

# Interaction of MEQ protein and C-terminal-binding protein is critical for induction of lymphomas by Marek's disease virus

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Marek's disease virus (MDV) is an oncogenic herpesvirus that induces fatal T cell lymphomas in chickens. With more than 20 billion doses of vaccine used annually, vaccination constitutes the cornerstone of Marek's disease control. Despite the success of vaccination, evolution of virulence among MDV strains continues to threaten the effectiveness of the current Marek's disease vaccines. MDV-encoded protein MEQ (MDV EcoRI Q) probably acts as a transcription factor and is considered to be the major MDV oncoprotein. MEQ sequence shows a Pro-Leu-Asp-Leu-Ser (PLDLS) motif known to bind C-terminal-binding protein (CtBP), a highly conserved cellular transcriptional corepressor with roles in the regulation of development, proliferation, and apoptosis. Here we show that MEQ can physically and functionally interact with CtBP through this motif and that this interaction is critical for oncogenesis because mutations in the CtBP-interaction domain completely abolished oncogenicity. This direct role for MEQ–CtBP interaction in MDV oncogenicity highlights the convergent evolution of molecular mechanisms of neoplastic transformation by herpesviruses because Epstein–Barr virus oncoproteins EBNA 3A and 3C also interact with CtBP. We also demonstrate that the nononcogenic MDV generated by mutagenesis of the CtBP-interaction domain of MEQ has the potential to be an improved vaccine against virulent MDV infection. Engineering MDV with precisely defined attenuating mutations, therefore, represents an effective strategy for generating new vaccines against this major poultry disease.

viral oncogenesis | vaccines | transcriptional repression

Herpesviruses are important pathogens associated with a wide range of diseases in humans and animals. Marek's disease virus (MDV) is one of the most contagious and highly oncogenic herpesviruses known (1). Apart from being an economically important disease affecting poultry health and welfare (2), Marek's disease (MD) has contributed significantly to our understanding of herpesvirus-associated oncogenicity. MD lymphomas have many biological parallels with lymphoid neoplasia associated with human herpesviruses such as Epstein–Barr virus (EBV) (3), and recent studies have suggested MD as a natural model for lymphomas overexpressing Hodgkin's disease antigen, CD30 (4). Unlike in humans, the control of many herpesvirus diseases in veterinary medicine is achieved through vaccination. Despite the success of the vaccination strategy in reducing losses caused by MD during the last 30 years, a continuing trend in increasing virulence of MDV strains is threatening the effectiveness of the existing MD vaccines (reviewed in ref. 5). A fundamental understanding of the molecular mechanisms of MD oncogenesis is therefore important to the development of more sustainable control strategies and to the cessation of the continuing trend in the evolution of MDV virulence (2). The transcriptional regulator MEQ (MDV EcoRI Q) is considered to be the major viral oncoprotein of MDV (6). MEQ has a nuclear distribution and can induce transcriptional activation or

repression depending on its dimerization partner and DNA-binding specificity (7).

MEQ sequence shows a consensus Pro-Leu-Asp-Leu-Ser (PLDLS) motif known to bind CtBP (C-terminal-binding protein), a highly conserved cellular factor with roles in the regulation of development, proliferation, and apoptosis. CtBP was initially identified as a cellular protein that interacts with the C terminus (amino acids 225–238) of adenovirus E1A oncoproteins. Although the precise significance of this interaction remains unknown, it is essential for the immortalization of primary rodent cells by E1A but also has been reported to negatively modulate E1A-mediated transformation, tumorigenicity, and metastasis (8, 9). Human CtBP also mediates the repressor and transforming activities of the oncoprotein Evi-1 (10).

Most of the factors that interact with CtBP are transcriptional regulators and bind to CtBP through a conserved Pro-X-Asp-Leu-Ser ("PXDSL") CtBP-binding domain that is necessary and probably sufficient for the interaction (11, 12). It has recently been shown that CtBP-containing protein complexes include several factors that coordinate enzymatic events that convert transcriptionally active chromatin to a repressive or silent state (13). A second mammalian CtBP was recently described and its two family members, CtBP1 and CtBP2, are largely homologous. Both proteins act as transcriptional corepressors, but they may have distinct tissue distributions and developmental roles (14). The proteins referred to as CtBP in this paper are the human and chicken CtBP1.

There is good evidence that CtBP is involved in the regulation of proliferation. The PXDSL amino acid motif is found in a cellular protein called CtIP (CtBP-interacting protein). CtIP bridges an interaction between the retinoblastoma tumor suppressor protein (pRb) and CtBP, forming a complex that can repress E2F-regulated genes and thus participates in the regulation of the cell proliferation cycle (15, 16).

We showed earlier that the EBV nuclear antigen, EBNA 3C, can bind CtBP *in vitro* and in cells through a PLDLS motif located in its C terminus (17). Subsequently, we found that EBNA 3A also binds CtBP through a previously unrecognized, nonconsensus bipartite motif located in the C terminus of EBNA 3A (18). Both EBNA 3A and 3C are essential for the efficient transformation of human B cells by EBV, and both can also act as "immortalizing" oncogenes in the rescue of primary rodent

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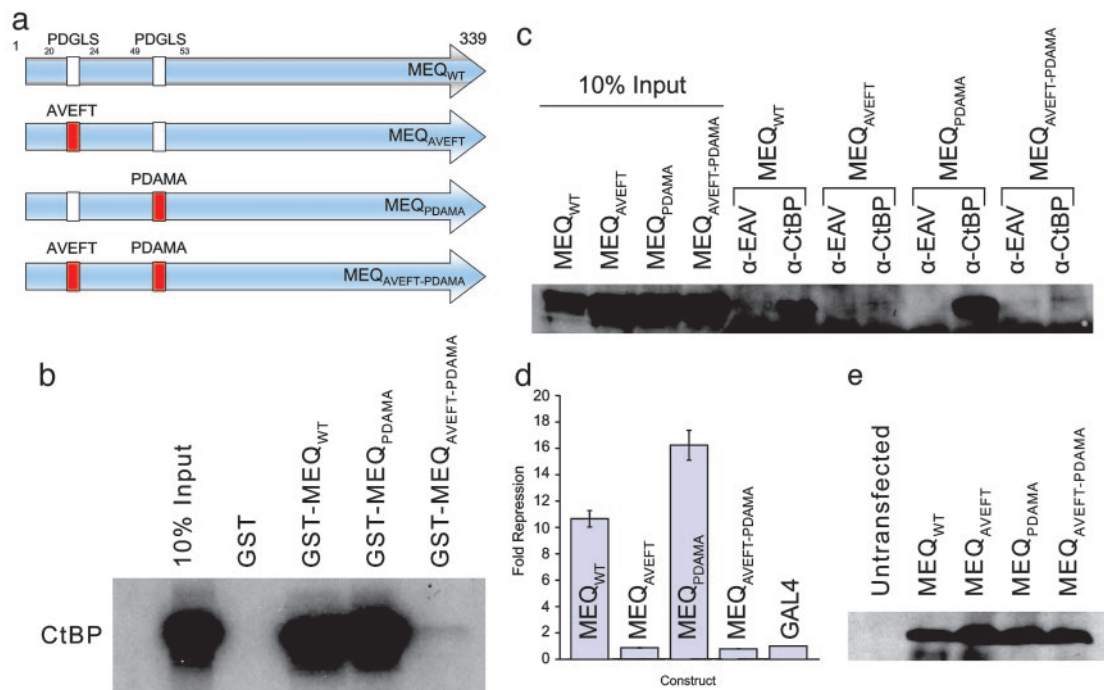
Abbreviations: MD, Marek's disease; MDV, MD virus; EBV, Epstein–Barr virus; MEQ, MDV EcoRI Q fragment; CtBP, C-terminal-binding protein; CtIP, CtBP-interacting protein; pRb, retinoblastoma tumor suppressor protein; BAC, bacterial artificial chromosome; chCtBP1, chicken CtBP1; RIR, Rhode Island Red; pfu, plaque-forming units.

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**Fig. 2.** MEQ binds to chCtBP1 through the PLDLS motif and induces transcriptional repression. (a) Illustration of the mutations in the CtBP-binding motifs. (b) Interaction of GST-MEQ<sub>1-170</sub> fusion protein with [<sup>35</sup>S]methionine-labeled chCtBP1 demonstrated by SDS/PAGE. (c) Extracts of DF-1 cells cotransfected with chCtBP1 and MEQ constructs immunoprecipitated with rabbit (anti-CtBP or unrelated anti-endogenous avian retrovirus) antibodies and detected with anti-MEQ antibody. (d) Mean ± SD luciferase activity (expressed as fold repression over that of the GAL4DBD construct) of extracts of DF-1 cells cotransfected with the firefly luciferase GAL4 reporter, *Renilla* plasmid, and GAL4DBD-MEQ<sub>1-174</sub> fusion constructs. (e) Western blotting of protein extracts with mouse anti-GAL4DBD antibody.

cipitated by the anti-CtBP serum, confirming that the PLDLS motif is not only essential, but also sufficient for the interaction with CtBP (Fig. 2c).

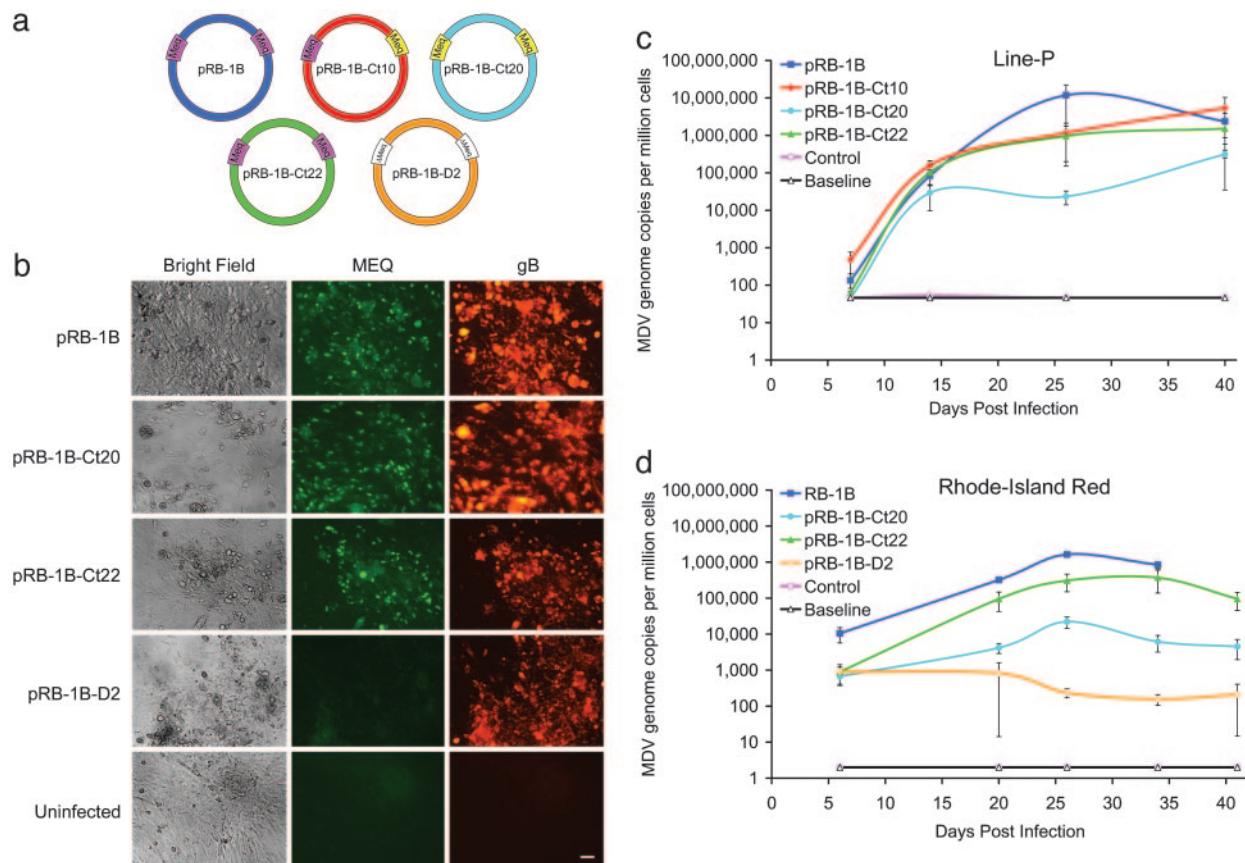
Because CtBP is a well characterized transcriptional corepressor, we wanted to establish whether the MEQ–CtBP interaction would affect the transcriptional repression function of MEQ. Therefore, we carried out luciferase reporter assays using MEQ constructs containing the N-terminal amino acids 1–172 cloned in-frame with the GAL4 DNA-binding domain. DF-1 cells were cotransfected with a GAL4-responsive luciferase reporter and the fusion constructs of wild-type MEQ (MEQ<sub>WT</sub>), MEQ<sub>AVEFT</sub>, MEQ<sub>PDAMA</sub>, or MEQ<sub>AVEFT-PDAMA</sub> together with *Renilla* control plasmids. Luciferase assays on the cell lysates showed that both MEQ<sub>AVEFT</sub> and MEQ<sub>AVEFT-PDAMA</sub> failed to induce repression, correlating with their inability to interact with CtBP. On the other hand, MEQ<sub>WT</sub> and the MEQ<sub>PDAMA</sub> repressed basal transcription from the GAL4-responsive promoter by 10- to 16-fold (Fig. 2d), indicating that a transcriptional repression function of MEQ is linked to the ability to interact with CtBP.

**Construction and Characterization of Mutant MDV.** Having demonstrated the interaction of MEQ and CtBP, we wanted to examine whether this interaction could be functionally important in MDV-induced oncogenicity. The availability of an infectious BAC clone (pRB-1B5) of the highly oncogenic RB-1B strain of MDV (19) provided us with the opportunity to use reverse genetics to construct mutant CtBP-interaction-defective viruses. We made use of the markerless replacement technique (20) in the pRB-1B5 clone to construct four MDV mutants (Fig. 3a). In the first mutant pRB-1B-Ct10, one copy of the MEQ gene was mutated to MEQ<sub>AVEFT-PDAMA</sub>. The procedure was repeated to make the second mutant pRB-1B-Ct20 in which MEQ<sub>AVEFT-PDAMA</sub> was introduced in both copies of the MEQ gene. In a two-step procedure, we also made a full revertant pRB-1B-Ct22 construct in which the wild-type sequences were restored. In

addition, a MEQ-deletion mutant, pRB-1B-D2, in which both copies of the MEQ gene were completely and precisely deleted, was also constructed. To confirm that the mutations did not affect the expression of MEQ or the replication of MDV, chicken embryo fibroblast cultures were transfected with the BAC DNA to allow reconstitution of the virus. MDV-specific plaques staining positive for glycoprotein B were detected in cells transfected with each of the BAC DNA clones. MDV plaques also showed positive staining for MEQ (except in pRB-1B-D2), confirming that the mutations in the CtBP-binding motifs of MEQ did not affect the *in vitro* virus replication or MEQ expression in chicken embryo fibroblast cells (Fig. 3b).

We then examined the *in vivo* proliferation of wild-type and mutant viruses using a MEQ-specific real-time TaqMan quantitative PCR that measures the MDV genome copy numbers in peripheral blood leukocytes of experimentally infected chickens (21). In the first experiment, we used inbred congenic line P (B<sup>19/19</sup>) chickens selected for their high susceptibility to MD (22). MDV genome copy numbers in all of the groups showed a steady increase during the course of infection, with pRB-1B, pRB-1B-Ct10, and pRB-1B-Ct22 groups registering 1 × 10<sup>6</sup> copies per million cells (Fig. 3c) at 40 days postinfection. Birds from the group infected with pRB-1B-Ct20 carried a lower virus load (3 × 10<sup>5</sup> copies per million cells), indicating that these mutations may also have some influence on the replication of MDV *in vivo*. However, in experiments in Rhode Island Red (RIR) birds (an outbred population of chickens with a heterogeneous genetic background), viral genome copy numbers (determined by using MDV pp38 gene-specific quantitative PCR) of pRB-1B-Ct20 virus were much higher than those of the MEQ-deletion mutant pRB-1B-D2 virus (Fig. 3d), indicating that compared with the deletion of MEQ, the mutations in the CtBP-binding domain do not completely interfere with virus replication in chickens.

**In Vivo Pathogenicity Studies of Mutant MDV.** Because the oncogenicity of EBV proteins EBNA3A and EBNA3C and cellular



**Fig. 3.** Replication of RB-1B viruses *in vitro* and *in vivo*. (a) Schematic illustration of the BAC clones: wild type (pRB-1B, dark blue), MEQ<sub>AVEFT-PDAMA</sub> single copy mutant (pRB-1B-Ct10, red), double mutant (pRB-1B-Ct20, light blue), revertant (pRB-1B-Ct22, green), and  $\Delta$ MEQ mutant (pRB-1B-D2, orange). (b) MEQ and glycoprotein B expression in MDV plaques from transfected BAC DNA. (Scale bar: 20 nm.) (c) TaqMan quantitative PCR showing the mean  $\pm$  SE of viral genome copy numbers in peripheral blood leukocytes of inbred line P chickens ( $n = 6$ ) at different days postinfection. (d) Mean  $\pm$  SE of viral genome copy numbers in the peripheral blood leukocytes of outbred RIR chickens ( $n = 6$ ).  $\Delta$ MEQ deletion mutant (pRB-1B-D2) was also included in this experiment.

oncoprotein Evi-1 is associated with their interaction with CtBP (10, 17, 18, 23), we next determined the oncogenic potential of the CtBP interaction-defective MDV mutants. Experimental infection of 1-day-old line P chicks with pRB-1B virus resulted in the development of visceral tumors characteristic of MD in 80% of birds (Fig. 4a). Mutation of the CtBP-interaction motif in one copy of MEQ in pRB-1B-Ct10 virus did not appear to have any effect on oncogenicity because this group also developed similar levels (70%) of MD tumors. However, abolition of CtBP interaction in both copies of the MEQ gene resulted in the complete loss of oncogenicity because the birds infected with pRB-1B-Ct20 virus were similar to the uninfected control birds and did not show any gross or microscopic lesions during the course of the 60-day experiment. These results clearly indicated that the MEQ-CtBP interaction is critical for MDV oncogenicity. This finding was further supported by the rescue of the oncogenic phenotype in the revertant pRB-1B-Ct22 virus, which produced tumors in 60% of birds. The loss of oncogenicity of the pRB-1B-Ct20 virus was not restricted to the genetically susceptible inbred line P because the mutant virus also failed to induce tumors in RIR birds (Fig. 4b).

Because our studies demonstrated that the mutations within the CtBP-interaction domain of MEQ can completely abolish MDV oncogenicity, we asked whether pRB-1B-Ct20 virus could be used as a vaccine against a virulent MDV. To explore this possibility, we immunized groups of 1-day-old line P chicks with 1,000 plaque-forming units (pfu) of either the commercially available CVI988/Rispens vaccine or the pRB-1B-Ct20 virus and challenged the chicks with virulent RB-1B virus. Compared with

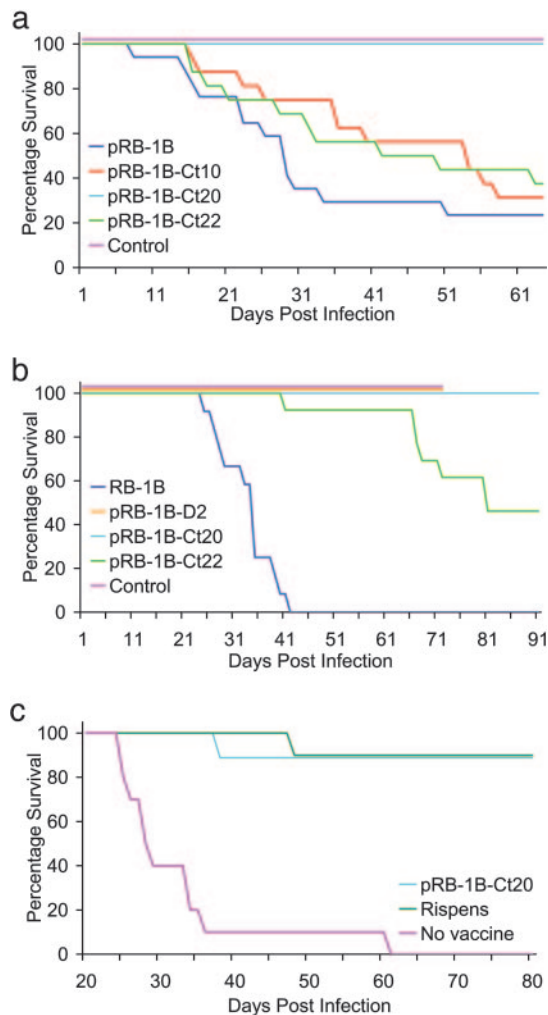
100% disease in unvaccinated control birds, 90% of birds vaccinated with both CVI988/Rispens vaccine and the pRB-1B-Ct20 mutant virus were protected against virulent MDV infection (Fig. 4c).

## Discussion

MDV is one of the most contagious herpesviruses known, inducing rapid onset T cell lymphomas in chickens. The acute nature of MD lymphomagenesis strongly indicated a direct role of MDV-encoded genes in the induction of tumors. Among the 100 or so known viral proteins encoded by the MDV genome (24), MEQ is considered to be the major oncoprotein. MEQ, encoded within the repeat regions of the MDV genomes of serotype 1 strains of MDV, is expressed in all tumor cells and lymphoblastoid cell lines derived from lymphomas (25). A direct role of MEQ in the induction of tumors has been recently demonstrated by using MEQ-deletion mutants of MDV, which failed to induce tumors (6).

Despite these observations, molecular mechanisms of MEQ-induced transformation are not completely understood. As a basic leucine zipper protein, MEQ is capable of forming dimers either with itself or with other leucine zipper proteins such as c-Jun, JunB, ATF, Fos, CREB, and C/EBP, and depending on the dimerization partner, MEQ appears to target distinct promoters, inducing either transcriptional activation or repression (7). Recent studies on the transcriptional regulation by MEQ have suggested that although MEQ/Jun heterodimers function as activators of transcription on some of the viral promoters (e.g., MEQ promoter),





**Fig. 4.** Genetic disruption of the MEQ–CtBP interaction leads to complete attenuation of MDV. (a) Percentage survival in inbred line P birds ( $n = 15$ ) infected with MDV. (b) Percentage survival in outbred RIR birds ( $n = 12$ ) infected with MDV. The pRB-1B-Ct20 virus showed complete loss of oncogenicity in both lines of birds. (c) Percentage survival of line P chickens vaccinated with commercial Rispens vaccine or pRB-1B-Ct20 virus ( $n = 9$ ) after being challenged with virulent RB-1B virus. The minimum number of birds in each experimental group is shown in brackets.

MEQ-MEQ homodimers have a repressor effect on other viral promoters such as the bidirectional pp14/pp38 promoter (7). CtBP is a highly conserved corepressor of transcription that is involved in diverse processes (see Introduction). The identification of a putative bipartite CtBP-binding motif in the MEQ sequence prompted us to investigate whether the function of MEQ depends on its interaction with CtBP. Data obtained from colocalization, GST pull-down, and immunoprecipitation assays demonstrated that MEQ physically interacts with CtBP and that the  $_{20}PLDLS_{24}$  motif is essential for the interaction with CtBP. However, the  $_{49}PDGLS_{53}$  sequence appeared to be unnecessary for the interaction because mutations in this domain did not have any measurable effect on the binding in either GST pull-down or immunoprecipitation assays. The results from the luciferase reporter assays indicated that the physical interaction of MEQ and CtBP may also be functionally relevant because both the MEQ<sub>AVEFT</sub> and MEQ<sub>AVEFT-PDAMA</sub> mutants that were unable to interact with CtBP also failed to repress transcription.

The present study provided a unique opportunity to examine the functional significance of the MEQ–CtBP interaction during

oncogenicity in a natural disease model in the target animal. This opportunity has been possible because of the availability of the infectious BAC clone of the highly oncogenic RB-1B strain of MDV (19), which allows the application of mutagenesis techniques for the rapid generation of mutant viruses. Pathogenicity studies in susceptible inbred and outbred chickens clearly demonstrated that MEQ–CtBP interaction is crucial for the induction of lymphomas because the pRB-1B-Ct20 virus with mutation in the MEQ genes that abolished the interaction with CtBP was completely nononcogenic. Mutation of both copies of MEQ was required for the total loss of oncogenicity because pRB-1B-Ct10 virus was still oncogenic. A revertant pRB-1B-Ct22 virus, in which the wild-type sequence was restored, regained its ability to induce lymphomas, further confirming that interaction with CtBP is critical for transformation. Although previous studies have implicated MEQ in MDV oncogenicity (6, 25), this study demonstrates a potential molecular mechanism for induction of T cell tumors by MDV. A direct role for MEQ–CtBP interaction in MDV oncogenicity may also highlight the convergent evolution of molecular mechanisms in cell transformation by oncogenic herpesviruses. The gammaherpesvirus EBV encodes two essential nuclear proteins, EBNA3A and EBNA3C, that require interaction with CtBP to act as immortalizing oncoproteins (17, 18). The molecular mechanisms of MEQ-induced transformation through recruitment of CtBP remain to be identified. MEQ might function in tumorigenesis and/or the establishment of latency in T cells by recruiting CtBP and associated repression/silencing complexes (13) to cellular gene(s), and the outcome may be down-regulation of critical functions required for the inhibition of proliferation or the induction of apoptosis. However, we cannot exclude the possibility that modulation of viral genes by MEQ–CtBP complexes is a prerequisite for MDV latency in T cells and hence their subsequent transformation.

The observation that MDV with mutant MEQ replicated less efficiently *in vivo* is intriguing. Complete deletion of MEQ results in almost no replication, but even the Ct20 mutant appears to be impaired when compared with wild-type MDV RB-1B. It is still unclear whether MEQ plays a role in MDV replication *per se* or whether it alters the ability of virus to establish latency in cells that subsequently support virus replication. A comprehensive histological investigation to compare the temporal and spatial distribution of various RB-1B mutants in infected birds should be undertaken. Such an investigation should help answer the unresolved question of whether even a modest reduction in Ct20 replication in chickens contributes to its complete lack of oncogenicity.

MD is the first virus-induced cancer in any species against which a live attenuated vaccine was used successfully, nearly 40 years ago (26). Despite the success of the vaccination strategy in reducing losses from MD, vaccines themselves are thought to be responsible for driving the virulence, and there is an urgent need to develop more effective vaccines to halt the pathogen race toward higher virulence (2). The complete loss of oncogenicity of pRB-1B-Ct20 virus prompted us to investigate the use of this virus as a live vaccine against virulent MDV. The protection levels of 90% against a virulent RB-1B challenge were equal to that of the current most widely used and effective CVI988/Rispens vaccine, indicating that the mutant virus is able to induce immunity despite the attenuation of its oncogenicity. Although further extensive trials will be required to develop MEQ mutant virus into a widely used vaccine against MD, the present study demonstrates the principle that novel vaccines can be developed by introducing precisely defined mutations based on the knowledge gained from the study of fundamental mechanisms of pathogenesis.

## Materials and Methods

**Cells and Viruses.** Primary chicken embryo fibroblast cells prepared from 10-day-old, specific-pathogen-free embryos (obtained from flocks maintained at the Institute for Animal Health, Compton, U.K.) were used for the propagation and analysis of growth curves of the viruses. A DF-1 cell line derived from line 0 chicken embryonic fibroblasts (27) was used for immunoprecipitation and reporter assays. The very virulent RB-1B strain of MDV originally obtained from K. A. Schat (Cornell University, Ithaca, NY) and the BAC-derived pRB-1B5 viruses (19) were used in pathogenicity tests.

**Plasmids.** Full-length MEQ amplified by PCR from the pRB-1B5 (19) was cloned into pcDNA3.1(+) vector (Invitrogen) to generate the pcDNA3.1-MEQ construct. The AVEFT, PDAMA, and AVEFT-PDAMA mutations in the CtBP-binding motif of MEQ were introduced by PCR. Plasmid pcDNA3.1-chCtBP1 contains the cDNA encoding the full-length *Gallus gallus* CtBP1 from line 0 chickens. The N-terminal amino acids 1–170 of MEQ fused in-frame with GST in pGEX2T (Amersham Pharmacia) vector and the N-terminal amino acids 1–172 of MEQ fused in-frame with Gal4-DBD in pCI-GAL4 (a kind gift from M. Parker) were also used. pST76K-SR-MEQ2.2 contains cDNA encoding MEQ and 1.2 kb of flanking sequence from pRB-1B5 in pST76K-SR (a kind gift from M. Messerle).

**GST Pull-Down and Immunoprecipitation.** These assays were performed essentially as described in refs. 17 and 18.

**Immunofluorescence Labeling.** MSB-1 cells were fixed in 4% paraformaldehyde for 1 h, and after washing with PBS, they were permeabilized by treatment with 0.1% Triton X-100 for 15 min. Washed cells were stained with a 1:1,000 dilution of rabbit anti-human CtBP serum (18) or a 1:100 dilution of mouse anti-MEQ monoclonal antibody FD7 and detected with Alexa 488-conjugated goat anti-rabbit and Alexa 568-conjugated goat anti-mouse antibodies (Molecular Probes), respectively. Cells were stained with DAPI (1:10,000) and viewed by using a Leica (Wetzlar, Germany) TCS SP2 confocal laser scanning microscope.

**Repression Assays.** DF-1 cells were cotransfected with a firefly luciferase GAL4 reporter, *Renilla* control, and GAL4DBD

fusion constructs of MEQ. Luciferase activity, measured 48 h after transfection by using the luciferase detection reagent kit (Promega), was expressed as fold repression over that of the GAL4DBD construct.

**Construction of Mutant Viruses.** Mutagenesis of the BAC clone pRB-1B5 (19) was carried out by using the MEQ construct (with the CtBP-binding mutation) in the shuttle vector pST76K-SR (20). DNA from the pRB-1B5 BAC and the mutant MEQ construct were electroporated into *Escherichia coli* DH10B cells (Invitrogen), and mutant clones were generated by a two-step markerless replacement technique (20). Single-copy mutant (pRB-1B-Ct10) was selected by restriction digestion analysis of PCR products. For the construction of BAC clones with mutations in both copies of the MEQ (pRB-1B-Ct20), the process was repeated with the same pST76K-SR construct. A full revertant (pRB-1B-Ct22) was constructed by repeating the process twice on the double mutant using the wild-type MEQ shuttle vector construct. The same procedure was also followed for constructing a MEQ double deletion mutant (pRB-1B-D2) in which both copies of MEQ were deleted. Mutations in each of the clones were confirmed by sequence analysis. To generate virus stocks, DNA from each of the mutant clones was transfected into chicken embryo fibroblast cells by using Lipofectamine (Invitrogen).

**Animal Studies.** All experiments were carried out in accordance with the U.K. Home Office guidelines using specific-pathogen-free inbred line P (B<sup>19/19</sup>) and outbred RIR chickens obtained from the Poultry Production Unit of the Institute for Animal Health. For pathogenicity studies, 1-day-old birds were infected with 5,000 pfu of the cell-associated virus stocks by the intra-abdominal route. The MDV-infected birds and the mock-infected control birds were maintained in separate rooms. For the protection study, groups of 1-day-old line P birds were intraabdominally vaccinated with 1,000 pfu of pRB-1B-Ct20 or CVI988/Rispens vaccine and challenged with 1,000 pfu of RB-1B virus at 9 days postvaccination. All birds that developed the disease or were killed at the end of the experiment were examined postmortem for any gross/microscopic lesions.

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