered. It has been known for some time that insertions of up to 59 amino acids, comprising a variable number of repeats of four prolines (PPPP) in the proline-rich central region of the protein, are found in strains of low virulence[100,101]. By contrast, more virulent strains exhibit modifications of the repeated PPPP motif in which the second proline is changed to alanine or glutamic acid[100]; the impact of these alterations on MDV virulence and its host range remains to be determined and is the focus of ongoing studies in several laboratories.

Despite the fact that Meq has a clear role in oncogenesis, its transforming properties — in comparison to retroviral oncoproteins such as v-Src — are weak. In this context, the question of the involvement of cellular and/or viral cofactors in virus-induced transformation seems logical. An interesting discovery was made in the IR_L/TR_L region of the MDV genome, namely the existence of a viral homologue of telomerase RNA (TR), termed vTR, which has extensive secondary structures that are similar to those of the so-called EBERs (Epstein-Barr encoded RNAs), small RNAs that are produced during latent EBV infection[102]. Together with a protein subunit with reverse-transcriptase activity (TERT), TR forms the functional core complex of the ribonucleoprotein enzyme telomerase. Telomerase activity is crucially involved in maintaining the physical integrity of eukaryotic chromosomes and has been implicated in cellular immortalization and tumorigenesis. MDV vTR has 88% sequence identity to chicken telomerase RNA (chTR) and was pirated from the chicken genome. Functional analyses have shown that vTR can reconstitute telomerase activity by interacting with chicken TERT (chTERT) more efficiently than chTR[103]. By contrast, a single-nucleotide mutation in the vTR sequence in the widely used vaccine strain CVI988-Rispens resulted in a substantial loss of functionality of vTR, strengthening the case for an association between vTR and the oncogenicity of MDV[102,103].

vTR has an important role in MDV-induced T-cell lymphomagenesis, as shown by generating and analysing mutant viruses that lacked either one or both copies of the diploid *vTR* gene (Trapp *et al.*, unpublished observations; FIG. 4; TABLE 1). *vTR*-negative mutants from the highly oncogenic MDV strain RB-1B were significantly impaired in their ability to induce lymphomas in MDV-susceptible birds, and tumour incidences were reduced by >60% when compared with parental virus or mutants lacking only one copy of *vTR*. Strikingly, solid neoplasms in individual birds infected with the *vTR*-negative viruses were also significantly smaller in size and less disseminated. Although tumour formation was clearly impaired in the absence of *vTR*, chickens

still succumbed to chronic MD, as demonstrated by wasting of birds (TABLE 1). The detailed molecular mode of action of vTR in MDV pathogenesis remains to be revealed, but it clearly possesses transforming properties. Currently, it is speculated that vTR might have a role in genome integration by aiding in the generation of telomeric elongations at the ends of the viral genome as a prerequisite for integration, in enhancing the survival of (latently) infected cells by its anti-apoptotic properties, and/or in promoting tumour-cell dissemination and homing to various organs by upregulation of cell-surface adhesion molecules such as integrin-αv[104].

Conclusions

Almost a century after the first description of Marek's disease, research on this syndrome, which is prevalent worldwide, is at a crossroads. The necessary tools are now available to allow in-depth characterization of the molecular events leading to primary infection, lytic replication, the establishment and maintenance of the latent state and, eventually, tumour formation and dissemination. MD research is therefore poised to have an important impact on fundamental cancer biology, particularly with regard to the pathogenesis of, and intervention in, aggressive virus-induced lymphoma. Special emphasis will be placed on elucidating the molecular details of T-cell transformation and the factors that govern tumour formation and dissemination in this natural virus–host model. Future research will certainly focus on the roles of Meq, vTR and possibly other virus-encoded small RNA structures such as microRNAs, which have been shown to be associated with latency and/or transformation in other (herpes)virus systems[105–107].

It is probable that the biggest challenge that lies ahead of the field, however, is the justified concern that the vaccines currently in use will soon reach the end of their useful lives. It is widely accepted that classical methods of improving MD vaccines "approach the threshold of efficacy" as stated by Witter, one of the founding fathers of MD vaccinology[108]. The only way out of this quandary is the rational design of a new generation of modified-live-virus vaccines as well as the development of more efficacious vaccine formulations and regimens. The recently generated infectious clones from different MDV strains will be instrumental in reaching this goal.

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Competing interests statement
The authors declare no competing financial interests.

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