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**Investigation of how host and viral
genes contribute to the pathogenicity
of avian oncogenic viruses**



**THE UNIVERSITY
of EDINBURGH**

Toby James Look

My thesis is submitted in the fulfilment for the degree of
Doctor of Philosophy (PhD)

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THE UNIVERSITY *of* EDINBURGH
The Royal (Dick) School
of Veterinary Studies



Declaration

I, the author of my PhD thesis, declare that the work generated herein is of my own unless stated otherwise. No part of my work produced for my PhD thesis has been submitted for any other degree or professional qualification.

Toby Look

Thesis Abstract

The avian oncogenic viruses include retroviruses such as Avian Leukosis virus (ALV) and herpesvirus Marek's disease virus (MDV) which together are major contributors to cancer in chickens worldwide. Both threaten the stability of poultry farming, causing significant economic losses and welfare implications. Increasing our knowledge of these diseases is critical to control ALV and MDV in poultry, allowing increased productivity and sustainable growth in the industry.

The causative agent, of Marek's disease, is MDV serotype-1 (*Gallid herpesvirus 2*, GaHV-2), a double-stranded DNA virus belonging to the family *Herpesviridae*. In non-vaccinated poultry, MDV infects 100% of immunologically naïve chickens and continues to evolve with increased virulence, making vaccination programmes more challenging. MDV infects CD4⁺TCRαβ⁺T-cells culminating in visceral lymphomas, neural lesions and paralysis, leading to death several days later. ALVs of the genus alpharetrovirus, are globally endemic in domestic chicken flocks and also replicate in related species. Lymphoid leukemia occurs in infected poultry. This B-cell lymphoma originates in the bursa of Fabricius and metastasizes to other internal organs. In industry, tumour mortality commonly causes a loss of 1-2% of birds with occasional losses >20%.

CRISPR-Cas9 gene editing was quickly established in many labs and opportunities to apply the basic CRISPR-Cas9 approach to a diverse array of biological problems followed. These include the investigation of gene-phenotype correlations in a single cell but also led to the ability to screen entire genomes for specific phenotypes. We utilised both single-cell and genome-wide screen editing techniques to investigate pathogenic determinates in MDV and ALV.

To investigate which genes of the MDV-1 vaccine strain CVI988 are essential or non-essential to virus growth and spread in cell culture, I designed and optimised an arrayed CRISPR-Cas9 screen to target all viral open reading frames. crRNA/tracrRNA transfection of fluorescent CVI988 infected DF1s enabled systematic analysis of gene essentiality across the whole MDV genome. Two identified genes as essential to viral replication and spread in DF1 cell culture were validated using CRISPR-Cas9 genome editing, providing further evidence that of the reliability of the assay and the requirement of MDV-19 and MDV-20 for viral replication and spread in DF1 cells. Furthermore, the non-essential genes identified maybe useful targets for recombinant virus generation. In the future, this methodology can be applied to other viruses.

MDV infection commonly results in latently infected T-cells, culminating in T-cell lymphomas. We aimed to investigate the essential host genes in a transformed chicken T-cell line (HP8 cells) using a Genome-Wide CRISPR Knockout (GeCKO) screen. We transduced a library of sgRNAs into HP8 cells and selected using the puromycin resistance marker, taking cell samples for up to 25 days. After removing genes that are considered essential in chickens, using previous chicken screen data and HP8 transcriptomic data to remove non-expressed genes, we shortlisted 339 hit genes that are required for the transformed phenotype of HP8 cells. The hits showed a significant mitochondrial signature; therefore, I investigated the use of mitochondrial inhibitors, metformin, chloramphenicol and A1210477. My data shows mitochondrial function is critical for the growth of HP8 cells and when mitochondria are perturbed, apoptosis increases, and MDV lytic genes are upregulated, suggesting a mechanism for viral reactivation upon mitochondrial stress. Furthermore, the gene IRF4 was shown to be critical for the proliferation of HP8 cells.

ALV subgroups are characterised by possessing unique entry receptors to the host cells. Two of the most pathogenically important ALVs, ALV-A (RCAS-A) and ALV-J (RCAS-J) were investigated using a CRISPR screen. Cas9-expressing DF1 cells were transduced with a library of sgRNAs, then infected with a green fluorescent protein (GFP) tagged RCAS-A or RCAS-J virus and sorted for non-GFP expression. The resulting cells were compared to an untreated control population, identifying genes that prevent RCAS-A/J infection and/or replication. Significant enrichment of guide RNAs targeting the respective receptors of each virus was observed. However, no other non-essential host genes appeared critical to infection with both RCAS-A and RCAS-J viruses.

gga-miR-21 is an oncogenic miRNA in chicken cells. It was proposed that the MDV oncoprotein Meq transactivates gga-miR-21, contributing to the oncogenicity of MDV. We deployed CRISPR-Cas9 gene editing to edit the gga-miR-21 promoter in HP8 cells and measured gga-miR-21 by RT-qPCR. Our results show that in HP8 cells, gga-miR-21 plays no significant role in the oncogenicity of HP8 cells. Additionally, Meq has been reported to bind the gga-miR-21 promoter causing its upregulation. Through knockout of Meq expression and overexpression, no correlation was seen between Meq and gga-miR-21 in HP8 cells. This suggests the gga-miR-21 interplay is not conserved across transformed MDV lymphocytes and while it may play a contributing role in some cell types it is not a critical mechanism in the oncogenic nature of MDV latently infected T-Cells.

Thesis Lay Summary

The avian oncogenic viruses include retroviruses such as Avian Leukosis virus (ALV) and herpesvirus Marek's disease virus (MDV) which together are major contributors to cancer in chickens worldwide. Both threaten the stability of poultry farming, causing significant economic losses and welfare implications. Increasing our knowledge of these diseases is critical to controlling ALV and MDV in poultry, allowing for increased productivity and sustainable growth in the industry.

All observable traits, good or bad are defined fundamentally by the genetics of that organism. Therefore, to learn about the pathogenic traits of interest, it is crucial to establish a genotype-to-phenotype (trait) link. To identify a phenotypic trait of interest CRISPR-Cas9 gene editing was used.

CRISPR-Cas9 gene editing technology is a method that enables precisely controlled gene editing. We used CRISPR-Cas9 to make gene knockouts (KO) of viral and host genes. Knocking out genes enables us to screen for observable traits of interest. Both single-cell editing and genetic CRISPR-Cas9 screens were used enabling the systematic analysis of all genes within an organism for a specific observable trait. My work presented in this thesis is broken down into four bodies of research:

First, I aimed to investigate the essential and non-essential genes in MDV vaccine strain CVI988. This work developed a methodology to systematically study the essentiality of viral genes, that can be applied to other viruses. This work also produced genome-wide information on the essential and non-essential genes of CVI988, helping to confirm the results of previous studies, but also highlighting which non-essential genes could be targeted in recombinant vaccine development. To do this I performed a screening method using CRISPR-Cas9 gene KO in single wells infected with the fluorescent CVI988 virus. First, a method was developed and optimised to screen the CVI988 virus to screen essentiality. Subsequently, a screen of all genes of CVI988 was performed. This produced comparative data of all gene essentiality across the CVI988 genome. Through gene editing of MDV latently infected cell lines, two essential genes were validated to prove the methodology worked.

Secondly, I aimed to unearth genes specific to the proliferation of MDV-infected T-cells. The primary pathology of MDV is the formation of cancerous T-cells, therefore, revealing host genes that contribute to the cancerous nature of MDV-infected cells will improve our understanding of how host genetics contributes to the development of cancer cells. To do this, I performed KOs of all chicken protein-coding genes in an MDV-infected tumorigenic cell line. The results showed several unique genes maybe

responsible for the cancerous nature of these cells. Many of these genes had a mitochondrial correlation. I then used mitochondrial inhibition to validate this suggestion and cell editing in isolation to confirm gene hits.

Next, I aimed to investigate the genes required for ALV-A and ALV-J infection and replication. ALV-A and ALV-J have their own receptors which are known to facilitate the entry of the viruses into host cells. Beyond, these receptors little is known about what host genes are required for viral infection and replication. To do this I performed a KO screen on all chicken cells combined following a fluorescent viral infection and then fluorescent activated cell sorting, this isolated any cells that resist viral infection. This identified the known receptors for ALV-A and ALV-J as the primary determinants of infection.

Finally, I aimed to determine the relevance of the gga-miR-21 expression regulated by oncoprotein Meq and the cancerous nature of MDV-infected cells. MDV latently infected T-cells often produce cancer in infected chickens. Meq is one of the major cancerous proteins produced by MDV. It had been reported that Meq and gga-miR-21 promoter interact to contribute to the cancerous nature of infected cells. Investigating the methods by which Meq contributes to producing cancerous cells will improve our understanding of viral-induced cancer biology. To do this I performed CRISPR-Cas9 gene editing of the gga-miR-21 promoter and used knockdown and upregulation of Meq to investigate the effects of gga-miR-21 on the transformation of MDV latently infected cells (HP8) and the involvement of Meq in regulating gga-miR-21 levels. I found that in HP8 cells Meq does not contribute to the increased levels of host gga-miR-21 levels and that gga-miR-21 promoter mutation does not significantly affect gga-miR-21 levels within HP8 cells.

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Abbreviations

aa	Amino Acid
ALV	Avian leukosis virus
AOV	Avian oncogenic virus
AP-1	Activator protein 1
BAC	Bacterial artificial chromosome
BAGEL	Bayesian Analysis of Gene Essentiality
bp	Base pair
Cas	CRISPR associated nuclease
cbl	cobalamin (vitamin B12)
CD4⁺	Clusters of differentiation 4
cDNA	Complimentary Deoxyribonucleic Acid
CEF	Chicken Embryo Fibroblasts
chNHE1	chicken Na ⁺ /H ⁺ exchange type 1
chTR	Chicken telomerase RNA subunit
CRISPR	Clustered regularly interspaced palindromic repeats
CRISPRi	Clustered regularly interspaced palindromic repeats interference
crRNA	CRISPR RNA
DLR	Dual-luciferase reporter assay
DNA	Deoxyribonucleic acid
DPI	Days post Infection
DSB	Double-strand break
dsRNA	Double-stranded RNA
dsRNA	Double-stranded RNA
EBV	Epstein-Barr virus or Human Herpesvirus 4
EDTA	Ethylenediaminetetraacetic acid
Env	Envelope
ER	Endoplasmic reticulum
FACS	Fluorescence Associated Cell Sorting
FBS	Foetal Bovine Serum
FCS	Foetal Calf Serum
FDR	False discovery rate
GaHV2	<i>Gallid Herpesvirus</i> 2 or Marek's Disease Type 1
GaHV3	<i>Gallid Herpesvirus</i> 3 or Marek's Disease Type 2
gB	Viral glycoprotein B
gC	Viral glycoprotein C
gD	Viral glycoprotein D
GeCKO	Genome-Scale CRISPR-Cas9 Knockout
Gga-miR-155	<i>Gallus gallus</i> microRNA 155
gH	Viral glycoprotein H
gL	Viral glycoprotein L
GO	Gene ontology
sgRNA	Guide RNA

HCF-1	Herpes simplex virus-associated host cell factor 1
HCMV	Human Cytomegalovirus or Human Herpesvirus5
HCV	Hepatitis C virus
HDR	Homology directed repair
HHV8	Human Herpesvirus 8
HIV	Human immunodeficiency virus
HRMA	High-resolution melting curve analysis
HSV	Human Simplex Virus
HSV-1	Herpes Simplex Virus Type 1
HSV-2	Herpes Simplex Virus Type 2
HVEM	Herpesvirus entry mediator
HVT	Herpesvirus of Turkeys
HVT	Herpesvirus of Turkeys or Melagrid Herpesvirus 1
IE	Immediate Early
IFN	Interferon
IL-2	Interleukin 2
IL-8	Interleukin 8
INDELS	Insertions and deletions
IRL	Internal Repeat Long
IRS	Internal Repeat Short
kDa	Kilodaltons
KO	Knock out
KSHV	Kaposi's sarcoma herpesvirus
LAT	Latency-associated non-coding RNA transcript
LB	Luria-Bertani
LFC	Log Fold Change
lncRNA	Long non-coding RNA
LORFs	Unique long ORFs
LTR	Long terminal repeat
mMDV	mild MDV
MeHV-1	Meleagrid Herpesvirus 1
MAGeCK	Model-based Analysis of Genome-wide CRISPR-Cas9 Knockout
MD	Marek's Disease
MDV	Marek's disease virus
MDV-1	Marek's Disease Virus Type 1 or <i>Gallid Herpesvirus</i> 2
MDV-2	Marek's Disease Virus Type 2 or <i>Gallid Herpesvirus</i> 3
Meq	Marek's EcoRI Q fragment protein
MHC I	Major Histocompatibility Complex class I
MHC II	Major Histocompatibility Complex class II
miR	MicroRNA
miR-155	MicroRNA-155
miRNA	microRNA
miRNAi	MicroRNA interference

MOI	Multiplicity of infection
mRNA	Messenger RNA
mRNA	Messenger RNA
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
ORF	Open reading frame
ORFs	Open Reading Frames
Ori_{lyt}	Origin of lytic replication
OXPHOS	Oxidative Phosphorylation
PAM	Protospacer adjacent motif
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
pp14	Phosphoprotein 14
pp24	Phosphoprotein 24
pp38	Phosphoprotein 38
q-RT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RCAS	Replication-Competent ASLV long terminal repeat (LTR) with Splice acceptor
REV	Reticuloendotheliosis virus
R-LORF	Repeat-long ORFs
RNA	Ribonucleic acid
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute medium
RRA	Robust ranking algorithm
RSV	Rous sarcoma virus
RT	Reverse transcription
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
RTR	Right terminal repeat
SDS	Sodium dodecyl sulfate
shRNA	Short Hairpin RNA
siRNA	Small interfering RNA
snRNA	Small nuclear RNA
SORFs	Unique short ORFs
TALEs	Transcription activator-like effectors
TCR	Toll-like receptor
TE	Buffer Tris-EDTA buffer
TERT	Telomerase reverse Transcriptase
TLR	Membrane-bound Toll-like receptors
tracrRNA	Trans-activating CRISPR RNA
Triton	X-100 t-Octylphenoxypolyethoxyethanol
TRL	Terminal repeat long
TRS	Terminal repeat short
TWEEN	20 Polyoxyethylenesorbitan Monolaurate
U_L	Unique long

Us	Unique short
USD	United States of America Dollars
UTR	Untranslated region
v	Virulent
vCXCL13	Viral chicken CXC ligand 13
vIL-8	Viral interleukin 8
VP16	Viral protein 16
vTR	Viral telomerase RNA subunit
vv	very virulent
vv+	very virulent plus
vv++	very virulent plus plus
ZF	Zinc fingers
GFP	Green Fluorescent Protein
gDNA	Genomic DNA

Chapter 1: Introduction

1.1 Avian Oncogenic Viruses

The avian oncogenic viruses include retroviruses such as Avian Leukosis virus (ALV) and the herpesvirus Marek's disease virus (MDV) which together are major contributors to cancer in chickens worldwide. Both threaten the stability of poultry farming, causing significant economic losses and welfare implications. Therefore, increasing our knowledge of both of these diseases is critical to the control of ALV and MDV in poultry, allowing for increased productivity and sustainable growth in the industry. The following sections will give details about these two viruses.

1.2 Marek's disease virus (MDV)

1.2.1 Overview

Marek's disease (MD) is a highly infectious viral neoplastic disease of chickens and other poultry. Despite being the first neoplastic disease that was shown to be preventable via live attenuated vaccines in the early 1970s, MD continues to threaten the sustainability of the poultry industry, which has given rise to a global financial burden of over 2 billion USD annually (Osterrieder *et al.*, 2006, Kennedy *et al.*, 2015). The causative agent, MDV serotype-1 (*Gallid herpesvirus 2*, GaHV-2), is a double-stranded DNA virus. In non-vaccinated poultry, MDV infection induces the transformation of CD4⁺TCRαβ⁺T-cells culminating in visceral lymphomas, neural lesions and paralysis approximately 28 days post-infection, leading to death several days later (Calnek, 2001). MDV is considered an excellent model for viral-induced cancers, similar to human viruses such as Epstein Barr virus (EBV), human immunodeficiency virus (HIV), Kaposi's sarcoma-associated herpesvirus (HHV8) and Hepatitis C virus (HCV), all of which are associated with tumours affecting different cell types (Esau, 2017). Consequently, this has driven significant progress in MDV pathogenesis studies (Burgess *et al.*, 2004, Kennedy *et al.*, 2015). Despite MD being controlled by a combination of vaccination, biosecurity measures and selection for genetic resistance, it has been observed that the vaccination of birds has significantly contributed to the evolution of more virulent strains (Davison and Nair, 2005, Read *et al.*, 2015).

1.2.2 MDV Taxonomy and Genome Structure

Within the *herpesvirales* order is the family of *Herpesviridae* which includes pathogenic viruses to birds and mammals (Zaichick *et al.*, 2011). *Herpesviridae* is split among 3 sub-families Alpha-, Beta- and

Gammaherpesvirinae. Within Alphaherpesvirinae are the genera *Mardiviruses*, *Itlovirus*, *Simplexviruses* (which include herpes simplex virus type 1 and herpes simplex virus type 2) and *varicelloviruses*. Beta- and Gammaherpesvirinae both contain human pathogenic viruses, within Betaherpesvirinae most notably contain cytomegalovirus (HHV-5) and the Gammaherpesvirinae sub-family contain Epstein-Barr virus (HHV4) and Kaposi's sarcoma associated viruses (HHV8) (Zaichick *et al.*, 2011). MDV-1 or *Gallid herpesvirus 2* belongs to the family *Herpesviridae*, subfamily Alphaherpesvirinae and the genus *Mardivirus*, which infect avian hosts. Within the *Mardivirus* genus, there are several viruses; *Gallid alphaherpesvirus 2* (GaHV-2, MDV-1), *Gallid alphaherpesvirus 3* (GaHV-3, MDV-2) and *Meleagrid alphaherpesvirus 1* (MeHV-1, HVT). GaHV-2 contains all pathogenic strains as well as the vaccine strain CVI988/Rispens. Both GaHV-3 and MeHV-1 demonstrate antigenic cross-reactivity to GaHV-2 which has been successfully exploited in several vaccines (Nair, 2004) (table 1). MDV strains have been further classified based on their pathogenicity with each pathotype representing a significant increase in pathogenicity and categorised as mild (mMDV), virulent (vMDV), very virulent (vvMDV) and very virulent plus (vv+MDV) pathotypes (Witter, 1997).

Genus <i>Mardivirus</i>	Alternate Nomenclature	Comments and selected Species
<i>Gallid alphaherpesvirus 2</i>	Marek's Disease Virus serotype 1 (MDV-1)	Contains all pathogenic strains such as RB-1B and Md5 but also contains the low pathogenic vaccine strain CVI988 Rispens.
<i>Gallid alphaherpesvirus 3</i>	Marek's Disease Virus serotype 2 (MDV-2)	Avirulent strains such as the vaccine strain SB-1.
<i>Melagrid alphaherpesvirus 1</i>	Herpesvirus of Turkeys (HVT)	Avirulent HVT FC126 vaccine strain.

Table 1. Taxonomy of *Mardivirus* genera showing alternative nomenclature and example strains. (Gatherer *et al.*, 2021).

The *Mardivirus* genome's organisation resembles that of Human Herpesvirus 1 (HSV-1), with two unique sequences, a unique long (U_L) and a unique short (U_S). Each of these is flanked by an inverted internal repeat (IR_L and IR_S) and terminal repeat (TR_L, TR_S) (Ajithdoss *et al.*, 2009). The sequences IR_L and TR_L as well as TR_S and IR_S are identical in sequence. The inverted and terminal repeats align in inverse orientation to one another, forming TR_L-U_L-IR_L-IR_S-U_S-TR_S genomic structure (figure 1) (Lee *et al.*, 2000, Davison *et al.*, 2004). The genome sizes vary between strains; HVT strain FC-126 is

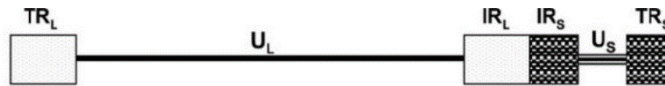


Figure 1. Organisation of the serotype 1 Marek's Disease Virus (MDV) genome. The MDV genome is constructed of a unique long (U_L) region flanked by inverted repeats, terminal repeat long (TR_L), internal repeat long (IR_L) and a unique short region (U_S) flanked by inverted repeats, internal repeat short (IR_S) and terminal repeat short (TR_S) (Lupiani *et al.* 2004).

159,160-bp (Afonso *et al.*, 2001), MDV-2 strain HPRS24 is 164,270-bp (Izumiya *et al.*, 2001), MDV-1 strain Md5 is 177,874-bp (Tulman *et al.*, 2000) and MDV-1 strain CVI988 is 178,311-bp (Spatz *et al.*, 2007).

Within the ~170,000-bp genome are 103 functional genes in addition to several potential splice isoforms of viral transcripts (Tulman *et al.*, 2000, Sadigh *et al.*, 2020). Most genes of MDV contain significant homology to HSV genes; however, there are several genes mainly located within the TR_L and IR_L regions which are unique to MDV itself, named LORFs (U_L , ORFs), R-LORF (Repeat-Long ORFs) and SORFs (U_S , ORFs) with 12 LORFs, 16 R-LORFs and 4 SORFs being identified thus far (Davison *et al.*, 2004). The origin of replication (Ori_{lyt}) in the TR_L and IR_L lies between phosphoprotein 38 (pp38) and pp14 (Camp *et al.*, 1991). The neighbouring genes to the Ori_{lyt} can be classified as those involved in latency (Meq, IL8 and pp14) and those involved with early lytic replication (pp38, pp24 and BamHI-H encoded protein) (Parcells *et al.*, 2003).

Despite the similarities to HSV including the ability to establish latent infection in host cells, MDV possess some striking differences. HSV mostly produces cell-free virions whereas MDV is strictly cell-associated within the host (although cell-free infectious MDV is released from the skin). Further differences include that MDV infects the bursa of Fabricius and the thymus by a distinctly separate route from that of HSV whereas HSV commonly infects neurons of sensory gangli and that MDV-1 consistently forms lymphomas whereas HSV commonly infects the (Davison *et al.*, 2004, Rosato and Leib, 2015).

Some of MDV's unique genes were quickly attributed to its oncogenic nature. One of the most notable is R-LORF7, otherwise known as MEQ, which is perhaps the most studied of all. Meq contains homology to the Jun/Fos family of transcription factors displaying transactivation and repression activities; however, most importantly MEQ is highly associated with the neoplastic nature of MD (Qian *et al.*, 1995). R-LORF2, "viral IL-8" (vIL-8) contains significant homology to mammalian and avians IL-8 and is involved in the recruitment of lymphocytes to infected cells to aid in the transmission of viral particles to new host lymphocytes (Boodhoo *et al.*, 2016). The promoter of R-

LORF14a, known as pp-38, spans across the origin of replication and has been linked to lytic viral replication (Boodhoo *et al.*, 2016).

Currently, 14 unique viral microRNAs (miRNA) have been located in MDV-1 specifically within the regions flanking Meq and within the latency-associated transcript (LAT) region (Burnside *et al.*, 2006, Burnside *et al.*, 2008). 18 miRNAs have been identified in MDV-2 and 17 in HVT but no conservation is seen between viruses. Despite no conservation between Mardiviruses, there appears to be strong conservation between strains (Yao *et al.*, 2007, Morgan *et al.*, 2008). Several studies have linked MDV miRNAs in pathogenicity, for example, MDV-1-miR-M4 was identified as a homolog of hsa-miR-155, a multifunctional miRNA involved in immune system regulation and oncogenicity (Zhao *et al.*, 2009a). However, the true extent of miRNAs in pathogenicity is not fully understood.

1.2.3 MDV History and Vaccination

MDV was named after the Hungarian veterinarian József Marek who first described the disease as polyneuritis in 1907 (Marek, 1907). The disease predominantly caused neurological signs and inflammation; however, by the end of the 1950s, the virus had evolved and was now predominantly a neoplastic disease, causing visceral T-cell lymphomas, a likely consequence of more industrialised farming practices (Benton and Cover, 1957, Witter, 1997). It was not until 1967 and 1968 that two groups independently isolated the virus (Churchill and Biggs, 1967, Nazerian and Burmester, 1968). In the early 1970s, the virus was sufficiently controlled by vaccination with the related avirulent *alpha*herpesvirus HVT (Churchill *et al.*, 1969). However, HVT (the first-generation MD vaccine) and all subsequent vaccines do not prevent superinfection by virulent MDV strains (Witter *et al.*, 1976). Therefore, it is likely MDV vaccinology has facilitated an evolutionary pressure for more virulent viruses to evolve, augmented by the periodic introduction of increasingly efficacious vaccines (Read *et al.*, 2015).

The introduction of HVT in the 1970s was followed by the emergence of very virulent (vv) MDV strains. To protect against vvMDV strains, bivalent vaccines (second-generation vaccines) containing HVT and non-oncogenic MDV-2 (GaHV-3) SB-1 strain were introduced in the 1980s and 1990s.

The subsequent emergence of very virulent plus (vv+) strains led to the widespread use of third-generation of vaccines including the attenuated non-oncogenic GaHV-2 strain CVI988/Rispens (Rispens *et al.*, 1972). It is predicted the use of the CVI988/Rispens vaccine may eventually lead to the evolution of very virulent plus plus (vv++) strains. However, the genetic selection of birds for increased resistance to MD may also have contributed to the evolution of virulence (Bertzbach *et al.*, 2020).

The correlation between strain virulence and the introduction of new vaccines may lack hard evidence but, at the same time, it is hard to ignore its significance. Currently, the gold standard for vaccination is the use of CVI988/Rispens with many commercial farms now choosing to combine with

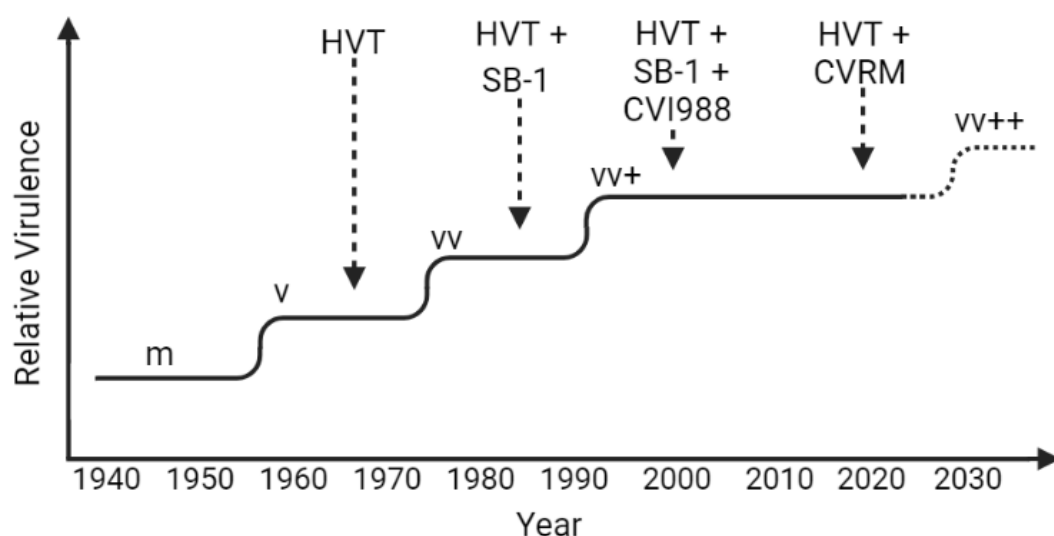


Figure 2. Stepwise evolution of Marek's disease virus isolates history and future predictions. Vaccine introductions are rough guides and differed between countries (Witter, 1998, Jarosinski *et al.*, 2006). HVT: Herpesvirus of Turkeys; SB-1: Gallid *alphaherpesvirus* 3 strain; CVI988: Gallid *alphaherpesvirus* 2 strain; CVRM: a CVI988 strain with a REV LTR insertion. All are vaccinal strains used to combat pathogenic Marrk's disease virus strains. Created in BioRender.

recombinant HVT vaccines that protect against MDV whilst also including protection against other poultry viruses. It was initially thought that the non-oncogenic nature of CVI988 was due to an insertion in the Meq oncogene (see section 1.6.7), resulting in the expression of a long Meq (L-Meq) protein. However, this has since been disproven, as a recombinant MDV (rMDV) strain carrying a long Meq induced high mortality and tumour incidences (Sato *et al.*, 2022). Still, little is known about the correlates for an effective MDV vaccine which has impaired further vaccine development. Nonetheless, in the last decade, insertion of the reticuloendotheliosis (REV) long terminal repeat (LTR) into the genome of CVI988 has generated a recombinant vaccine virus candidate named CVRM. When compared to CVI988, CVRM

has shown increased replication rates *in vitro* and showed equal or better protection than CVI988 (Lupiani *et al.*, 2013). A modified CVRM has now been commercialised by Boehringer-Ingelheim and is in use as the Prevexxion® RN vaccine. Over the coming years, it will be revealed whether the CVRM offers increased protection to vv+ MDV strains *in vivo* and whether like the vaccines before it, it will drive the evolution of more pathogenic strains. Another recent development in the vaccinology of MDV has come from the engineering of a recombinant vaccine virus from the vv+MDV strain 686BAC clone, where a double deletion of *meq* and *vIL8* genes formed an attenuated virus (686BAC-ΔMeqΔvIL8) which has shown similar replication levels to the parent virus and equivalent protective levels to CVI988 (Liao *et al.*, 2021b).

1.2.4 MDV Replication

It is considered that herpesvirus replication is fairly conserved throughout the family. The viral envelope expresses several glycoproteins, that attach the virus particle to a target cellular membrane which then triggers endocytosis, releasing the capsid into the cytoplasm. Dynein/dynactin motors transport the capsid along tubulin microtubules towards the nucleus (Dohner *et al.*, 2002). Subsequently, the capsid binds nuclear pores and the dsDNA is released into the nucleus (Ojala *et al.*, 2000). The next stage involves the circularisation of the linear dsDNA to form covalently closed circular DNA (Yao *et al.*, 1997). However, short telomeric repeats (sTMR) in the MDV genome also facilitate integration into telomeric regions of host chromosomes. MDV sTMR has been shown to have an important function in the replication, pathogenicity, and tumour formation of MDV (Greco *et al.*, 2014). All viral transcripts are produced by the cellular RNA polymerase II, which transcribes genes in a defined cascade, regulated by a tegument protein VP16 (HSV1) or UL48 (MDV-1) brought in during viral fusion (Boehmer and Nimonkar, 2003, Davison *et al.*, 2004). VP16, in association with several other proteins, activates RNA polymerase II to transcribe the immediate early (IE) genes. IE genes ICP0, ICP4, ICP22 and ICP27 orchestrate further transcription (Boehmer and Nimonkar, 2003).

Genome replication is initiated at the origin of replication. Within MDV there are two replication origins located in the repeat-long regions (Davison *et al.*, 2004). Both contain identical sequences, with three UL9 binding sites which are involved in the unwinding of supercoiled DNA and transcriptional activation (Lehman and Boehmer, 1999). These UL9 promoters include pp38, pp24 and BamHI gene family transcription factors for MDV-1, and include c-MYC and MEQ-enhancer binding sites (Nazerian and Lee, 1976, Bradley *et al.*, 1989, Qian *et al.*, 1996), all of which culminate in fine-tuning of gene expression and replication. UL9, through interaction with UL8, recruits the viral helicase primase which establishes the replication fork. Viral DNA

polymerase is recruited through UL30 to preform DNA replication (Boehmer and Nimonkar, 2003).

During the lytic replication phase of circularised viral DNA, long concatamers are cleaved and packaged into preformed capsids in a process conserved among herpesviruses (Homa and Brown, 1997). Within *alpha*herpesviruses, latency is often established; in the 5'-3' orientation, their latent genes are expressed such as Meq and vIL-8. During reactivation of MDV-1 from latency, viral genes are expressed from the origin of replication in the 3'-5' direction and pp38 is expressed (Parcells *et al.*, 2003). Therefore reactivation has been associated with pp38 expression, but pp38 is not essential for virus replication (Baigent *et al.*, 1998, Reddy *et al.*, 2002b). In *alpha*herpesviruses, latency is accompanied by a lack of detectable viral replication (Boehmer and Nimonkar, 2003).

1.2.5 MDV Life Cycle

MDV reproduces rapidly, completing a replicative cycle within 18-20 h. The infection starts when viral glycoproteins (gD, gC and gB) interact with their cognate host cell receptors to facilitate fusion, penetration and delivery of the viral particle to the host cell cytoplasm. Initial infection is thought to target naïve lung epithelial cells and occurs via the inhalation of virus particles in contaminated dust or dander, shed from productive infection in feather follicle epithelial cells (Abdul-Careem *et al.*, 2009). MD pathogenesis occurs in four stages; an early cytolytic phase at 2-7 dpi - described as a semi-productive lytic viral replication in lymphocytes, followed by a latency phase between 7-10 dpi in CD4⁺ T-cells, resulting in the systemic spread. Viral reactivation in CD4⁺ T-cells stimulates an immunosuppressive and cytolytic phase at 18 dpi, and eventually, around 28 dpi a proliferative phase is often seen in cutaneous tissues and culminates in tumour formation originating from transformed CD4⁺ T-cells (Boodhoo *et al.*, 2016).

Initial infection of lung epithelial cells is followed by macrophage infection either simultaneously or subsequently after lung epithelial infection. Either way, infected macrophages transport MDV to primary and secondary lymphoid organs (Barrow *et al.*, 2003). Moreover, virally infected cells produce the viral chemokine vIL-8, which recruits B-cells to the site of infection, further facilitating viral transmission to CD4⁺ T-cells (Engel *et al.*, 2012). The role of vIL-8 has been shown to be essential in the cytolytic infection of B-cells (Parcells *et al.*, 2001).

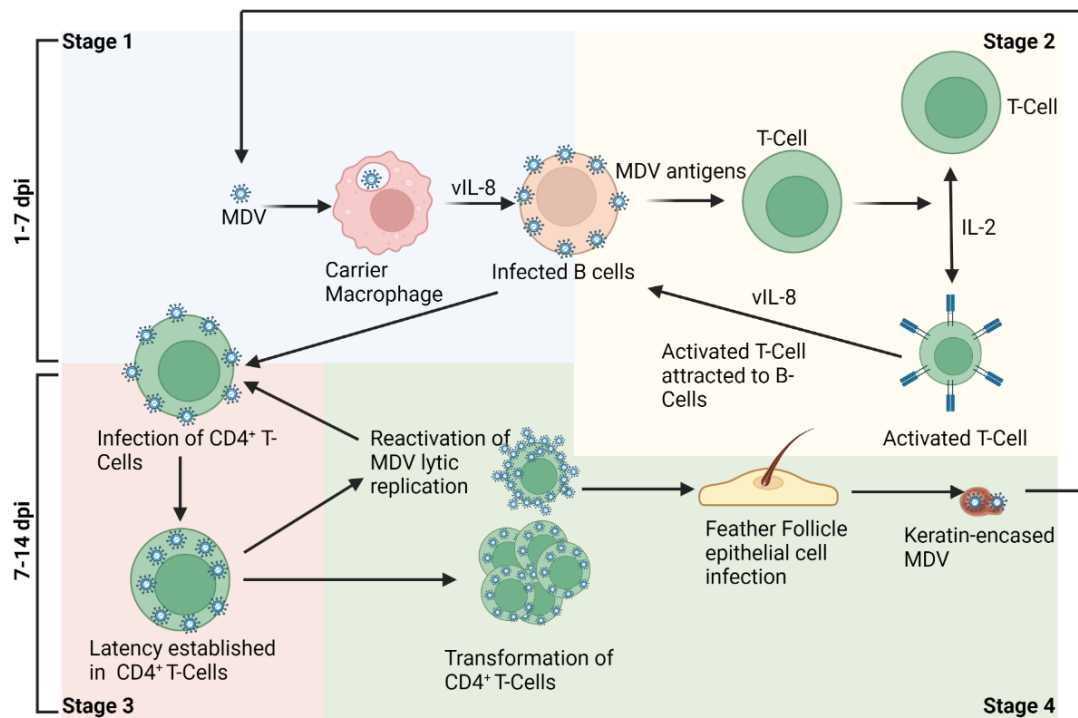


Figure 3. The natural *in vivo* Marek's disease lifecycle is divided into 4 stages. It is thought that macrophages within the lung transfer Marek's Disease Virus (MDV) to B-cells which occurs between 3 and 6 days post infection in the first phase. This is followed by the chemoattraction of CD4⁺ T-cells in the second stage. During the second stage between 5-10 days post infection a latent infection is established in activated CD4⁺ T-cells. The third stage is distinguished by reactivation of MDV between 10 to 14 days post infection. The fourth stage around 14 days post infection often will be characterised by lymphomas the extent of which depends on the virulence of the infecting strain. At this stage MDV will replicate within the feather follicle epithelium (FFE) cells and is shed in chicken dander as keratin encased MDV. These particles are then available for inhalation to repeat the infection cycle. IL: Interleukin; MDV: Marek's disease virus. Adapted from Jarosinski *et al.* 2006). Created in BioRender.

Semi-productive lytic viral replication subsequently occurs primarily in B-cells either recruited to the lung or virally infected by macrophage transmission in lymphoid organs (Boodhoo *et al.*, 2016). This is mainly controlled by the viral IE pp38 gene, which is only expressed in lymphocytes (Boodhoo *et al.*, 2016). Lytic MDV infection of B-cells results in a rapid decline in circulating B-cells and the ability to produce antibodies at the site of infection. Consequently, MDV-infected chickens become immunosuppressed facilitating viral spread and increasing susceptibility to co-infection with other pathogens (Biggs and Nair, 2012). In addition to infected lung epithelial cells, infected B-cells can recruit T-cells through the production of vIL-8, and

transmit MDV particles to naïve T-cells, the preferential target of MDV (Parcells *et al.*, 2001, Engel *et al.*, 2012).

Thereafter, the MDV-infected T-cells enter a latency state at 7-10 dpi, where MDV remains undetected by the immune system. Such an infection can be maintained for life and the virus continues to replicate within lymphocytes. Latency is achieved through the absence of viral or tumour antigen expression and downregulation of MHC class I to aid in immune evasion (Calnek, 2001, Boodhoo *et al.*, 2016). During the latency phase, CD4⁺ T-cells develop a fully transformed phenotype in all sites of the immune system involved in the primary and secondary lines of defence. The neoplastic transformation of CD4⁺ T-cells is mainly related to viral oncogenic proteins such as MEQ, which regulate oncogenes and tumour suppressor genes. Transformed neoplastic cells can act as a reservoir of MDV, consequently infiltrating and infecting the peripheral nerve interspace. At this stage, proteins such as pp14 stimulate neuropathy. Consequently, birds succumb to paralysis and eventually die. Alternatively, reactivation from the latent phase results in the uncontrolled proliferation of lymphocytes, predominantly caused by the action of MEQ on T-cell signalling (Boodhoo *et al.*, 2016). The resulting lymphoma metastasises to several organs, the nervous system, skin and eyes. Continued lymphoma eventually causes death.

Assuming birds survive the lymphoproliferative stage during the latent infection a cutaneous infection usually results, identifiable by PCR of blood cells and feather tips (Abdul-Careem *et al.*, 2009). This is dependent on the strain virulence; for example, very virulent plus+ (vv+) strains result in mortality before the lymphoproliferative phase, but infection by less virulent strains will progress. Cutaneous infection is the major source of environmental contamination from scales and feather debris shed by infected chickens (Abdul-Careem *et al.*, 2009). This is the only stage where it is believed MDV supports the production of cell-free viral particles, which are expelled from feather follicle epithelial cells (Harmache, 2014). Cell-free virus particles possess a highly protective envelope and when released can persist for months in poultry houses. The highly infectious MDV particles subsequently infect naïve birds through horizontal transmission (Burgess *et al.*, 2004).

1.2.6 MDV Pathogenesis

MDV encodes over 100 genes that are involved in the virus lifecycle or pathogenicity. Included are several genes associated with virulence, pathogenicity and tumour formation (Bertzbach *et al.*, 2019). The most extensively studied factors include the oncoprotein Meq, vTR, vIL-8/vCXCL13, RLORF4, RLORF5a, pp14 and pp38 (Bertzbach *et al.*, 2018, Bertzbach *et al.*, 2020).

1.2.6.1 pp14

Phosphoprotein 14 (pp14, MDV6, MDV75) is a 14 kDa polypeptide expressed with immediate early kinetics and is dispensable for virus formation and tumorigenesis (Bertzbach *et al.*, 2018). However, it is associated with neurovirulence in MDV infection (Tahiri-Alaoui *et al.*, 2013). *In vivo*, pp14 deletion studies have shown a significant reduction in clinical signs compared with the WT virus (Tahiri-Alaoui *et al.*, 2013). Much of the mechanism of pp14 action and how it contributes to MDV pathogenesis is yet to be fully understood.

1.2.6.2 pp38

Phosphoprotein 38 (pp38, MDV73) is a 38 kDa protein expressed with immediate early kinetics. pp38 is transcribed as a full-length mRNA as well as two spliced variants (SplA and SplB) (Li *et al.*, 2006). The full-length pp38 expression is associated with the early lytic replication and the two spliced variants are expressed during the establishment of latency. pp38 transitional expression means that, along with pp24, pp38 is often considered a biomarker for the latent to lytic switch (Roy *et al.*, 2021). This transition of pp38 expression has been linked to the metabolic activity of the cell and may contribute to the transformation of lymphocytes (Li *et al.*, 2006). Since pp38 is associated with the lytic-latent switch and early cytolytic infection of lymphocytes, it is not surprising that experimental deletions of pp38 results in decreased tumour incidence (Reddy *et al.*, 2002b). However, evidence suggests that pp38 is not involved in the maintenance of the transformed phenotype of latently infected T-cells (Zhang *et al.*, 2019a). An additional role that may contribute to tumorigenesis is pp38 inhibition of apoptosis either through a direct block or inhibition of cytotoxic T-cells (Gimeno *et al.*, 2005).

1.2.6.3 RLORF4

RLORF4 (MDV3.4/78.3) is located within the TRL/IRL and was brought to prominence when it was observed that several attenuated MDV strains lack R-LORF4 highlighting its potential role in pathogenesis (Jarosinski *et al.*, 2005a). RLORF4 deletion studies saw increased virus replication and spread *in vitro* but virus load decreased *in vivo* as well as decreased tumour formation (Jarosinski *et al.*, 2005b). To further these studies it has been seen that RLORF4 deletion inhibits the activation of NF- κ B leading to decreased Type I interferon and promotes IFN- β and IL-6 production *in vitro* and *in vivo*, therefore suggesting RLORF4 functional role in immune system modulation leading to reduced viral titres in infection studies (Liu *et al.*, 2019).

1.2.6.4 Viral IL8/ vCXCL13

vIL8 is a CXC chemokine secreted from MDV-infected cells that play an important function in the infection of target cells and pathogenesis. It was identified as the functional ortholog of chicken CXC ligand 13 (CXCL13) and binds to the cellular CXC receptor 5 (CXCR5) (Haertle *et al.*, 2017). Initially, MDV infects lung epithelial cells and local macrophages. During early natural

infection, vIL-8 is secreted, which recruits B-cells, the predominant target for lytic replication. At the same time, vIL-8 also recruits CD4⁺ and CD25⁺ T-cells which are often associated with latent infection and tumorigenesis (Engel *et al.*, 2012). When vIL-8 is abrogated in birds infected through natural transmission, tumour incidence and pathogenesis are completely abolished, demonstrating the requirement for specific cell types to establish infection (Engel *et al.*, 2012).

1.2.6.5 Viral Telomerase RNA (vTR)

One requirement of an oncogenic virus is the avoidance of cellular senescence or apoptosis. Telomerase is a ribonuclear protein complex that is involved in the maintenance of the telomeres present at the end of eukaryotic chromosomes, protecting them from progressive degradation (Blackburn, 2000). Due to the inability of DNA polymerase to copy to the end of a DNA strand, chromosomes would become unstable and programmed cell death would occur if there was not a telomerase to reconstitute the chromosomal ends. The telomerase complex consists of two major constituents, a telomerase reverse transcriptase (TERT) that catalyses the extension of telomeric DNA through the addition of (TTAGGG)_n and a telomerase RNA (TR) that provides a template for TERT and the extension of telomerase (Collins, 2008). MDV encodes a homolog of chicken TR (chTR) with 88% nucleotide sequence conservation and is critical for efficient MDV tumorigenesis (Fragnet *et al.*, 2003, Trapp *et al.*, 2006). vTR incorporates itself into the telomerase complex resulting in increased telomerase activity compared to chTR. However, vTR oncogenesis is independent of its role in the telomerase complex and cellular TR overexpression has been seen to have equivalent tumour formation suggesting cellular TR can compensate for vTR loss (Kaufer *et al.*, 2010, Kheimar *et al.*, 2019).

1.2.6.6 MDV Telomeric Repeats

MDV preferentially infects CD4⁺ T-cells where it integrates into the telomeres of host chromosomes, usually integrating into multiple chromosomes (Kaufer *et al.*, 2011). Integration of MDV is facilitated by telomeric repeats (TMR) that are situated at either end of the virus genome and the junction between IRL and IRS. Sequence mutation or reduction in length of the TMRs have shown TMRs are critical for virus replication and tumour formation (Kaufer *et al.*, 2011, Greco *et al.*, 2014).

1.2.6.7 Meq

One of the most studied genes of MDV, due to its relation to oncogenicity is the MDV Eco Q-encoded protein otherwise known as Meq or RLORF7 is a gene unique to MDV-1 which encodes a polymorphic 339 amino acid (aa) protein. The MDV genome contains two copies of Meq - one in the TRL and one in the IRL region (MDV5 and MDV76) (Spatz *et al.*, 2007). As well as wild-type Meq there are several Meq variants (resulting from insertions, deletions and point mutations) found in MDV strains.

Meq, a basic leucine zipper protein, forms homodimers, but can also heterodimerise with the cellular c-Jun protein. The resulting dimers bind response elements such as AP-1 which also exists in the Meq promoter itself, leading to a rapid increase in MEQ upon initial translation (Stik *et al.*, 2013). In the 339-aa MEQ protein, the first 1-120 aa form a basic amino acid-rich DNA binding domain, and the latter 121-339 aa form a proline-rich transactivation motif at the carboxy-terminal (Ajithdoss *et al.*, 2009). MEQ is expressed in the lytic stages, during latency most genes are not expressed however, Meq is one of the few viral genes consistently expressed in all MDV-transformed cells, giving evidence for its function in latency and transformation (Lee *et al.*, 2000, Arumugaswami *et al.*, 2009). Meq is dispensable for replication but reports consistent with the oncogenic behaviour of MEQ include, (i) when highly expressed, MEQ results in the transformation of rat fibroblast cells and protects against apoptosis (Liu *et al.*, 1998); (ii) transfection of antisense MEQ into an MDV tumour cell line is capable of reverting the transformed phenotype in these cells (Xie *et al.*, 1996); (iii) MEQ Knockout experiments show MEQ is crucial in MDV induced lymphomas *in vivo* and is involved in latency; (iv) only MDV-1 serotype encodes full-length MEQ (Lee *et al.*, 2000). Finally, although the CVI988/Rispens vaccine strain encodes a functional MEQ protein, this virus is non-oncogenic (Lupiani *et al.*, 2004). CVI988/Rispens encodes a long MEQ (LMEQ) through having a 177-bp (59 amino acids) in-frame insertion of a proline-rich repeat in the transactivation domain (Spatz *et al.*, 2007, Chang *et al.*, 2002a). The predicted amino acid sequence compared between the very virulent strain Md5 and CVI988 revealed 2 and 4 aa differences in the DNA binding and transactivating regions respectively (Ajithdoss *et al.*, 2009). This alteration has been identified to encode a weak transactivator MEQ protein, which only transactivates in the presence of chicken c-Jun rather than the transforming ability of MEQ alone (Ajithdoss *et al.*, 2009, Chang *et al.*, 2002b).

Several pathways have been revealed to be influenced by Meq which include: (i) Meq interacts with p53 suppressing its transcriptional activity, thus, suppressing apoptotic signalling (Deng *et al.*, 2010). (ii) Meq interacts with HSP70, a protein well established as an inhibitor of apoptosis and promoting cell survival (Zhao *et al.*, 2009b). (iii) Meq interacts with retinoblastoma tumour suppressor protein pRB which forms a complex with CtBP that represses E2F-regulated genes influencing the progression of the cell cycle (Brown *et al.*, 2006). Meq also regulates MDV gene expression, transactivating latent gene expression and inhibiting lytic MDV gene promoters such as ICP4, pp38 and pp14 (Bertzbach *et al.*, 2018). Taken together it is clear why Meq is often referred to as the 'Major Oncogene' and is likely the single gene most strongly associated with MDV virulence.

1.2.7 gga-miR-21

One of the most important pathogenic miRNAs in both human and chicken oncogenesis is miR21 which is found to be upregulated in both species as well as other mammals (Stik *et al.*, 2013, Feng and Tsao, 2016). miR21 targets tumour suppressor genes involved in proliferation, apoptosis and tissue invasion and gga-miR-21 has been seen to be regulated by the MDV primary viral oncogene Meq (Stik *et al.*, 2013, Feng and Tsao, 2016).

miRNAs are small non-coding RNAs with an average length of 22 RNA bases. They play a significant role in gene expression regulation, most commonly binding the 3' untranslated region of target messenger RNA to suppress expression (O'Brien *et al.*, 2018). There is great interest in miRNAs derived from homology studies of humans and numerous studies have highlighted genes involved in the lymphoproliferative process of MDV. Of interest have been miRNAs, such as gga-miR-219b (Zhao *et al.*, 2017), MDV-1-miR-M4 (Zhang *et al.*, 2019b), gga-miR-26a (Xu *et al.*, 2010), gga-miR130a (Han *et al.*, 2016) and gga-miR-21 (Stik *et al.*, 2013).

Traditionally, tumour-inducing viruses have been associated with viral oncoproteins such as MEQ but, increasingly, attention has shifted to the role of miRNAs in tumorigenesis (Yao *et al.*, 2009). Viral miRNAs are a key characteristic of herpesviruses. MDV-1 encodes 25 miRNAs, derived from 13 precursor miRNAs located within the *meq* oncogene region and LAT region of the terminal and inverted repeat long and short regions of the genome (Burnside *et al.*, 2006, Yao *et al.*, 2008). Another characteristic of herpesviruses is their latent phase which has long been associated with the neoplastic nature of these viruses. This led to the theory that miRNAs are essential in the equilibrium between latent and productive viral infection, including the regulation of host immune surveillance and evasion. Herpesvirus miRNAs have been reported to mediate the transition from latent to lytic infection via the control of IE genes (Boss *et al.*, 2009). EBV infection induces the cellular miR-155 which is well established to play critical roles in immune regulation, and lymphocyte homeostasis including the immortalisation of B-cells (Muylkens *et al.*, 2010). The functional ortholog of gga-miR-155, MDV-1-miR-M4 has been shown to be highly expressed during both lytic and latent MDV replication and is crucial in MDV-induced tumorigenesis (Zhao *et al.*, 2011). This demonstrates a key role for miRNAs in gene expression with key implications in disease. This is nowhere more obvious than in MDV-transformed cell line MSB-1 where the viral “microRNAome” accounted for over 60% of sequences (Yao *et al.*, 2008).

It has been reported that compared to retrovirus-transformed T cell lines, MDV-transformed cell lines show significant upregulation of miRNA levels; although this maybe a consequence of the number of viral genomes in cell lines analysed, it cannot be debated that there is a clear differential expression of viral miRNAs (Yao *et al.*, 2009). A second study supports these

findings, reporting that copy numbers of miRNAs such as miR-M4 are higher in tumours induced by vv+MDV strains than those induced by vvMDV strains (Morgan *et al.*, 2008). Together, these studies present evidence that miRNAs are involved in tumorigenesis and virulence of MDV.

Although virally encoded miRNAs are associated with MDV tumourigenesis, host miRNAs are also differentially expressed. Host-encoded miRNAs can act in *cis* (on host-encoded genes) or *trans* (on viral target genes) to alter gene expression and thus induce phenotypic changes. miR-146b, miR-454, miR-7b, miR-34a, miR-18a, miR-133a, miR-29a and gga-1et-7d are just some of the host miRNAs which were upregulated in MDV-transformed T-cells compared to non-infected T-cells (Yao *et al.*, 2009). In the MSB-1 T-cell line latently infected with MDV, it was reported that nearly a third of miRNAs matched the *Gallus gallus* (Red Jungle Fowl) genome and was noted that one of the most abundant was gga-miR-21 (Yao *et al.*, 2008). Additionally, gga-miR-21 has been highly associated with expression in various vertebrate tumours including overexpression during MDV lymphomagenesis (Fujita *et al.*, 2008, Padhi and Parcells, 2016).

In humans, the gga-miR-21 homolog hsa-miR-21 transcription is mediated via promoter transcription factors, for example, AP-1, STAT3 and FoO3a (Loffler *et al.*, 2007, Fujita *et al.*, 2008, Wang and Li, 2010). AP-1 transcription factors are also known to induce T-cell activation after antigen signalling and co-stimulatory signals (Barski, 2017). In the MDV-transformed MSB-1 cell line, it has been reported that MEQ can bind response elements, some of which (such as the AP-1 sites) exist in the gga-miR-21 promoter region (Stik *et al.*, 2013). The binding of MEQ to gga-miR-21 AP-1 promoter response elements resulted in the transactivation of the gga-miR-21 gene, which was subsequently observed to mediate latency and apoptosis, thus controlling the formation of the transformed phenotype (Stik *et al.*, 2013). In a further example, although not statistically significant, very virulent Md-5-MEQ activated *MMP-3* expression to a higher level than that of both CVI988-MEQ and CVI988-LMEQ (Ajithdoss *et al.*, 2009). *MMP-3* is required for the breakdown of extracellular matrix proteins and the invasion of tissues, b-zip proteins such as MEQ binds AP-1 response elements within the *MMP-3* promoter transactivating the gene.

It has been reported that MEQ transactivates the gga-miR-21 gene by binding to its promoter, therefore, this interaction may contribute to the regulation of tumour suppressors or oncogenes. gga-miR-21 is overexpressed during infection with the virulent RB-1B strain but not with the non-oncogenic strain CVI988/Rispens, suggesting a reduction in the transactivation by the major oncogene *meq* (Stik *et al.*, 2013).

gga-miR-21 negatively regulates the tumour repressor protein Programmed cell death protein 4 (PDCD4) (Young *et al.*, 2010). This has also been reported in chickens by Stik *et al.* (2013), therefore, gga-miR-21 expression

is thought to prevent the recruitment of viral defence mechanisms resulting in higher viral titres and reduced ability to regulate cell division (Stik *et al.*, 2013). Therefore, research into gga-miR-21 may unearth one reason why MEQ contributes to MDV pathogenicity.

1.3 Avian leukosis virus (ALV)

1.3.1 Overview

ALVs of the genus Alpharetrovirus, are globally endemic in domestic chicken flocks and will replicate in related species, such as quail, turkey and pheasants. Transmission can occur through both vertical and horizontal means. Lymphoid leukosis occurs naturally in infected poultry where this B-cell lymphoma originates in the bursa of Fabricius and metastasises to other internal organs. In industry, tumour mortality commonly causes a loss of 1-2% of birds with occasional losses >20% (Merck Sharp & Dohme (UK) Limited). ALVs are classified into different subgroups (A – K) based on the properties of the envelope that determine the host range, interference and neutralisation profiles. Subgroup-J (ALV-J) causes myelocytomatosis and is a significant problem in broiler breeders, due to increased mortality, reduced weight gain, immunosuppression, and eradication costs. ALV-A and ALV-B predominantly infect layer and breeder chickens and are associated with lymphoid leukosis, or erythroblastosis resulting in reduced egg production weight gain and loss of breeding potential. All 3 subgroups represent significant economic losses in the industry (McMullin, 2020). Control is focused on removing infected individuals due to no current vaccine or treatment against any subgroup (Dai *et al.*, 2015).

In infected birds, immunological responses are usually limited to the neutralising antibodies which bind the specific envelope glycoprotein of that subtype. The lack of response observed in birds is attributed to the presence of proviruses which stimulate antigenic tolerance (Beemon, 2021). Therefore, ALV is generally controlled through eradication in breeding stocks and detection and culling of infected birds in the field. Thus, there is a clear requirement for vaccinations or resistant chicken lines to supplement control measures.

Aside from culling to control, several other techniques maybe of use, including antivirals and engineering-resistant genes. Sun *et al.* (2019) have shown the effectiveness of Sargassum fusiforme polysaccharide (SFP) as an antiviral (Sun *et al.*, 2019). Meanwhile, antibodies are successful at curtailing both ALV-A and ALV-J (Qiu *et al.*, 2014, Meng *et al.*, 2018). Additionally, gene deletion or modifications maybe an alternative route to protect chicken flocks. The deletion of Trp38 amino acid from ALV-J receptor chicken Na⁺/H⁺ exchange type 1 (chHNE1) was effective at conferring resistance to ALV-J in

DF1 cells (Kucerova *et al.*, 2013, Lee *et al.*, 2017a). Also, Koslova *et al.* (2020) have shown the deletion of W38 from chNHE1 conferred resistance *in vivo* (Koslova *et al.*, 2020). Subsequently, Koslova *et al.* (2020) have shown knockout of the *tva* receptor confers resistance to ALV-A and the newly emerging ALV-K with no obvious pathological implications (Prikryl *et al.*, 2019, Koslova *et al.*, 2021). However, it is yet to be revealed if there are any long-term consequences (Koslova *et al.*, 2021).

1.3.2 ALV entry receptors and RCAS vectors

Retroviruses enter a host cell via a cognate receptor on the surface of a cell and their associated envelope glycoproteins. This culminates in the fusion of the viral envelope and host cell membrane, depositing the virion core to the cell's cytoplasm (Hughes, 2004). ALVs are considerably similar in their sequence, and antigenic makeup and have similar envelopes. However, tropisms vary, based on well-defined regions within the envelope glycoproteins. Resulting in specific viral-host cell receptor interactions, a unique feature for retroviruses (Dorner *et al.*, 1985).

ALVs, as with other retroviruses, harbour 4 components within their single-stranded RNA genome and follow the structure 5'–*gag*–*pro*–*pol*–*env*–3' with Long Terminal Repeats (LTRs) flanking either end. Evolution and mutation of the ALV *env* genes led to several subgroups being identified from chickens, A-E, J and K, with subgroups A, B and J being the most common pathogenically and ALV-A most common in commercial flocks (Bai *et al.*, 1995).

Subgroups A and B cause B-cell lymphomas within the bursa of Fabricius which commonly metastasises to organs such as the kidney, liver and spleen, causing death within several months (Beemon, 2021). Subgroups C and D-induced diseases are relatively rare. Members of subgroup E are endogenous viruses not associated with diseases. Recently, the ALV-J subgroup has presented the greatest threat to the poultry industry since its emergence and spread during the 1980s (Payne and Nair, 2012). Myeloid leukaemia induced by ALV-J first broke out in 1997-1998 in Western Countries in Broiler Breeders culminating in a loss of 1/3 of Broiler chickens, a year later it had spread to China and in subsequent years ALV-J had reached most other chicken species (Gao *et al.*, 2010). Despite ongoing eradication programs involving the culling of infected birds, ALV-J has not been fully eradicated, and sporadic outbreaks have still been reported. For example, in early 2018 an epidemic was simultaneously reported in >6 provinces of China (Wang *et al.*, 2019). ALV-J evolves rapidly, enabled by sequence variability in the hypervariable region of the envelope surface protein and 3'UTR indicating the potential for further outbreaks (Feng *et al.*, 2016). Like other retroviruses, ALV encodes specialised enzymes encoded from the *pol*

segment, reverse transcriptase (RT) and Integrase (IN) (Coffin, 1987). RT functions to transcribe the ssRNA genome into dsDNA known as the provirus. Once a dsDNA provirus is formed IN performs integration of the provirus into the host genome. The development of erythroid, myeloid and lymphoid tumours in chickens results from insertional mutagenesis. The flanking LTRs contain enhancer and promoter-like motifs, culminating in the insertional activation of gene expression at the site at which the virus is integrated (Greuel *et al.*, 1990). Examples of proto-oncogenes which ALV regulate include *c-myc*, *c-erbB* and *c-bcl-2* (Tam *et al.*, 1997, Gong *et al.*, 1998).

The Replication-Competent ALV long terminal repeat (LTR) with a Splice acceptor often shortened to 'RCAS' are a collection of laboratory based retroviral vectors from the SR-A strain of Rous Sarcoma virus (RSV) of the family avian leukosis viruses (ALVs). In most cases, retroviruses that have transduced a cellular oncogene are replicative incompetent. However, RSV is replication-competent due to the presence of a full set of viral genes, in addition to the *src* oncogene. The replication competence of RSV despite the insertion of the *src* oncogene has allowed the development of RCAS vectors capable of accepting foreign genes of interest at this site (Hughes, 2004). The use of RCAS vectors however is hindered as their replication is limited to avian cells, although mammalian systems can be manipulated to express the specific receptors for the RCAS subgroups. RCAS vectors belonging to the different ALV subgroups carry specific *env* sequences capable of binding to appropriate entry receptors on avian cells. The cellular receptor for envelope A is TVA (Bates *et al.*, 1993).

1.3.1.1 TVA receptor

TVA is part of the low-density lipoprotein receptor (LDLR) family. These comprise a single transmembrane glycoprotein, which primarily operate as cell surface endocytic receptors for binding and internalising extracellular ligands (Li *et al.*, 2001). The receptor TVA function was unknown until its mammalian ortholog was uncovered, known as the CD320 gene with a function in the uptake of cobalamin (cbl, vitamin B12)/transcobalamin II complex in humans (Elleder *et al.*, 2004, Quadros *et al.*, 2009). Cbl is an essential vitamin for all animals including chickens and is critical in metabolic cycles and methylation of proteins and nucleic acids (Quadros *et al.*, 2009). TVA has since been confirmed as the Cbl receptor in chickens *in vitro*, and *in vivo* TVA also acts as the receptor for Cbl-saturated transcobalamin but this is not limited to just TVA-mediated internalisation (Krchlikova *et al.*, 2021).

Subgroup ALV-K also utilises the TVA receptor, confirmed by precise editing of amino acids at the c-terminal loop of the LDL-A module of TVA. Upon editing DF1 cells showed complete resistance to ALV-K, by blocking the interaction with gp85 of ALV-K, whilst B12/cobalamin uptake was unhindered (Li *et al.*, 2023). A similar study has used amino acid substitutions to reveal

key residues which block ALV-A gp-85 interaction with TVA receptor, also demonstrating editing DF1 cells could confer resistance to ALV-A whilst B12/cobalamin uptake is not affected (Chen *et al.*, 2022).

Transcobalamin has similarities to haptocorrin, a molecule that can bind Cbl in the gastrointestinal tract in humans and transport Cbl to the blood, therefore, transcobalamin may substitute the role of haptocorrin in birds (Lopes-Marques *et al.*, 2014). Such metabolic changes have been attributed to neuropathology in mouse models (Arora *et al.*, 2017). Therefore, KO or editing of TVA in chickens maybe expected to affect Cbl uptake and present some pathologies however; none have been reported, but this is yet to be thoroughly investigated over time (Koslova *et al.*, 2021).

A parallel Cbl uptake method seems to be present, as Cbl-transcobalamin uptake appears above background levels when TVA is KO, similarly the same is observed in CD320 (TVA ortholog) KO mice and humans with CD320 defects where no pathologies have been reported (Koslova *et al.*, 2021, Chen *et al.*, 2022, Li *et al.*, 2023). Therefore, a degree of compensatory Cbl uptake may lessen pathologies across a variety of species.

1.3.1.2 chNHE1

ALV-J infection of chicken cells is known to be facilitated by the specific interaction of ALV-J envelope protein (Env J) with chNHE1 (Chai and Bates, 2006, Lee *et al.*, 2017a, Koslova *et al.*, 2020). The chNHE1 catalyses the exchange of intracellular H⁺ for extracellular Na⁺ acting to regulate pH and cell volume homeostasis (Reshkin *et al.*, 2000). ALV-J infection has been linked to upregulation and increased activity of chNHE1 expression contributing to intracellular alkalinisation thought to be necessary for oncogenic transformation (Feng *et al.*, 2016).

1.3.3 Life Cycle of Avian Retroviruses

Retroviruses are encapsulated by a lipid bilayer. Protruding from the bilayer are Env glycoproteins which bind to specific proteins on host cells to initiate infection. These viral entry receptors are often ubiquitous allowing avian retroviruses to infect a broad range of tissue types and cells (Williams *et al.*, 2004). A two-step process follows; the viral glycoprotein undergoes a conformational change when interacting with the target cell membrane, triggering viral uptake through endosomal encapsulation. The low endosomal pH triggers the release of the capsid into the cytoplasm (Barnard and Young, 2003). The cytoplasmic location allows reverse transcription of viral RNA to occur, resulting in dsDNA provirus (Telesnitsky, 2010).

The dsDNA associates itself with integrase and other viral proteins to form a pre-integration complex. Alpharetrovirus integrase contains a nuclear

localisation signal, facilitating the active transport of the pre-integration complex into the nucleus through the nuclear pore (Justice and Beemon, 2013). The integrase can now act on the nuclear DNA to integrate the provirus DNA into the host cell's genomic DNA. The location of integration for ALV favours areas of active transcription (Justice and Beemon, 2013).

The integrated viral DNA is then transcribed by RNA polymerase II starting at viral LTRs which contain promoters and enhancer sequences. A 100-nucleotide direct repeat forms a stem-loop structure to direct the nuclear export of the transcript. The viral transcript templates the synthesis of three polyproteins, gag, pol and Env. Gag is further processed through phosphorylation and acetylated at the N-terminus while Env is further processed in the endoplasmic reticulum adding glycosylation and subsequently folding to form a trimer (Palmiter *et al.*, 1978, Ficht *et al.*, 1984). The Env trimer binds its specific receptor and is trafficked to the cell membrane for secretion by the secretory pathway. The binding of the viral particle to the receptor acts to block superinfection (Einfeld and Hunter, 1988).

1.4 CRISPR-Cas9 and CRISPR screens

1.4.1 CRISPR-Cas9 origins, development and uses

First developed in the 1970s, genetic engineering quickly integrated itself as an established tool to study gene function and enable biotechnological advancements. Such advancements have had an unprecedented impact on human health, food security and as research tools. These have included revolutionary advancements, for example, insulin-synthesising bacteria (Ladisich and Kohlmann, 1992) and vitamin A enriched golden rice to name a few (Ye *et al.*, 2000). Within the life sciences, technologies that enable the deletion, insertion and editing of DNA sequences of cells or whole organisms have facilitated gene function analysis. However, for many years these processes were costly and time-consuming.

Initially, gene editing using site-specific targeted gene editing used modifiable DNA-binding proteins. Four major classes of DNA binding proteins were defined: meganucleases (Smith *et al.*, 2006), zinc finger (ZF) nucleases (Urnov *et al.*, 2005), transcription activator-like effectors (TALEs) (Christian *et al.*, 2010) and RNA-guided DNA endonuclease Cas9 (Cong *et al.*, 2013, Mali *et al.*, 2013). Meganucleases, ZF and TALE proteins mechanistically recognise specific DNA sequences through protein-DNA interactions. However, neither of these established themselves in the market due to several problems including low efficiency. They could only target a single site at any one time and designing protein constructs was time-consuming, laborious, expensive and required skilled staff in protein design.

These challenges limited the uptake of the technology. The simplicity of the combined use of Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) immediately superseded these DNA-Protein interactions. The development of CRISPR-Cas9 culminated from over a decade of research on repetitive sequences seen commonly in bacteria and the majority of archaea (Hsu *et al.*, 2014). CRISPR consists of a repeating sequence of bases, interrupted by spacer sequences which are derived from past viral invaders. These organisms contain particular Cas enzymes which integrate exogenous protospacer sequences into the CRISPR loci. Between each exogenous piece of DNA are spacers that allow discrimination between self and non-self-recognition. Importantly each exogenous protospacer sequence integrated into a CRISPR array is a sequence from the viral invaders' genomic DNA that is adjacent to a protospacer adjacent motif (PAM) sequence, for example for *Streptococcus pyogenes* PAM sequence is 5'-NGG-3' (Anders *et al.*, 2014). The PAM sequence is key to differentiating host-integrated protospacer sequences from an exogenous viral sequence which includes the PAM sequence.

The CRISPR-Cas lineup consists of three systems (Type I, II and III). The rarest of the systems Type II is not seen in archaea and only ~ 5 % of bacteria and has become the most regularly used system. The type II system consists of a trans-activating CRISPR RNA (tracrRNA) which hybridises with the sequences of the integrated repeats also CRISPR RNAs (crRNA) to form an RNA complex that is enzymatically processed by endogenous RNase III and other yet unknown nucleases. The crRNA consists of the integrated DNA of invading bacteriophage and the tracrRNA forms the stem-loop structure which binds and localises the Cas protein to the specific DNA target site (Shmakov *et al.*, 2015). Due to the mechanistic simplicity and potential for easy RNA programming, this subsequently led to most commercially available genetic engineering CRISPR technologies being based on type II systems. In 2012 it was discovered that both crRNA and tracrRNA could be combined into a single guide RNA (sgRNA) (Jinek *et al.*, 2012). Abolishing the need for maturation steps required for the crRNA and tracrRNA respectively. Therefore, multiple sgRNAs could be easily designed to direct specific Cas9 cleavage and genetic manipulation. In January 2013 the Zhang lab published the first method using CRISPR-Cas9 to genetically edit the genome in human and mouse cells (Jiang *et al.*, 2013). The advances that followed have led us to numerous methods to study all aspects of the genomic code not only non-homologous end joining (NHEJ) mutation via frameshift insertion/deletions (INDELs) mutations that can induce loss of protein function, but specific homology-directed repair (HDR) has enabled successful studies of DNA traits in the context of cells or organisms (Jinek *et al.* 2013). With the success of the aforementioned studies and techniques, a plethora of CRISPR-based genomic, epigenetic and transcriptional manipulation techniques have followed.

The CRISPR sgRNA consists of around 25 nucleotides that can be specifically designed to target a specific DNA sequence by base pairing. Over previous technologies, the nucleotide interactions greatly enhanced specificity, and ease of design and allowed multiplexed targeting by introducing a large cohort of sgRNAs to a single target cell. Additionally, the diversity of functions that CRISPR-Cas9 technology can facilitate is vast, with the capability to fuse any modular enzymatic component to an active or deactivated Cas9.

Aside from studies of singular or multiple epigenetic modifications on gene transcription or base and gene modifications on gene function. One of the greatest advancements CRISPR technology has provided is the mass editing of genomes in cells or organisms due to its inherent simplicity, low cost and specificity. These benefits lead to large-scale functional screens. In such an approach, rather than the transfection of a single sgRNA into a population of cells, CRISPR screens involve the introduction of hundreds to thousands of sgRNAs each with a unique target into a population of cells (Shalem *et al.*, 2014). Such screens have become pertinent to unbiased genotype-phenotype studies and now form the basis of high throughput assays to functionally screen many genes concurrently.

It is clear CRISPR has rapidly established itself as a crucial tool in the field of molecular biology, but also therapeutic applications are some of the most revolutionary (Dunbar *et al.*, 2018). Rapid advancement has facilitated many scientific discoveries however, CRISPR is not without its problems. Perhaps one of the most publicised criticisms is the ethical use of CRISPR in human, animal and plant genetic engineering. These ethical and environmental concerns are propounded by CRISPR's capability to genetically alter an entire population or species - so-called 'gene drives' offer the potential to spread parasite-resistant mosquitos in the fight against malaria. However, without engineered reversal strategies and significant research on the wider environmental impact, they remain controversial (Gantz *et al.*, 2015, Hammond *et al.*, 2016).

Many of the ethical concerns and technological problems are associated with *in vivo* experiments and treatments. However, in most molecular biology applications including CRISPR screens in mammalian cell lines, methodologies are well-validated and reproducible (Doench, 2018, Dempster *et al.*, 2019).

1.4.2 CRISPR-Cas9 Screens

Characterising genomic function is fundamental to several fields of biomedical research including cancer screens, drug-drug interactions, drug sensitivity and resistance, gene therapy, regenerative medicine applications and infectious disease (Ford *et al.*, 2019). The genotype-phenotype

relationship underpins research into gene functionality. However, genomic data sets represent thousands of genes each with many polymorphisms, therefore high throughput methodologies were sort after. The advent of CRISPR-Cas9 genome editing technologies and its first mammalian application in 2013 revolutionised the field of gene editing (Mali *et al.*, 2013). Due to the adaptability, ease of use and rapid perturbation of genomes CRISPR-Cas9 proved easy to scale up to simultaneously test thousands of genetic changes.

The ability to perturb gene function forms the foundations of elucidating the functionality of all genes. Since the development of CRISPR screens, unbiased genome-wide genetic studies have been designed to reveal genetic vulnerabilities and fitness genes which were first systematically used to study acute myeloid leukaemia (AML) (Tzelepis *et al.*, 2016). The first CRISPR screens developed were the loss-of-function or drop-out screens, whereby they would concurrently perturb thousands of genes in a pooled format. Given that sgRNAs are stably integrated into the genome through viral infection, hits can be identified using positive or negative selection for a specific phenotype followed by massively parallel sequencing and cross-referencing to the sgRNA library. With clear benefits, pooled screens have become frequently used to study genetics in mammalian cells. Using CRISPR-Cas9 to conduct comprehensive analyses of genomes is a robust tool with little additional infrastructure required and the technology can be implemented in most laboratories.

Large-scale systematic loss of function screens have become a frequent method to identify cancer vulnerabilities or genes essential for cancer cell proliferation or survival (Kiessling *et al.*, 2016, Ma *et al.*, 2017b, Wang *et al.*, 2017).

CRISPR-Cas9 screens are not just limited to cancer research, as such screens have also been successful in infectious disease studies revealing essential, susceptible and resistant genes for a specific disease (Doerflinger *et al.*, 2017). The gathered information can subsequently be used to determine drug targets or guide genetic engineering to develop resistance against the infecting pathogen (Doerflinger *et al.* 2017).

1.4.3 sgRNA library design

The process of gene editing first begins with the design of a sgRNA or a library of sgRNAs for the target gene/genes. Firstly, when designing a sgRNA library, the genome used needs to be considered, and genomes are constantly evolving from their initial characterisation. These changes include the identification of new genes, the reclassification of others, for example from pseudogenes to genes or vice versa, the inclusion of exons or introns and the identification and redefining of single nucleotide polymorphisms

(Doench, 2018). Therefore, for each new sgRNA design, the latest annotation of the genomic DNA should be used, increasing confidence in downstream analysis.

For completeness, a screen should cover a whole genome, especially if the downstream assay is not limited by the number of possible hits. The number of sgRNAs designed to target each gene can vary. The use of more (8-10 sgRNAs) maybe beneficial for the detection of genes with weaker phenotypes since this improves statistical power that hits emerge without false positives. However, fewer sgRNAs are often used because the larger library can be screened at the same cost and requires fewer cells, while still uncovering genotype-phenotype correlations.

A study by Hart *et al.* (2017) evaluated the effect of increasing the number of sgRNAs targeting the same gene on the total number of hits; they found that by increasing the number of sgRNAs the hit rate increased. Although at a diminishing rate, the greatest increase in hits was seen when using 4 sgRNAs (Hart *et al.*, 2017b). However, another, study suggests that 6 sgRNAs offer the best trade-off between redundancy and cell requirements (Ong *et al.*, 2017). The same study also investigated the influence of replicates on the identification of hits. As expected, the number of hits increases with the replicate number but again eventually plateaus. The greatest performance in hit identification was seen at 2 replicates. Therefore, it can be concluded that 4 sgRNAs per target and 2 replicates will produce efficacious hits while minimising the use of reagents, costs and time. This redundancy in sgRNAs is essential for an effective screen as multiple sgRNAs can also lessen the chance of sgRNA drop-out that may occur at multiple stages. For example, failure to be, amplified, produced in bacteria, produced as a virus or transduced into cells leads to false negatives (Doench *et al.* 2018). Increasing the number of sgRNAs theoretically improves accuracy, but library size can be limited; DNA synthesis is inherently error-prone and therefore increasing library size increases the chance of off-target sgRNAs (LeProust *et al.*, 2010). Although, libraries greater than 10^7 in number can be transformed and propagated in bacteria, the cell culture demands of mammalian cells for screening are often impractical for most laboratories, making libraries greater than 100,000 sgRNAs often less feasible (Ford *et al.*, 2019).

A final consideration is the use of controls in a screen, of which there are two options; non-targeting sgRNAs or sgRNAs that target known or suspected non-essential genes. Although minor differences between methods, Hart *et al.* 2017 report that sgRNAs targeting genes without a growth phenotype are more robust than non-targeting sgRNAs as a negative control for genome-wide screens of cell fitness.

sgRNAs are designed with bioinformatics computational tools which use specialist algorithms to identify sgRNA targets adjacent to PAMs. There are many algorithms available for sgRNA design and data sets have been compared across algorithms (Haeussler *et al.*, 2016). Although each method has drawbacks, computational analysis greatly enhances the chance of a

successful screen. These PAM sequences are fixed determinants which may limit possible sgRNA targets. However, as it has been found that every gene has multiple potential target sequences, these limitations may only be observed if designing sgRNAs for smaller genomic elements such as promoters (Montague *et al.*, 2014). There is ongoing research into identifying more relaxed PAM sequences, for example, the Sp-CAS9-NG, which recognises NG sequence or Cas12a endonuclease which recognises T-rich domains (Kim *et al.*, 2017a, Nishimasu *et al.*, 2018, Collias and Beisel, 2021). The tools used for sgRNA design are evolving rapidly as CRISPR technologies become more frequently used, thus using up-to-date methods is recommended. The use of multiple programmes can be useful to benefit from the advantages of each programme (Doench *et al.*, 2014, Heigwer *et al.*, 2014, Montague *et al.*, 2014).

In CRISPR-Cas9 screens, the greatest issue is decreased cell viability, as a result of off-target sgRNAs that when bound to an unintended site can activate the DNA damage response and cell apoptosis. This is an artefact of mismatch tolerance, a beneficial trait to bacteria to tolerate mutations. Therefore, the potential exists for a skewed readout of gene essentiality (Lino *et al.*, 2018). Thus, the library design is a crucial consideration for a screen.

When designing a sgRNA library there are two important factors to the construction of a high-quality library and to mitigate predetermined bias. The first one is the identification of a unique target sequence to limit off-target Cas9 activity. This is usually restricted by the placement of PAM sites in the genome and then selected via BLAST algorithms to minimise off-target activity (Costa *et al.*, 2017). The seed region (10-12 bp next to the PAM sequence) is most critical to high-fidelity binding and is a consideration in many algorithms (Semenova *et al.*, 2011). The second is to predict the sites with the highest genome editing efficiency, using methods equal to those above. Since no correlation exists between on-target sgRNA and the likelihood of off-target effects, generally sgRNAs are designed to best suit the target and thus inherently off-target effects are minimised (Doench, 2018). Off-target binding of sgRNAs, is the topic of several reviews by Zhang *et al.* (2015) and Tsai *et al.* (2016) for more clarity (Zhang *et al.*, 2015, Tsai and Joung, 2016). Techniques to reduce deleterious off-target effects are rapidly evolving and therefore the latest methods should always be used where possible (Cho *et al.*, 2014).

These are not the only considerations for a high-fidelity screen. When targeting a gene there are several potential sgRNA binding sites, and the selection of target sites should not be randomly chosen. The structure of the target gene should be considered, for example, the position of downstream start codons, as a sgRNA targeting upstream of a start codon may not inactivate the gene. Similarly, sites near the carboxyl end of a protein may still allow the majority of a protein to be synthesised, in addition to sites associated with SNP (Hough *et al.*, 2017) and sites near the end of the coding region (Doench *et al.*, 2014). Avoiding these pitfalls ensures the greatest chance that each sgRNA effectively disrupts the target gene.

Alternatively, spliced exon isoforms can prevent completeness in a study. However, usually, a single representative transcript is selected for sgRNA library construction (Doench *et al.* 2014). Another consideration is continuity across cell lines; generally, continuity is maintained due to the fidelity of NHEJ across cell lines and species and thus is usually disregarded (van Overbeek *et al.*, 2016).

The term genome-wide is often intended to refer to protein-coding genes only, therefore overlooks a large percentage of genes that may have a regulatory function. If these regions want to be studied this will often require custom library design, which is crucial due to the propensity for these regions to vary more frequently between cell lines. Although many parameters can be considered, our understanding of sgRNA design is still limited and behind that of comparable siRNA design (Costa *et al.* 2017).

1.4.4 Delivering Cas9 and the sgRNA Library

Selection of the optimal delivery system for Cas9 and the library is integral to achieving high editing efficiencies and accuracy. Considerations include cell type, if the screen is pooled or arrayed and cargo size (Ford *et al.* 2019).

Cas9 at 162 kDa is a large protein and combining its delivery with sgRNA lentiviral vector leads to low lentiviral titre due to its size. Thus, commonly Cas9 is delivered before the sgRNA library, which as a result can be delivered at a higher titre (Sanjana *et al.*, 2014). Expression of Cas9 in this manner requires quantification of Cas9 activity as it is possible that differences in expression, stability and localisation could remove the selectable marker adjacent to the Cas9 protein. Therefore, Cas9 expression should be periodically tested before the sgRNA library is introduced (Doench *et al.* 2014). Nevertheless, the study of Shalem *et al.* (2014), one of the first genome-wide loss of function studies, successfully used the lentiCRISPR-Cas9 construct approach. Alternatively, a stably expressing cell line of your cells of interest can be produced which benefits from higher Cas9 expression and are less likely to drift in their performance over time. However, it is important that the integration of Cas9 does not affect the phenotype of interest, for example, proliferation. The success of this form of Cas9 delivery was first demonstrated by Wang *et al.* (2014). The studies by Sahelm *et al.* (2014) and Wang *et al.* (2014) were the first proof of principle studies to establish both methodologies.

The next step involves the transfection of the library of sgRNAs into a population of cells, which has primarily utilised lentiviruses. Lentivirus has gained this position for several reasons. The integration trait of lentiviruses into the genome permanently means that this serves as a tag for the sgRNA construct delivered to each cell, thus later can be read by NGS. This trait also makes lentivirus effective for transduction into primary cells which can be difficult to transfect (Ford *et al.* 2019). Lentivirus also possess a large cargo capacity, meaning they can hold large genes or multiple gene cassettes and have extensive tropism (Song, 2017).

The pool of lentiviruses created harbouring the sgRNAs is transduced to the target cells at a low multiplicity of infection (MOI = 0.3) to reduce the chance of lentivirus-sgRNA coinfection. Multiple integration events into the same cell can add background noise during analysis, therefore this is mitigated via a low MOI (Doench, 2018). Cells are then regularly passaged to maintain high representation (500-1000x), in the hope of reducing false positives and negatives from erroneous skew (Doench *et al.* 2018).

1.4.5 Arrayed and pooled screening

The studies of Shalem *et al.* (2014) and Wang *et al.* (2014) were some of the first to use genome-wide loss of function screens and demonstrate its benefits over siRNA in human cell lines. Both studies presented proof of principle methodologies which enables positive or negative selection. CRISPR loss-of-function screens can take one of two forms: arrayed or pooled. Pooled screening technique involves the delivery of the lentiviral

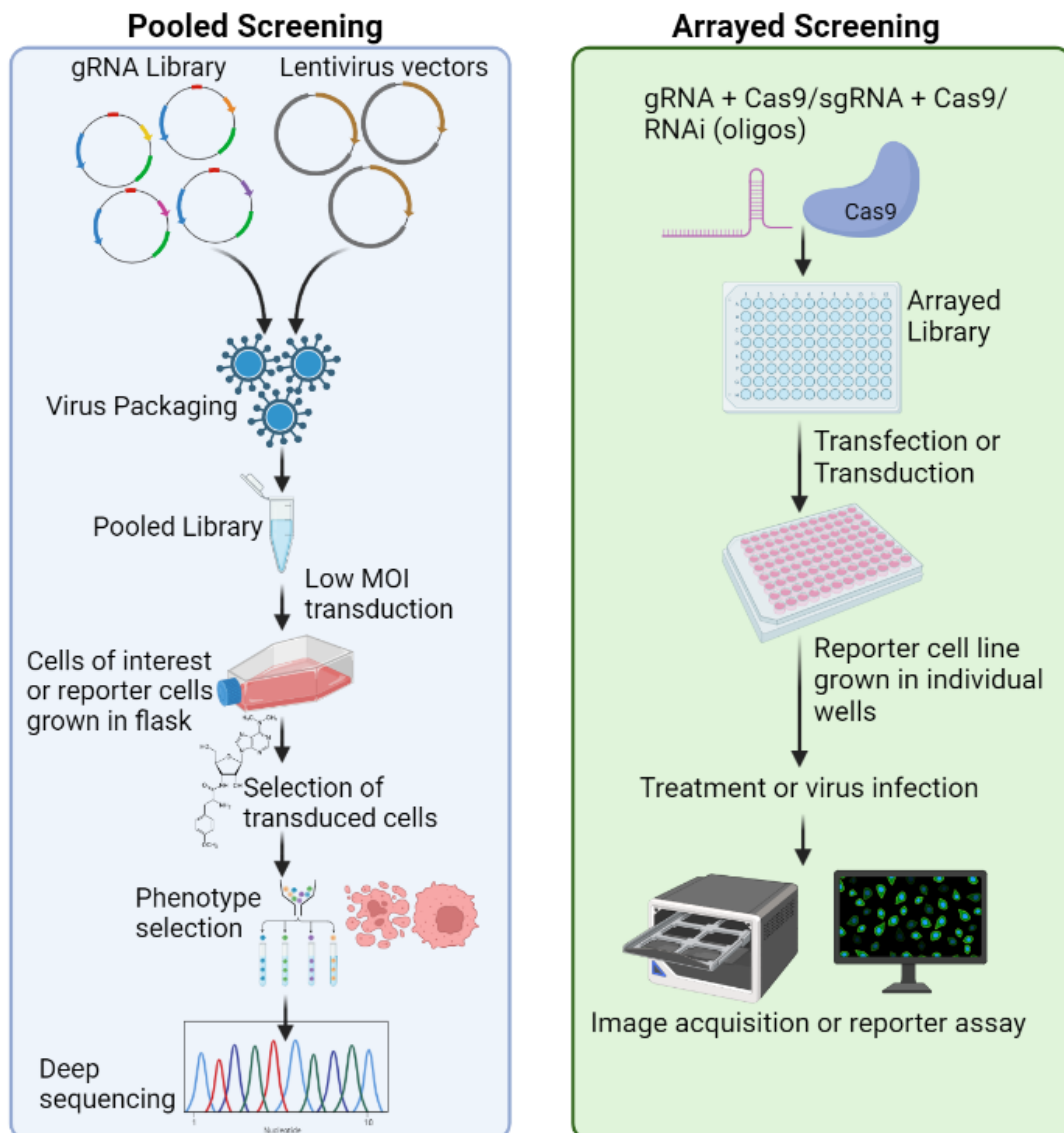


Figure 4. A generalised diagram of pooled and arrayed screening methods.

Briefly pooled screening involves the construction of a lentivirus sgRNA library, transduction to a large pool of homozygous cells at a low MOI to ensure 1 sgRNA only transduces 1 cell. This is followed by selection using an antibiotic selectable marker for the lentivirus transduced cells, thereafter phenotypic selection is performed often these include proliferative phenotypes or viral infectivity. Deep sequencing is then used to reveal the hit genes. Arrayed screens involve the delivery of CRISPR components targeting a specific gene to a single well of cells, followed by phenotypic measurements that can be designed specifically to the gene of interest being perturbed. Deep sequencing is not required as the sgRNAs added to each well are recorded at start of the assay. Created in Biorender.

sgRNA cassette into a flask of cells at low MOI before selection for a specific phenotype. The results are then obtained from deep sequencing of the genomic DNA for enrichment or depletion compared to a control unselected sample. The pooled screening technique involves the delivery of the lentiviral sgRNA cassette into a flask of cells at low MOI before selection for a specific phenotype. The results are then obtained from deep sequencing of the genomic DNA for enrichment or depletion compared to a control unselected sample. This limits pooled screens to simple readouts such as proliferation. On the other hand, arrayed based techniques involve the delivery of sgRNA to a discrete population of cells thus mitigating the possibility of several sgRNAs infecting the same cell. Rather than selection and sequencing for arrayed screens DNA sequencing is not required since the well can be cross-referenced to the sgRNA library. Since each well contains a unique mix of viruses, vectors and reagents, phenotype analysis is tailor-made to each well. Therefore, the output is a ranked phenotypic score dependent on the sgRNA target gene, for example, cell morphology or fluorescence and other complex phenotypes (Agrotis and Ketteler, 2015). Additionally, transcriptome data can be obtained using RNA sequencing, a useful tool for cancer screens, since cancers regularly possess gene overexpression. Therefore, arrayed screens can provide further insights into the effect of a perturbation and are also more suited to primary cells or neurons (Costa *et al.* 2017). Several disadvantages exist including the requirement of specialist equipment and possible lab automation for large screens, costly, lower throughput, quality control is difficult, editing efficiency maybe low and well variation can be high (Costa *et al.* 2017). Interestingly, Datlinger *et al.* (2017) have taken the benefits of pooled screens' high throughput and arrayed screens benefit of varied readouts to study the transcriptome profile, one of the most extensive analyses of cellular response to a perturbation. In this technique, a pooled screen is combined with single-cell RNA sequencing and the use of sgRNA barcodes to distinguish each cell's perturbation the use of sgRNA barcodes to distinguish each cell's perturbation (Datlinger *et al.*, 2017).

However, to date for the majority of applications, in particular cancer vulnerability genes, the pooled screening approach has most often been applied, since pooled screening requires a simple setup, can be conducted in

most laboratories with basic cell culture facilities, is cost-effective, can comprehensively analyse an entire genome and is suitable for the identification of cell viability, proliferation or drug resistance genes. Disadvantages do exist, such as the large number of cells required, the inability to study primary cells, the requirement of NGS and a selection step (Costa *et al.* 2017).

Pooled screens have been successful in several fields, for example, the identification of cancerous tumour suppressor genes (Katigbak *et al.*, 2016), oncogenic genes (Kiessling *et al.* 2016), and host dependency factors for viral infection (Park *et al.*, 2017). Initially, CRISPR screens had primarily experimented on human cell lines which soon expanded into the mammalian cell but also in *Drosophila* cells (Bassett *et al.*, 2015) and zebrafish (Shah *et al.*, 2015). CRISPR-Cas9 gene editing has also been used successfully to modify chicken cell lines and embryos (Zuo *et al.*, 2016), and a CRISPRa screen has been shown to successfully identify Pan-avian Influenza Virus Inhibitory Host Factors (Heaton *et al.*, 2017), although to date no pooled CRISPR dropout screen has been performed on chicken cell lines.

1.4.6 Pooled screening - viability screens

CRISPR-Cas9 viability screen's primary objective is to identify essential genes, in other words, these screens look to find genes required for cell survival or proliferation and at a molecular level to unearth genetic vulnerabilities within the cells of interest. These genetic vulnerabilities are therefore of interest therapeutically as their inhibition can lead to reduced viability.

All types of screens depend on a subsequent assay (or screen) that distinguishes cells that display the phenotype of interest from those that do not. This is performed using a positive or negative selection screen to identify sgRNA editing which has resulted in the desired phenotype.

The simplest form of screening is to identify genes affecting cell fitness. Mutations that reduce cell fitness will be depleted as selection progresses. By the end of the screen, some mutations may be completely absent from the cell pool, therefore, these screens are often referred to as negative selection or drop-out screens. At the genomic scale, tens of millions of cells are required to prevent depletion from occurring by chance and the library needs to be represented numerous times over, to provide sufficient sensitivity to detect sgRNA depletion from the cell population (Agrotis and Ketteler, 2015).

Negative selection screens are most regularly utilised in cancer studies to identify the genetic drivers of proliferation (Luo *et al.*, 2008). Therefore, essential genes become depleted in the pool of CRISPR-sgRNA targeted cell population while cells, where their survival is unaffected, outgrow them.

On the contrary to negative selection screens, in positive selection screens the cells of interest are enriched over time. Selective pressure for such screens may use specific culturing conditions (Rotem *et al.*, 2015) or infectious agents (Ma *et al.*, 2015). A positive selection screen will generally result in the elimination of most cells. For this reason, compared to negative selection screens, where most cells survive, positive selection screens are often very robust. Due to the high number of cells required pooled negative selection screens are limited to cells which have a reasonable proliferation potential, ruling out the ability to study primary cells or neurons. However, a single screen can identify both positive and negative selection phenotypes. For example, a viability screen on a cancerous cell line can unearth the depletion of oncogenes but also the enrichment of tumour suppressor genes. Therefore, after selection, a comparison to the sgRNA representation before selection will reveal hit genes, the strongest gene candidates will be those which have been selected by multiple sgRNAs.

1.4.7 Use of Flow Cytometry and FACS in screening

The fundamental approach of a pooled screen is the ability to separate the cell of the desired phenotype from a general population. Fluorescence-activated cell sorting (FACS) is an effective method of achieving this when paired with fluorescent markers (Ma *et al.*, 2017b). Some screens may employ the use of a fluorescent gene downstream of a gene of a specific phenotypic trait, therefore when the sgRNA perturbations are applied the cell positive or negative for the specific trait will be distinguishable by fluorescence. Such techniques have been widely utilised including in a study of the cattle herpes virus BoHV-1 (Tan *et al.*, 2020b). Others may simply use a protein marker displayed on the cell surface which may include fluorescent pathogens (Li *et al.*, 2020). These screens are dependent on a reporter assay which can amplify or detect with adequate sensitivity to distinguish at least two populations (Yu and Yusa, 2019).

1.4.8 Problems with CRISPR-Cas9 and their associated screens

The high throughput screens niche was occupied by RNAi for example, to study cancer gene dependencies. CRISPR-Cas9 quickly established itself as the method of choice for gene editing mainly due to the easy adaptability of the 20-bp protospacer sequence that could be designed to target any desired sequence, which led the way to CRISPR being implemented in genome-wide screens. When directly comparing RNAi and CRISPR dropout cancer screens the CRISPR methodology has been shown to identify a significantly higher number of essential genes and shows a significantly lower false-negative rate, therefore providing a more comprehensive analysis (Munoz *et*

al., 2016). Although CRISPR is the technology of choice for gene editing and screens, knockouts generated with standard Cas9 and CRISPR-Cas9 screens have several disadvantages and considerations worth thought:

- Paracrine effects are inherent in a pooled format since cross-talk between cells cannot be controlled. A particular gene KO phenotype may not be easily displayed due to compensatory effects from neighbour cells. Thus, a genome-wide screen may not embody a genotype-phenotype analysis of all genes (Ford *et al.* 2019).
- Pooled screens also limit the phenotypes that can be investigated for proliferation and survival although this can be expanded with the use of FACS that can sort fluorescent makers and cell surface markers (Ford *et al.* 2019).
- Essential genes cannot be studied since KO will cause the death of the cells being studied.
- It is possible that INDELS do not cause a frameshift mutation or fail to alter protein function or production sufficiently to alter gene function. Costa *et al.* (2017) suggest this maybe as high as 1 in 3.
- When phenotypic redundancy is present cells may not express a particular phenotype.
- When a gene is naturally not expressed at rest, the phenotype cannot be devolved.
- A caveat to these screens is that only a few have used culture conditions reflective of the solid-state tumour they represent.
- Mutation relies on host repair mechanisms to generate INDELS, therefore targeted mutations to genes involved in these processes may reduce the editing efficiency.
- Using primary cells is challenging due to the tens of millions of cells required for a CRISPR screen, therefore screens lend themselves to studying cell lines derived from tumours.
- Non-mendelian diseases and synthetically lethal genes cannot be identified with standard pooled screening. Most human diseases are polygenic, therefore dual CRISPR-Cas9 KO systems are in development (Wong *et al.*, 2016).
- Pooled screens often require weeks to complete, allowing time for selection to complete, during which the population of cells is passaged several times to maintain the relative representation of sgRNA (Doench, 2018).
- KO studies fail to uncover the gain of function mutations.
- A large segment of the genome is made up of introns. These regions often contain enhancers and regulatory elements, for example, non-coding RNA. For the most part, these regions are understudied, but in cancers, these sequences of DNA are known to be dysregulated (Huarte, 2015). Oncogenes can be regulated by these enhancers

(Datlinger *et al.* 2017) and long non-coding RNA (lncRNA) which when perturbed can show altered proliferation phenotypes (Zhu *et al.*, 2016).

- Regularly, cancer cell lines are aneuploid, often having an enhanced number of chromosomes over the WT; therefore, cells can experience non-targeted toxicities from CRISPR-Cas9 KO editing (Wang *et al.*, 2015). Furthermore, aside from the study of cancerous cell lines the use of immortalised cells prevents robust translation to physiological conditions, as the unregulated division may mask certain phenotypes, for example, the accumulation of a protein and clonal variability. This lack of continuity in the transition from *in vitro* to *in vivo* studies has led some to perform *in vitro* screens before moving to primary cell screens (Chen *et al.*, 2017, Kramer *et al.*, 2018).
- sgRNA barcodes used for gene identification are not without fault. sgRNAs which have been integrated may not have necessarily induced INDEL mutation. Nonetheless, with the high efficacy of CRISPR-Cas9 recruitment and enzymatic activity, it is assumed all target genes are KO with well-designed sgRNAs. Despite this, most downstream analyses will only shortlist genes where more than one sgRNA has produced the same phenotype. This is due to the lack of understanding of sgRNA off-target effects and their efficiency in the setting of a large library (Agrotis and Ketteler, 2015). If well-studied genes are known to impact the phenotype of interest these can be useful internal controls or if absent internal controls can be added to measure the success of a screen.

Considering the diversity of protocols and reagents now available for CRISPR Cas9 knock-out screens one may question the reproducibility of such screens. Recently Dempster *et al.* (2019) compared two pan-cancer CRISPR-Cas9 gene dependency studies. Despite the two studies being carried out at separate institutes using their own set of personnel and using significantly different protocols and reagents the study found a significant level of concordance between results. To further this work through repeats at both institutes it was identified experimental repeats are most greatly influenced by the sgRNA library and selection (Dempster *et al.*, 2019). Therefore, CRISPR-Cas9 genome-wide knockout studies are a reproducible and robust method for the identification of essential genes in cancer.

1.4.9 CRISPR screen analysis

Like many other studies we implemented a pooled CRISPR-Cas9 screen methodology to collate hit genes and subsequent experiments to validate each hit, often tailored to the functionality of that specific gene. Most commonly, 2 populations are compared, the treated population whereby gene knockout, inhibition or activation has taken place and been selected for

a specific phenotype. In contrast, the control population is either defined as a population whereby no CRISPR effect had taken effect or is induced, and the antagonist of the desired population is taken. The control population is usually the initial or earliest time point population (Wang *et al.*, 2014, Wang *et al.*, 2015). The guides targeting essential genes will be depleted in a time-dependent manner with genes having little impact on viability only dropping out after 2-3 weeks of selective culture, the point of maximum separation. On the contrary, if a selective marker is used these sgRNAs will be enriched in the cell population.

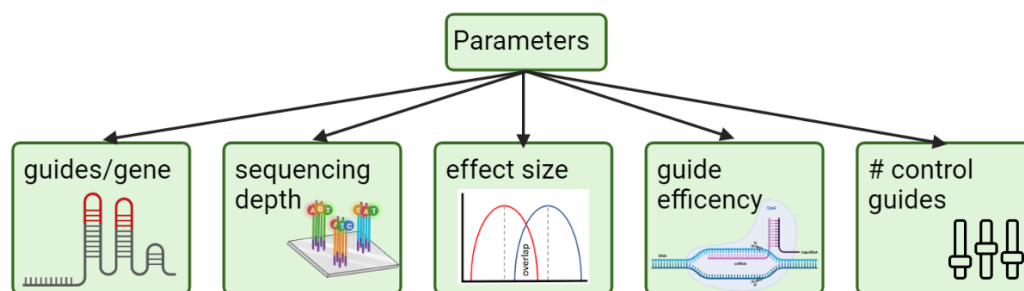


Figure 5. Overview of parameters that can be varied in a CRISPR dropout screen analysis. The number of guides per gene can vary between libraries, often a minimum of 4 per gene are used but up to 10 can be used to improve the statistical power of downstream analysis. The higher the sequencing depth can increase the likelihood of identifying hit genes, particularly those with lesser effect on the phenotype of interest. The effect size shows how meaningful a relationship is between two variables or the difference between two groups. The larger the effect size the more likely that observation is likely to be real. Therefore, altering the effect size parameters can vary the number of hits collected in a screen. Guide efficiencies are maximised to limit the off-target effects and the number of guides required per gene to gain statistical significance. Control guides targeting non-essential, or no regions of the genome can be added to improve statistical analysis. Adapted from Bodapati *et al.* 2020. Created in Biorender.

Screens are typically summarised by a normalised log fold change statistic for each gene, calculated from the abundance of that sgRNA in the treated population compared to the abundance in the control population and normalised such that the majority (mode or mean) log fold change is 0. This assumes that most genes do not correlate with the phenotype (Bodapati *et al.*, 2020). Normalisation can be further enhanced using internal non-targeting control guides which are often included in sgRNA libraries and are intended not to cut anywhere in the genome; comparative studies suggest greater than 300 are used in most cases (Bodapati *et al.*, 2020). A set of essential and non-essential genes has been established as a reference control for human cell lines, helping to assess the performance of these

positive and negative control guides (Hart *et al.*, 2014). However, their use in livestock animals has not been investigated.

Challenges remain in analysis, such as sgRNA efficiency, possibly a consequence of local sequence effects or epigenetic factors (Isaac *et al.*, 2016). This caveat is often combated using multiple (4-10) sgRNAs per target gene and increasing the breadth of PAM sites that can be used (Cui *et al.*, 2018). Other biases include varying gene effect sizes which can lead to only the identification of hits with large effect size and cell death from excessive cutting in copy number regions (Meyers *et al.*, 2017, Daley *et al.*, 2018). A smaller effect size maybe seen where only subtle variations are seen in a screen, thus would require a greater number of guides and a higher sequencing depth. These are just a few factors that complicate analysis although CRISPR screen data analysis algorithms do aim to combat some of these issues.

Another consideration when screening is the sequencing depth applied. A low sequencing depth will correlate to a low signal-to-noise ratio; thus it is generally recommended to use a sequencing depth of >500 reads per sgRNA in the library, although evidence suggests 25 reads per guide maybe sufficient (Joung *et al.*, 2017, Bodapati *et al.*, 2020). Both sequencing depth and the number of guides per gene can influence false discovery rates. However, in this regard sequencing depth is of less importance compared to the use of several sgRNAs, which will improve both technical and biological variation rather than technical alone, thus improving statistical power (Bodapati *et al.*, 2020).

False positive candidates have been attributed to CRISPR-induced DSB followed by lentiviral integration that contains the promoter elements of the vector into the genomic target location leading to upregulation of the target gene (Manjon *et al.*, 2022).

One programme widely used for CRISPR screen analysis for essential genes is Model-based Analysis of Genome-wide CRISPR/Cas9 Knockout (MAGeCK) (Li *et al.*, 2014). This method takes raw read counts and fits them to a negative binomial model to test if the initial sgRNA counts vary significantly from the end condition counts. Subsequent sgRNA-level *p*-values are collated at the gene level using a ranking algorithm and return a list of genes with their false discovery rates. MAGeCK has been used extensively across CRISPR screening methodologies and is well-maintained with regular updates. Additionally, in a recent review and a comparative analysis across several CRISPR screen data analysis software packages, MAGeCK was recommended (Bodapati *et al.*, 2020, Hanna and Doench, 2020). In addition to gene hit analysis and ranking, the data from genes enriched/depleted can be assimilated via their pathway interactions. Allowing collection of data on the enrichment or depletion of specific biological pathways thus ranking of pathways in terms of their essentiality can be

performed, for example, gene set enrichment analysis (GSEA) (Subramanian *et al.*, 2005, Liberzon *et al.*, 2015) or STRING (Szklarczyk *et al.*, 2017).

It is clear computational techniques dominate the CRISPR screens from the design of guides to the interpretation of the sequencing data. Although much of the computational workflows have become more standardised and seamless to perform it is essential to remain critical of the limitations this software may have. Since many choices exist, care in these decisions has the potential to increase the power and outcome of a screen.

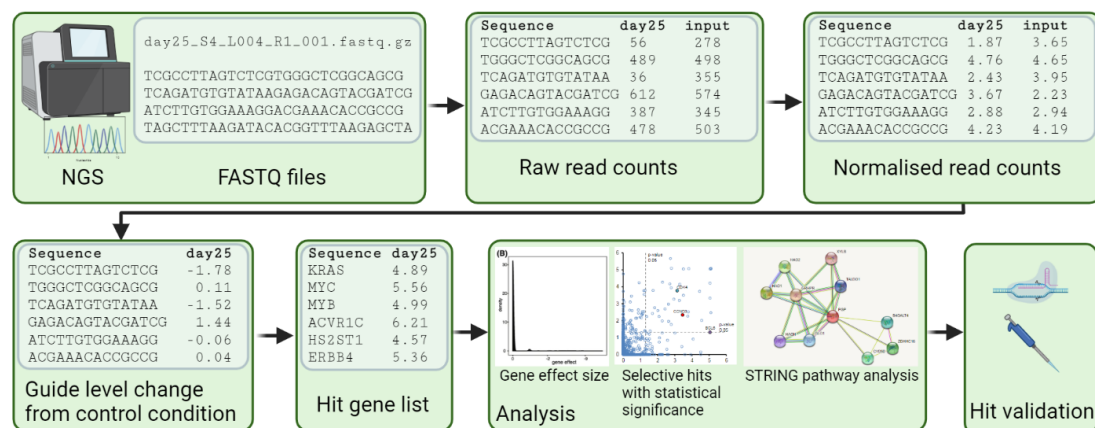


Figure 6. Workflow example for analysis of pooled CRISPR/Cas9 screens. Next-generation sequencing (NGS) returns FASTQ files for each sample. Initially, a sequencing run with multiple samples required demultiplexing to obtain FASTQ files associated with each sample. The number of reads per guide is counted and normalised by log transformation and comparison to control guides in the library. Guides are then matched to their corresponding gene, ranked, and hit genes identified. Further analysis can be performed for example to identify enriched/depleted pathways. Finally, it is recommended to validate hits through a smaller targeted pooled screen or investigations into specific gene genotype-phenotype relationships. Figure generated in BioRender and adapted from (Hanna and Doench, 2020).

1.4.10 CRISPR screens to identify viral determinates of infection

The toll of viruses on worldwide health has been clear, especially highlighted through the recent pandemic of SARS-CoV2 and epidemics such as Zika virus and Ebola virus in the last decade. Enhancing our biological understanding of viral host interactions is critical to identify anti-viral therapeutic targets or improving vaccinology campaigns. The vast improvement of CRISPR-Cas9 screens over previous technology (RNAi) has

permitted CRISPR screens to investigate viral infection. The majority of the initial CRISPR screens used cytopathic virus to identify genes associated with infection and viral replication, utilising survival as a phenotypic readout, leading to the enrichment of viral-resistant cells (Ma *et al.*, 2015, Haga *et al.*, 2016, Marceau *et al.*, 2016, Orchard *et al.*, 2016, Savidis *et al.*, 2016, Zhang *et al.*, 2016, Kim *et al.*, 2017b, Lin *et al.*, 2017). Some of these screens also used flow cytometry to enrich for viral resistant cells using a virus-activated reporter gene (Heaton *et al.*, 2017, Park *et al.*, 2017). Many of these screens recovered hits of known receptors and membrane-localised proteins associated with viral binding and entry. These established factors involved in viral infection provide strong positive control candidates and provided proof the methodology is effective at isolating host cell gene dependencies from viral infection.

1.4.11 CRISPR screens in livestock species

CRISPR screens in livestock species have been slower to establish due to reagents, protocols and sgRNA libraries being first optimised for use in humans; it has taken time to design methodologies compatible with livestock species. Nonetheless, publications of CRISPR screens in livestock species have been increasing since 2020. To our knowledge, there are no CRISPR screens have been conducted in chickens or chicken cell lines. However, CRISPR screens have been conducted on other livestock species, including cattle and pigs. One of the first livestock CRISPR screens was in cattle and aimed to investigate important host genes for Bovine Herpes Virus Type 1 (BoHV-1) replication (Tan *et al.*, 2020b). To do so Tan *et al.* (2020) utilised GFP-tagged BoHV-1 virus and FACS sorting to sort cells which inhibited infection and cells that permitted higher levels of viral infection. The screen identified the two membrane tethering complexes EARP and GARP and when reduced in MDBK cells BHV-1 virion production was reduced. Aside from the aforementioned study to date no other study has used CRISPR screen to investigate cattle.

Pig screens are more numerous, with 3 studies published to date. The first of these established a knockout library for screening functional genes using diphtheria toxin as the selective agent to induce cell death. Their study identified previously validated genes involved in diphtheria toxin resistance providing proof of principle for the use of a genome-wide knockout library in pig cells (Yu *et al.*, 2022). The second study to utilise the CRISPR screen in pigs was that of Tse *et al.* (2022) who applied a CRISPR library to pig cells followed by swine acute diarrhoea syndrome coronavirus (SADS-CoV) infection to identify the host entry factors. The screen uncovered placenta-associated 8 protein (PLAC8) as an essential host factor for SADS-CoV infection (Tse *et al.*, 2022). Another study used a similar approach but used Porcine reproductive and respiratory syndrome virus (PRRSV) as the

selective agent. The study found several genes including KxDL Motif Containing 1(KXD1), Proteasome 26S Subunit, Non-ATPase 3 (PSMD3) and Galectin 2 (LGALS2) to be enriched post selection. Together these studies of CRISPR screens in livestock have been successful in proving the benefits of genetic screens in identifying novel host determinates for viral infection.

1.5 Aims of PhD

The oncogenic viruses MDV and ALV are a significant threat to the commercial poultry industry necessitating the use of vaccines and resistant poultry strains. The outcome of these infections is the formation of lymphomas which culminate in reduced weight gain or egg production if not mortality. Research on these viruses has been extensive with a focus on viral pathogenic genes which includes several viral oncogenes. However, much less is known about the contribution of host genetics to viral replication, spread and oncogenesis. CRISPR-Cas9 screening techniques allow the unbiased analysis of many genes simultaneously for various phenotypes and as of now few CRISPR screens have been performed on viruses and even fewer in chickens and none in relation to MDV and ALV infection. Therefore, the main aim was to perform genome-wide screens on host cells and viral genes to uncover genes involved in viral infection and oncogenicity.

MDV has been extensively studied for genes that are critical in MDV oncogenesis and to a lesser extent viral replication. Many inferences made on the genes required for viral replication have been sourced from other herpes viruses such as the *alphaherpesvirus* counterpart HSV-1. Since the advent of CRISPR-Cas9 gene editing it is now much more feasible to knock out genes on a wide scale and screen for a chosen phenotype. Given this new technology and the fact no systematic analysis had ever been performed on gene essentiality for viral replication and spread *in vivo*. It was aimed to complete an arrayed CRISPR screen to identify and rank the MDV viral genes in terms of essentiality for viral replication and spread in cell culture. Once the screen had identified both essential and non-essential genes, I aimed to validate essential genes by making viral genetic deletions in MDV latently infected transformed cell line MSB-1 and subsequent triggering of MDV lytic reactivation. The more essential genes are the less likely lytic reactivation would occur. It was also planned to validate non-essential genes through CVI988 viral gene deletions, since a non-essential gene deletion in CVI988 would not be suspected to effect CVI988 growth in cell culture.

MDV primary target is CD4⁺ T-cells, MDV will subsequently form a latent infection resulting in the transformation of these cells. These MDV transformed T-cells are used extensively in MDV research, two of these such cell lines are named HP8 and MSB-1. Much is already known about viral genes that contribute to the oncogenic phenotype of transformed T-cells for

example the Meq oncoprotein. However, much less is known on how the hosts genes contribute to the transformation of MDV latently infected cells. Therefore, it was aimed to screen HP8 cells using a GeCKO approach aiming to uncover the essential genes required for the transformed phenotype. Once genes had been identified which were both essential and specific to HP8 cells I aimed to validate these findings outside of a pooled screen through CRISPR-Cas9 gene perturbations and proliferation measurements. Additionally, we aimed to compare the finding of our study with those of Ma *et al.* (2019) who screened latent EBV transformed cell lines.

After the first two screens were preformed, it was investigated how CRISPR-Cas9 screens could be used to study other avian oncogenic viruses and their hosts. Another oncogenic pathogen of Chickens is ALV which also cause T-cell lymphomas. Several sub-type of ALVs exist where their tropism is determined by specific cellular receptors. Aside from these receptors little is known surrounding their requirement of host genes for entry and replication. ALVs effectively infect DF1 cells in cell culture, the lab based strains RCAS viruses have been previously tagged with GFP. Therefore, it was aimed to use a GeCKO screen approach in combination with FACS to investigate the essential genes required for the infection and replication of RCAS viruses. Subsequently, I aimed to validate any genes of interest through CRISPR-Cas9-mediated editing in DF1 cells outside a pooled setting.

The final project thesis aim involved the investigation of the gga-miR-21 Meq interaction. It is well established that Meq is the major oncoprotein of MDV and is largely responsible for the transformation of T-lymphocytes. Previously it has been proposed that gga-miR-21 is upregulated in MDV infected tissues *in vivo*, and is present a higher levels in virulent MDV compared to a vaccinal MDV strain (Stik *et al.* 2013). Therefore, we aimed to further investigate whether Meq interacts with gga-miR-21 promoter to elicit its upregulation in the MDV transformed T-cell line HP8 and weather this interaction is involve in the continued proliferation of HP8 cells.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Media

No.	Media	Components	Source	Usage
1	CEF media	SQ water	The Pirbright Institute Central services unit	CEF culture
		10 % 10x 199 (+glutamine)	Gibco, 21180-021	
		10 % Tryptose phosphate broth	Gibco,11500546	
		Heat (56°C) inactivated foetal calf serum, 10 % for growth media or 5 % for maintenance media	Sigma-Aldrich, F0926 (HI FCS)	
		2.7% 7.5% sodium bicarbonate	Sigma-Aldrich, S8761	
		1 % 100x penicillin and streptomycin	Gibco 15140-122 (10,000 U/ml)	
2	DF1 media	Dulbecco's modified eagle's medium (with 4500 mg/L glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate)	D6429-500ML	DF1 cell culture
		1% 100x penicillin and streptomycin	Gibco, 15140-122 (10,000 U/mL)	
		10% (or 5%/2%) heat (56°C) inactivated Foetal Bovine Serum	Sigma-Aldrich, F0926	
3	MSB-1 Media	RPMI-1640	Sigma-Aldrich, R8758	HP8 and MSB-1 cell culture
		10% Tryptose phosphate broth (TPB)	Fisher Scientific, 11500546	

		10% heat (56°C) inactivated Foetal Bovine Serum	Sigma-Aldrich, F0926	
		1% 10mM Sodium pyruvate	Sigma-Aldrich S863	
		0.1 % 50mM 2-mercaptoethanol	Gibco 31350	
		1% 100x penicillin and streptomycin	Gibco 15140-122 (10,000 U/mL)	
4	Freezing media	80 % of DF1 / media/MSB-1	N/A	DF1, HP8 and MSB-1 cells
		10 % heat (56°C) inactivated Foetal Bovine Serum	Sigma-Aldrich F0926	
		10 % Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich D8418	
5	OptiMEM	OptiMEM Reduced Serum Medium	Gibco, 11058-021	Transfections and electroporation
6	LB broth	Luria Bertani (LB) broth	Gibco, 10855021	Bacterial culturing
		100 µg/mL Ampicillin	Sigma-Aldrich-A5354	
7	SOC media	Super optimal broth with catabolite repression	Invitrogen 15544034	Bacterial transformation
8	SOB media	Tryptone (20g per L)	Oxoid, LP0042	Bacterial culturing
		Yeast extract (5 g per L)	Oxoid, LP0021	
		Sodium chloride (0.5 g per L plus extra to adjust pH to 7)	Alfa Aesar, A12313	
		Potassium Chloride, 1M (2.5 mL per L)	Sigma-Aldrich, 60142	
		Magnesium Chloride (added after autoclaving)	BDH, 101494V	
9	LB agar plate	Luria Bertani (LB) broth	Gibco 10855021	Agar plates

		100 µg/mL Ampicillin	Sigma-Aldrich, A5354	
		Select Agar	Invitrogen, 30301049	

Table 2. List of Media

2.1.2 Reagents

No.	Reagent	Component	Source	Usage
1	PBSa	Ca ⁺² - Mg ⁺² free phosphate buffered saline (PBS), pH 7.4	The Pirbright Institute Central services unit	Cell washes, membrane washes and as a control solution
2	PBST	PBSa, Tween (20) 1%	Sigma-Aldrich, P3563	Membrane wash
3	Versene	(0.53 mM) aqueous solution of the tetrasodium salt of ethylenediaminetetraacetic acid Na ₄ EDTA	The Pirbright Institute Central services unit	Cell trypsinization
4	Trypsin	2.5 % Trypsin	Gibco, 15090046	Cell trypsinization
5	4 % PFA	4 % Paraformaldehyde (PFA) solution in PBS	Chem Cruz, SC-281692	Cell fixation
6	DMSO	Dimethyl Sulfoxide	Sigma-Aldrich, D8418	Cryopreservation
7	Puromycin	1 mg/µL puromycin	Sigma-Aldrich, P8833	Transfection and lentivirus transduction selection
8	Ampicillin	100 µg/mL Ampicillin	Sigma-Aldrich, A5354	Bacterial selection
9	Blasticidin S HCl solution	10 mg/mL Blasticidin S HCL	Santa Cruz, CAS 3513-03-9	
10	Squishing Buffer	10 mM Tris-HCl pH 8.2	Sigma-Aldrich, 93622	Genomic DNA extraction
		1 mM EDTA, 25 mM NaCl	Sigma-Aldrich,	

			324504	
		200 µg/mL Proteinase K	Sigma-Aldrich, P5568	
		25 mM NaCl	Sigma-Aldrich, S5150	
		Deionized water	The Pirbright Institute Central services unit	
11	Agarose gel	UltraPure™ TBE Buffer, 10X (1X TBE in deionised water)	Invitrogen, 15581044	Gel electrophoresis
		DNA Agarose	Invitrogen, 16500500	
12	Agarose gel stain	GelRed™ Nucleic Acid Gel Stain (1X in deionised water)	Biotium, BT41003	Nucleic acid visualisation
13	DNA Ladder	1 kb Plus DNA Ladder	New England Biolabs, N3200	DNA base pair marker
14	Gel Loading Dye	Gel Loading Dye, Purple (6X), no SDS	New England Biolabs, B7025	DNA gel visualisation dye
15	Trypan Blue	Trypan Blue solution	Sigma-Aldrich, T8154	To count viable and dead cells
16	Polybrene	Polybrene Infection / Transfection Reagent	Sigma-Aldrich, TR-1003	To enhance infection
17	Lipofectamine 2000 reagent	N/A	Thermo Fisher, 11668027	Eukaryotic cell transfection reagent
18	TransIT-X2® Dynamic Delivery System	N/A	Mirus, MIR 6004	Eukaryotic cell transfection reagent

19	TransIT®-Lenti Transfection Reagent	N/A	Mirus, MIR 6600	Eukaryotic cell transfection reagent
20	Sodium Butyrate (NaB)			To induce lytic reactivation in HP8 and MSB-1 cells
21	MCL1 inhibitor	A 1210477	Tocris, 6409	Mitochondrial inhibitor
22	Metformin		Tocris, 2864	Mitochondrial inhibitor
23	Chloramphenicol		Sigma, R4408	Mitochondrial inhibitor
24	7-AAD		Abcam, AB228563	Necrosis Dye for Flow cytometry
25	Annexin V		Fisher Scientific, R37174	Apoptosis Dye for Flow cytometry

Table 3. List of Reagents

2.1.3 Enzymes

No.	Enzyme	Source	Buffer for reaction used	Source	Usage
1	Bbs-I	New England Biolabs, R3539	1X rCutSmart Buffer	New England Biolabs, B6004	sgRNA CRISPR-Cas9 cloning
2	HindIII-HF	New England Biolabs, R3104	1X rCutSmart Buffer	New England Biolabs, B6004	Concentrating sgRNA gDNA in HP8 CRISPR screen
3	Go Taq Polymerase (Promega M300)	Promega, M712	N/A		PCRs
4	LongAmp® Taq DNA Polymerase	New England Biolabs, M0323	1X Longamp Taq Buffer	New England Biolabs, B0323	PCRs
5	Q5 high-fidelity polymerase	New England Biolabs, E0555	1X Q5 reaction buffer	New England Biolabs, B9027	PCRs

6	NEBNext® Ultra™ II Q5® Master Mix	New England Biolabs, M0544	N/A		PCRs
7	Luna® Universal qPCR Master Mix	New England Biolabs, M3003	N/A		Sybr Green qPCR
8	ABsolute Blue® q-PCR Low Rox master-mix	Thermofisher, AB4318B	N/A		Primer-Probe qPCR

Table 4. List of Enzymes

2.1.4 Cells and Cell Lines

No.	Cells/Cell Lines	Description	Source	Cell Type	Culture conditions	Biosafety Level
1	Chicken Embryonic Fibroblasts	Primary embryonic chicken fibroblasts, prepared from 10-day-old Valo SPF embryos	The Pirbright Institute	Primary adherent fibroblasts	CEF media Incubated at 38.5 °C in 5% CO ₂	1
2	DF1 cell line	Naturally immortalised chicken fibroblast cell line	The Pirbright Institute	Adherent fibroblasts	DF1 media Incubated at 38.5 °C in 5% CO ₂ .	1
3	MSB-1 cell line	Chicken lymphoblastoid cell line. Derived from spleen lymphoma Transformant : <i>Gallid herpesvirus 2</i> (Marek's disease virus) (strain bc-1)	The Pirbright Institute	Suspended transformed T-cell line	MSB-1 media Incubated at 38.5 °C in 5% CO ₂	2

		(Nazerian and Witter, 1975).				
4	HP8 cell line	Chicken lymphoblastoid cell line. Transformant : <i>Gallid herpesvirus 2</i> (Marek's disease virus) (strain GA) (Akiyama and Kato, 1974).	The Pirbright Institute	Suspended transformed T-cell line	MSB-1 media Incubated at 38.5 °C in 5% CO ₂	2

Table 5. Cells and Cell Lines.

2.1.5 Plasmids

No.	Plasmids	Description	Source	Usage
1	pSpCas9(BB)-2A-Puro (PX459) V2.0	Cas9 from <i>S. pyogenes</i> with 2APuro, and cloning backbone for sgRNA (V2.0)	Addgene, Plasmid #62998	CRISPR/Cas9 editing
2	pX330A-1x2	Cas9 from <i>S. Pyogenes</i> with 2X cloning backbone for sgRNA	Addgene, Plasmid #58766	CRISPR/Cas9 editing
3	pcDNA-EGFP	Green fluorescence expression	Addgene, Plasmid #13031	Control for Transfections
4	lentiCas9-Blast	<i>S. pyogenes</i> Cas9 protein and blasticidin resistance. For use with 3 rd generation lentivirus systems	Addgene, #52962	Cas9 cell lines
5	pCMV-VSV-G	Envelope protein for producing lentiviral and	Addgene, #8454	Lentivirus production

		MuLV retroviral particles		
7	pCMV-HIV-gag/pol	Lentiviral packaging vector	Addgene, #12260	Lentivirus production
8	psPAX.2	lentiviral packaging plasmid	Addgene, #12260	Lentivirus production
9	pMD2.G	VSV-G envelope expressing plasmid	Addgene, #12259	Lentivirus production
10	pKLV2-U6sgRNA5(gBFP)-PGKGFP2ABFP-W	Cas9 activity reporter with GFP and BFP	Addgene, # 67984	Cas9 cell activity test

Table 6. List of Plasmids.

2.1.6 Molecular Kits

No.	Molecular Kit	Source	Usage
1	Monarch® Genomic DNA Purification Kit	New England Biolabs, T3010	Genomic DNA (gDNA) extractions
2	QIAquick PCR Purification Kit	Qiagen, 28104	PCR purification
3	QIAprep Spin Miniprep Kit	Qiagen, 12123	Plasmid preparations
4	QIAGEN Plasmid Midi Kit	Qiagen, 12143	Plasmid preparations
5	QIAGEN Plasmid Maxi Kit	Qiagen, 12162	Plasmid preparations
6	RNeasy Micro Kit	Qiagen, 74004	Cell RNA extraction
7	DNeasy Blood & Tissue Kit	Qiagen, 69504	Cell DNA extraction
8	Applied Biosystems™ High-Capacity RNA-to-cDNA™ Kit	Fisher Scientific, 4387406	cDNA preparation from RNA
9	Luna® Universal qPCR Master Mix	New England Biolabs, M3003	Sybr Green qPCR

Table 7. Molecular Kits.

2.1.7 Primers

2.1.7.1 General Primers

No.	Amplification region	Primers 5' → 3' (F – Forward, R - Reverse)	Size (BP)
1	Cas9	F- CATCGAGCAGATCAGCGAGT	275

	R- CGATCCGTGTCTCGTACAGG	
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Table 8. General Primers.

2.1.7.2 CRISPR-Cas9 Screen PCR Primers

1st PCR Primers	
Primer name	Sequence (5'-3')
Forward 1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCTTGTG GAAAGGACGAAACACCG
Forward 2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATCTTGT GGAAAGGACGAAACACCG
Forward 3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGATCTTG TGGAAGGACGAAACACCG
Forward 4	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGATCTT GTGGAAAGGACGAAACACCG
Forward 5	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCGATCT TGTGGAAAGGACGAAACACCG
Forward 6	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATCGATC TTGTGGAAAGGACGAAACACCG
Forward 7	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGATCGAT CTTGTGGAAAGGACGAAACACCG
Forward 8	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGATCGA TCTTGTGGAAAGGACGAAACACCG
Forward 9	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACGATCG ATCTTGTGGAAAGGACGAAACACCG
Forward 10	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTACGATC GATCTTGTGGAAAGGACGAAACACCG
Reverse 1	GTCTGGAGCATGCGCTTTAGCTGTCTCTTATACACATCTCC GAGCCACGAGAC
2nd PCR Primers	
Primer name	Sequence (5'-3')
ngs_l7_n 701	CAAGCAGAAGACGGCATAACGAGATTAAGGCGAGTCTCGT GGGCTCG*G
ngs_l7_n 702	CAAGCAGAAGACGGCATAACGAGATCGTACTAGGTCTCGTG GGCTCG*G
ngs_l7_n 703	CAAGCAGAAGACGGCATAACGAGATAGGCAGAAGTCTCGT GGGCTCG*G
ngs_l7_n 704	CAAGCAGAAGACGGCATAACGAGATTCCTGAGCGTCTCGTG GGCTCG*G
ngs_l7_n 705	CAAGCAGAAGACGGCATAACGAGATGGACTCCTGTCTCGTG GGCTCG*G
NGS_l5_ S502	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTTCG TCGGCAGCGT*C
NGS_l5_ S503	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCG TCGGCAGCGT*C

NGS_I5_S504	AATGATACGGCGACCACCGAGATCTACAC AGAGTAGATCG TCGGCAGCGT*C
NGS_I5_S517	AATGATACGGCGACCACCGAGATCTACAC GCGTAAGATCG TCGGCAGCGT*C
Note: 1st PCR primers (PCR1_F1 to PCR1_R) were ordered as regular oligos and index primers (idx_S502 to idx_N706) as Ultramers from IDT with the last base (*) phosphorothioated to prevent degradation. The barcodes are in bold.	

Table 9. CRISPR-Cas9 genome-wide screen primers.

2.1.7.2 CVI988 Screen Primers

MDV gene	Forward Primer	Reverse Primer	Unedited length	Edited length
MDV glycoproteins				
MDV-13/13.5	GCTGCTCGGTAGC ATGAAAA	ACGGTTGTACGCA ACTGTCT	732	425
MDV-22	GCCCTGGCTGAGT GTCTATC	GGATTGACTACCC CGCTGTT	1228	453
MDV34	ATTGAAACGCGTT GCGTACA	GCAAGTTGCATTG GATGGCT	1079	379
MDV57	TTGATCGTTGTTTC GAACCGC	ATAGCTGTGGCAT TCGTCGT	1258	348
MDV67	AACAGTGTTTTGC GGCCAAG	TCCGCGCAATGCA AATCATC	1175	415
MDV94	GGCGATGGAAGAA TCCCGAG	ACGACTGGGTGCT GCAATAA	802	387
MDV95	CAGATAGACGGCG TGTGTGA	ACTGCCTTGTGTT CGTGTCT	1185	319
MDV96	ATGGGACGAGGAC TGCAAAG	AAGCGGTATAACC CGAACCC	999	637
MDV40	ACACAGCATCATC TTCTGAGTCT	TGCACTATTTTAG GCGGAATTG	2595	1878/ 237
CVI988 screen				
MDV0.5/80.5	CCCCACCCCATC CAAAATA	CTGTGGGCCGGG GTCT	502	272
MDV5/76	AAGGGTCACCGTT AGGGGTA	CAGGGAGCAGAC GGACTATG	178	HRM
MDV-20	AACAAGCGAAAAT TCGGCGG	AAACCATATCAAC GATGGCAGC	842	163
MDV-21	ACCGATACCATTTT GGGACC	TAGACTATGCATC CAGCGCC	910	99
MDV-27	ATCGTTTAGGCGC GAGTCAT	ACAGCCCAACGTC GTATGAA	1035	249
MDV6.5(132 BP)/75.2	AAAGCACTACCGA AGTCGC	TGGAGGAATAAGG GGGTGGT	1298	1143

MDV-13/13.5	AGCTGCTCGGTAG CATGAAA	TGAAATATCGGCA GGCGCAA	479	172
MDV-28	GCGTGTCTTCTGTG GAGGGAT	AACAGGTGCTACG ACCTTACG	976	100
MDV31	GCAATGTCGTCCA TGTTCCG	GCGCCTACAGGTA GACTCTT	947	318
MDV37	CTCATGTGGCGTT TCCCTGT	TTCGTCGTTGCGT ATGCCTT	1100	198
MDV81.5/10 2.5	TTGAGTCAGGCAT TGCGGAG	TGTGGATCGGGAA TCGAGGA	248	137
MDV3/78	ATGCAGGCGTTGT TGCTAGT	AGCGTATTCCACA ACCTCCAG	110	HRM
MDV-2/79	CCGCTGTTTACTC GCTGACT	CTTATTGCGGAGC GTGCC	633	353
MDV84.5/99 .5	GAAGCTACGGTTC AAGTGCG	ATTTGCGGCTCTA GCGATGA	538	338
MDV3.4/78. 3	AATTTCCATTTCGAT GGGGCA	AGGGCGTATATAG CGCAAGC	505	197
MDV3.8/78. 1	ACAACAGTGGCAC ACATCAAAC	ACGAATATACAGG CACGGACC	411	174
MDV85/99	TCGACTAAGCACA AAAGGCTCT	TGCTTAACCGGCA GTACAGG	119	HRM
MDV56	TGCCAATTCAAAC GACACCG	GAAACACTCGCCC CCACTAA	1046	114
MDV-2.6/78.5	CATGAGCGTGCAC AAAGACG	GATTCTACCCGCA GGACTCG	268	152
MDV57.4	CGAGATGATCGTG CCAGTCA	AGTGTGGCTGGAT GCAATGA	315	139
MDV-19	CAACGCGAGGCAG AAGATTT	CATAGCGTTTGAT CACCGCAG	1349	663
MDV-11.5	AAATGTCAGCGTC CGCCTAA	TGCATTCAGCAGC AAGACCT	160	89
*genes that were targeted by a single guide high-resolution melt (HRM) curve analysis were used to access editing.				

Table 10. CVI988 gene screen primers.

2.1.7.3 qPCR Primers

Primer Target	Primer	Sequence (5'-3')
GAPDH	GAPDH-FP	AGAACATCATCCCAGCGTCC
	GAPDH-RP	CGGCAGGTCAGGTCAACA
c-MYC	c-MYC-F	CTCCCCAGCAAGAACTACGAT
	c-MYC-R	GCAGATGAAGCTCTGGTTGAC
c-REL	c-REL-F	CTGAACGTCGAGTCCTGTCTTTTCA
	c-REL-R	TCCACAGTTCTTATTCACACGGCAA
TP-63	TP-63-F	GATTGCACCTCCTAGCCACCTGATC
	TP-63-R	TGATGAGAATTGGGCGACGGTTCA
ICP4	ICP4-F	CGCCACACGAGAACACAATG

	ICP4-R	GGTTGGAGTAGAGCTGCAACT
gB	gB-F	GGTTCAACCGTGATCCGTCTA
	gB-R	CGATTCCTTCACCCCACT
pp38	pp38-F	GAAAACAGAAGCGGAATGCG
	pp38-R	CGATCCAAAGCGCTCATCTC
Meq	Meq-F	GGTCTGGTGGTTTCCAGGTGA
	Meq-R	GCATAGACGATGTGCTGA

Table 11. qPCR Primers.

Gene target and sgRNA reference number	sgRNA	5'→3' sgRNA cloning oligo	3'→5' sgRNA cloning oligo
TVA sg	CCGACTGCTACCC GCTGGAG	CACCGCCGACTGCTAC CCGCTGGAG	AAACCTCCAGCGGGTA GCAGTCGGC
TAOK1 sg4	ATTTGCGGGAGCA TACGGCA	CACCGATTGCGGGAG CATACGGCA	AAACTGCCGTATGCTC CCGCAAATC
TAOK1 sgB	TGATGTGCGCACC AACGAAG	CACCGTGATGTGCGCA CCAACGAAG	AAACCTTCGTTGGTGC GCACATCAC
LOC1070 49459 sg4	ATGGCATTATCA ATAAAGC	CACCGATGGCATTAT CAATAAAGC	AAACGCTTTATTGATAA ATGCCATC
PRPSAP 2 sg2	ATTTGCTGAACGT TTGCGGT	CACCGATTGCTGAAC GTTTGC	AAACACCGCAAACGTT CAGCAAATC
NF2 sg3	AGAAGCAACAATG GCCAACG	CACCGAGAAGCAACAA TGGCCAACG	AAACCGTTGGCCATTG TTGCTTCTC
CDKN2A sg3	CAGAATGCGGCG AAGCGTCC	CACCGCAGAATGCGGC GAAGCGTCC	AAACGGACGCTTCGCC GCATTCTGC
NANOG sg1	ATGACCCCTACAT TTCACCA	CACCGATGACCCCTAC ATTTCACCA	AAACTGGTGAAATGTA GGGGTCATC
BRAP sg3	GTGCGAGGGAGA TATGTGTC	CACCGGTGCGAGGGA GATATGTGTC	AAACGACACATATCTC CCTCGCACC
ASL2 sg1	TGTCTGAAGTTGA TATCCAG	CACCGTGTCTGAAGTT GATATCCAG	AAACCTGGATATCAAC TTCAGACAC
GSTK1 sg2	ACGTGGTATCTCC TTACTCC	CACCGACGTGGTATCT CCTTACTCC	AAACGGAGTAAGGAGA TACCACGTC
SLC9A1 sgA	CGGCGTCCAGAAT GATGGGA	CACCGCGGCGTCCAG AATGATGGGA	AAACTCCCATCATTCT GGACGCCGC
SLC9A1 sgB	GCCGGAGGCGAT GAGCCTAA	CACCGGCCGGAGGCG ATGAGCCTAA	AAACTTAGGCTCATCG CCTCCGGCC

IRF4 sg2	TGAATAGGTACCC AACTACA	CACCGTGAATAGGTAC CCAACTACA	AAACCTGTCTATCTGG TCGATCAGC
IRF4 sg4	CTGATCGACCAGA TAGACAG	CACCGCTGATCGACCA GATAGACAG	AAACTGTAGTTGGGTA CCTATTAC
IFR4 sgA	TGGGCACACAGTC TTTGTAG	CACCGTGGGCACACAG TCTTTGTAG	CACCGCACACAGTCTT TGTAGGGGT
TBRG4- sg2	TCAGGTGGTACGA CATGGCG	CACCGTCAGGTGGTAC GACATGGCG	AAACCGCCATGTCGTA CCACCTGAC
TBRG4- sg3	TCAGGTGTGGAAT AACACCT	CACCGTCAGGTGTGGA ATAACACCT	AAACAGGTGTTATTCC ACACCTGAC
TBRG4- sg4	TTCTGCCATGACA CCCATCG	CACCGTTCTGCCATGA CACCCATCG	AAACCGATGGGTGTCA TGGCAGAAC

Table 12. sgRNAs.

2.2 Methods

2.2.1 General Methods

2.2.1.1 Px459/Px330a plasmid cloning

All single-stranded oligos were delivered dry from Integrated DNA Technologies as ssDNA oligos with standard de-salting. These single-stranded oligos were annealed at 10 pmol/μl in a reaction mix constituting 10 pmol/μl of top strand oligo, 10 pmol/μl of the bottom strand, diluted in nuclease-free water. The annealing mix was incubated at 90 °C for 3 minutes and subsequently at 37°C for 1 h. The annealed oligos were designed so that when annealed overhangs of 5' CACC 3' and 3' AAAC 5' on either side are exposed which are complementary to the overhangs generated by plasmid PX459/Px330a restriction digested with BbsI. The Px459/Px330a contains restriction sites for the BbsI enzyme which linearises the plasmid generating overhangs of 5'-GAAGAC (N)2-3' on the upper strand and 3'-CTTCTG (N)6 - 5' on the bottom strand of the DNA respectively. Annealed oligos were ligated into cut Px459/Px330a plasmid using a ligation mix made up of 2 uL of each oligo, 1 μL T4 DNA Ligase, 2 μL T4 DNA Ligase Buffer (10X) and 100 ng of vector DNA, with the remaining topped up to 20 μL with nuclease-free water. The ligation mix was incubated at 37 °C for 2 h or until transformation.

2.2.1.2 Bacterial Transformation

All the bacterial transformations were performed using Subcloning Efficiency DH5α competent cells (Invitrogen, 18265017). Each transformation used 50

µl of competent cells (revived from -80°C) and thawed on ice. Plasmid DNA of the ligation mixture (10 ng – 100 ng) was added to the thawed cells and incubated on ice for 30 mins. Subsequently, the mixture was incubated at 42 °C for 1 min (heat-shock) and immediately transferred onto ice for 3 mins incubation before warmed SOC medium was added to the mixture, which was then incubated at 37 °C with shaking (at 220 RPM) for 1 h before plating onto pre-warmed LB agar plates containing 1 µg/mL Ampicillin and incubated overnight at 37 °C. The following day bacterial colonies were picked, and a colony PCR was performed using specific primers in tables 11 and 12. Once confirmed by PCR the specific plasmid-containing bacteria were propagated in LB broth containing 1 µg/mL Ampicillin. The bacterial cultures were harvested using the QIAGEN® Plasmid Mini, Midi and Maxi Kits, following the manufacturer's suggestions.

2.2.2 Cas9 Cell Construction

2.2.2.1 Lentivirus Production

Seed T-75 flask with 6.6×10^6 low passage 293FT cells and allow to adhere overnight at 37 °C 5 % CO₂. A transfection mixture was prepared by adding 6 µg lentiCas9-Blast, 1.2 µg pCMV-VSV-G, 4.8 µg pCMV-HIV-gag/pol, 36 µL Lipofectamine 2000 (Invitrogen) to 2 mL Opti-MEM (Invitrogen). Next, the total 7 ml 5% FCS containing Pen-Strep free DMEM was added slowly and the 1 ml of plasmid DNA-Lipofectamine mixture was added dropwise on top of the cells. After 24 h incubation at 37 °C 5 % CO₂, the media was changed to complete DMEM and incubated at the same conditions for a further 24 h when the first harvest was taken and an additional 24 h until the 2nd harvest. The supernatant was collated and spun down at 500 x g for 5 mins and subsequently filter through a 0.44 µm PES filter to remove cells and cellular debris. Filtered lentivirus supernatant was stored at -80°C until use.

2.2.2.2 Lentivirus Cas9- Blasticidin Transduction

The lentivirus supernatant was added 1:2 to MSB-1 media with 1 million cells in a 6-well plate plus 8 µg/mL polybrene. Plates were spininfected at 800 x g 32 °C for 2 h and further incubated at 38.5 °C 5 % CO₂ for 48 h. Thereafter, 10 ug/ml or 12.5 ug/ml blasticidin containing MSB-1 media was used for up to 2 weeks post-selection or until untransduced control cells had died completely, indicated by trypan blue staining.

2.2.2.3 Selection of Cas9-positive clones

Expression of Cas9 was confirmed in the mixed pool of Cas9-blasticidin cells using Cas9-F and Cas9-R primers in PCR (Table 2.1.6). Once confirmed the population of cells was then single-cell sorted into 96 well plates and allowed to grow up into single-cell clones in complete media plus 12.5 ug/mL blasticidin. Around 20-30 of these clones were then investigated. First a Cas9 activity assay using reporter pKLV2-U6sgRNA5(gBFP)-PGKGFP2ABFP-W

(Addgene #67984) was used, whereby Cas9 negative cells will express GFP and BFP and Cas9 positive only GFP. pKLV2-U6sgRNA5(gBFP)-PGKGFP2ABFP-W lentivirus was produced using the method described above. In addition, the lentivirus was concentrated through ultracentrifugation. Filtered lentivirus supernatant was centrifuged in 36 mL centrifuge tubes at 90,000 x *g* for 2 h at 4 °C and resuspended in 3 mL media and subsequently stored at -80 °C. Cells were then spininfected at 800 x *g* 32 °C for 2 h plus 12 µL/ mL polybrene and further incubated at 38.5 °C 5 % CO₂ for 48 h. After 1-2 passages and 96 h post-transduction cells were analysed using MacsQuant flow cytometry for BFP/GFP expression. Cells where GFP expression was predominant, were selected. Western blot analysis was used to confirm the result.

For the western blot approximately 1×10^6 HP8 cells and the control cells were harvested and mixed with 10 µL TruPAGE LDS sample buffer (Sigma), 4 µL TruPAGE™ DTT Sample Reducer and 26 µL water and heated at 95 °C for 8 min. These samples were separated on a 4-12 % TruPAGE precast gel, and the resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. Expression levels of Meq were detected using an anti-Meq monoclonal antibody (MAb) FD7 (Brown *et al.*, 2006) along with a loading control α -tubulin (Sigma-Aldrich). Following probing with the primary antibody overnight at 4 °C, the blot was incubated with secondary antibody IRDye®680RD goat anti-mouse IgG (Li-Cor). Blots were visualised using Odyssey Clx (LI-COR).

2.2.2.4 Sybr green RT-qPCR

RNA was extracted from cells using the RNAeasy kit and was diluted to 10 ng/µL, of which 9 µL was used per 20 µL reaction for the High-Capacity RNA-cDNA kit following the manufacturer's protocol. Sybr Green Lunar 2x master mix was used with the primers listed in tables 9-11 to make a working stock of each primer set. 19 µL was then aliquoted into each well of a 96-qPCR plate and 1 µL cDNA was added, performed in triplicates. The plate was subsequently sealed, vortexed and centrifuged before being loaded into the Quantstudio QS5 machine where standard curve Sybr Green qPCR was run following Sybr Green Lunar qPCR protocol. Results were analysed using Delta Delta Ct ($\Delta\Delta Ct$) method.

2.2.2.5 Growth, apoptosis assays, qPCR assays

A-1210477, metformin and chloramphenicol were used as mitochondrial inhibitors; subsequently, the growth and apoptosis of HP8 cells were recorded. 200,000 cells were seeded in 12-well plates and treated with increasing concentrations of inhibitor (A-1210477 0-20 µM, Chloramphenicol 0-2000 µg/mL and metformin 0-120 nM). Each condition was matched with the inhibitors solvent, A-1210477 = DMSO, Chloramphenicol = Ethanol/Isopropanol (95:5) and metformin = water. The cells were cultured for 3 days post-treatment and growth was analysed every 8 h in the IncuCyte

live cell imager. After 3 days the cells were treated with Annexin V (Fisher Scientific, R37174) and 7-AAD (Abcam, AB228563) according to the manufacturer's recommendations and fluorescence was analysed using MacsQuant 10 machine after 30 min incubation in the dark at room temperature. AVOL-1 cells were enumerated after 72 h of treatment using a countess cell counter (Fisher Scientific). At the endpoint, cells were harvested, and RNA was extracted using RNeasy Micro Kit which was used in RT-qPCR, as described in 2.2.2.4.

2.2.2.6 RT-qPCR to analyse viral and host gene changes after mitochondrial inhibition

200,000 HP8 cells were seeded in a 6-well plate and cultured with the mitochondrial inhibitors chloramphenicol (500 µg/mL) (control: 5 µL ethanol/isopropanol 95:5), metformin (100 nM) (control: 160 µL water) and A1210477 (7.5µM) (control: 7.5 µL DMSO). Sodium Butyrate (2.5 nM) was used as a positive control, as well as known positive and negative MDV DNA samples (MDV reference laboratory, The Pirbright Institute). After 48 h of culture, the DNA was extracted from the cells using DNeasy Blood & Tissue Kit and Luna SYBR green qPCR was performed as previously described using pp38 primers to detect MDV genomes and GAPDH as an internal control.

2.2.3 CRISPR-Cas9 screening

2.2.3.1 CRISPR- CAS9 library amplification

The pKLV2-Chicken CRISPR library was synthesised by Genewiz and the Grey lab (Roslin Institute) using the CRISPR sgRNA expression vector pKLV2-U6sgRNA5(BbsI)-PGKpuro2ABFP-W (Tzelepis *et al.*, 2016) which possesses puromycin and BFP markers. This consisted of a library of ~71,000 guides and targeted 4 guides per gene. The pKLV2-Chicken was amplified by performing three reactions in total. Each consisted of 25 µL Endura ElectroCompetent cells (Lucigen, 60242) electroporated with 10 ng plasmid (1 µL of a 10 ng/µL stock) in a prechilled 0.1 cm electroporation cuvette (Bio-Rad, #165-2089). The cuvette was electroporated (Bio-Rad #165-2098) at 1.6 Kv, 25 µF and 250 Ω. Each reaction was diluted in 1974 µL recovery media (ThermoFisher, 15544034), transferred to a 15 mL tube, loosely capped and incubated at 37 °C and 220 RPM for 1 h. Next all three reactions were pooled and added to a 500 mL super optimal broth (100 µg/mL ampicillin) and incubated at 37 °C and 220 RPM for 15 h. To estimate transformation efficiency, 50 µL was diluted in 450 µL and plated to 10-cm dishes containing 100 ug/mL Ampicillin agar in serial dilutions to 10⁻⁶. Plates were incubated at 37 °C overnight and subsequently counted. Our estimations predicted >15 x10⁷ total colonies, corresponding to 500-1000 colonies per sgRNA, which is most optimal. Plasmids were then extracted and purified from the super optimal broth bacterial culture using the Qiagen Plasmid Maxi Kit (Qiagen, 12162) and following the manufacturer's instructions.

2.2.3.2 Lentivirus production

Low passage number (< 6) 293FT cells were plated at a density of 7.5×10^6 cells/T-150 flask and allowed to adhere overnight until >80% confluent at 37 °C 5 % CO₂. The amplified pKLV2-Chicken lentivirus was generated via co-transfection of the amplified sgRNA CRISPR CAS9 library, psPAX.2 and pMD2.G. The ratio of pKLV2-Chicken/pdPAX.2/pMD2.G was 30 µg, 25 µg, and 10 µg respectively per T-150 flask. The two reagents used were Lipofectamine 2000 and TransIT-Lenti.

For the Lipofectamine 2000 transfection, 150 µL Lipofectamine 2000 and 7 mL warmed OptiMEM, were mixed and incubated at room temperature for 20 mins (Table 13). Thereafter the transfection mixture was added to the flasks for 6 h incubation at 37 °C at 5 % CO₂ and the media was replaced with 25 mL fresh DF1 media (10 % FBS). The viral supernatant was collected at 72 h and 96 h.

An alternative method was also used whereby 90 µL of TransIT-Lenti was diluted in 3 mL warmed OptiMEM and mixed well (table 13). DNA was added to warmed OptiMEM, mixed and added to the TransIT-Lenti solution. 293FT cell media was replaced with 18 mL fresh DF1 media (10% FBS) per T-150 flask. The Transfection DNA mixture was added dropwise to the cells and incubated at 37 °C at 5 % CO₂. The viral supernatant was collected 48 h after transfection.

The viral supernatant was kept at 4 °C, pooled and filtered through a 0.22 µm PES filter. The resulting supernatant was concentrated through ultracentrifugation at 90,000 x g for 2 h at 4 °C, resuspended and finally stored at -80 °C. Alternatively, a lenti-x concentrator (TakaRa, 631232) was used following the manufacturers' recommendations.

	Media	Reagent
Tube A	3.5 mL OptiMEM	150 µL Lipofectamine 2000
	3 mL OptiMEM	90 µL TransIT-Lenti
Tube B	3.5 mL OptiMEM	30 µg pKLV2-Chicken CRISPR Library
		25 µg psPAX.2
		10 µg pMD2.G (VSV-G)
	3 mL OptiMEM	30 µg pKLV2-Chicken CRISPR Library
		25 µg psPAX.2
		10 µg pMD2.G (VSV-G)

Table 13. Lentivirus Transduction Preparation Protocol.

2.2.3.3 MOI determination

1 million HP8 cells were seeded into a 6-well plate with 12 ug/mL polybrene and dilution of pKLV2-Chicken lentivirus in dilutions from 1/1, 1/5, 1/10, 1/20, 1/40 and 0 was added. After plates were spininfected at 900 x g at 32 °C for 2 h and subsequently cultured with lentivirus for two days, then 72-96 h later the cells were analysed using flow cytometry for the presence of BFP-positive cells. This was used to estimate percentage transduction efficiency.

2.2.3.4 gDNA extraction and preparation for NGS

For downstream sgRNA analysis, I used phenol/chloroform extraction protocol or a gDNA extraction kit. Next, the harvested gDNA was PCR amplified using NEBNext Ultra II Q5 polymerase (NEB #M0544), allowing each 50 µL reaction to hold 2 µg gDNA. Forward primers PCR1_F1-F10 were used in all reactions with one PCR1_R reverse primer (table 14). The thermocycling conditions are shown in table 15. Subsequently, the PCR reactions were pooled and purified using a PCR Purification Kit (Qiagen #28104) according to the manufacturer's instructions and an additional PCR 10 cycles for barcoding, using NEBNext Ultra II Q5 polymerase and a unique combination of one forward primer idx_Sxxx (table 9) and one reverse primer idx_Nxxx. The final PCR purification step was performed using a PCR Purification Kit (Qiagen #28104). Tape station (D1000) and Qubit was used for QC and then sequenced using Illumina Nextseq sequencing. 5 µL of each 25 µL PCR reaction was pooled and a PCR purification (Qiagen 28104) was

Component	25 µL reaction	Final Concentration
Q5 High-Fidelity 2X Master Mix	12.5 µL	1X
10 µM Forward Primer Mix (all 10)	1.25 µL	0.5 µM
10 µM Reverse Primer	1.25 µL	0.5 µM
Template DNA	1-2 µg genomic DNA	
Nuclease-Free Water	To 25 µL	

Table 14. PCR_1 Reaction Setup.

Cycle Number	Denature	Anneal	Extend
1	98 °C, 3 mins		
2-21	98 °C, 10 s	62 °C, 30 s	62°C, 60 s
22			72 °C, 2 min

Table 15. Thermocycling Conditions for PCR_1.

carried out. The elution was used in the 2nd PCR with 10 cycles for barcoding, using NEBNext Ultra II Q5 polymerase and a unique combination

of one forward primer (idx_Sxxx) and one reverse primer (idx_Nxxx) (table 16 and 17).

Component	50 μ L reaction	Final Concentration
Q5 High-Fidelity 2X Master Mix	25 μ L	1x
10 μM Forward Primer Mix (all 10)	2.5 μ L	0.5 μ M
10 μM Reverse Primer	2.5 μ L	0.5 μ M
Template DNA	50 ng genomic DNA	
Nuclease-Free Water	To 50 μ L	

Table 16. PCR_2 Reaction Setup.

Cycle Number	Denature	Anneal	Extend
1	98 °C, 3 mins		
2-11	98 °C, 10 s	65 °C, 15 s	65 °C, 1 min
12			65 °C, 2 min

Table 17. Thermocycling Conditions for PCR_2.

Quality controls were performed using TapeStation 2220 (Agilent) using D1000 tape and reagents (Agilent). The expected product is in the region of ~270-bp for PCR_1 and in the region of ~350-bp for PCR_2. The purified products from all samples of each experiment were pooled and submitted to Genetics Core at Edinburgh Clinical Research Facility for sequencing.

Sequencing (1x100) was performed on the NextSeq 2000 platform (Illumina Inc, #SY-415- 1002) using the NextSeq 1000/2000 P2 Reagents (100 cycles) v3 Kit (#20046811). PhiX Control v3 (#FC-110-3001) library was spiked into the run(s) at a concentration of 20% to help with cluster resolution and facilitate troubleshooting in case of any problems with the run. Base call data produced by the NextSeq 1000/2000 Control Software (Version 1.4.1.39716) is automatically uploaded to BaseSpace, a cloud-based data management and analysis service provided by Illumina. Here it is converted into FASTQ files to allow downstream analysis.

2.2.3.5 Gene set enrichment analysis

All gene set enrichment analyses on selected gene subsets were completed using Enrichr (<https://maayanlab.cloud/Enrichr/>).

2.2.4 HP8 Cell Screening

In total, 9.6×10^7 HP8-Cas9 cells were transduced in 6-well plates, containing 2 million cells per well, along with 12 $\mu\text{g/mL}$ polybrene and 400 μL of pKLV2-Chicken library lentivirus in a total of 2 mL MSB-1 media. Plates were spininfected for 2h at $900 \times g$ and 32°C and subsequently incubated at 38.5°C and 5 % CO_2 for 48 h. Achieving an MOI of ~ 0.15 . Thereafter, lentivirus media was removed and replaced with MSB-1 media. At 72 h post-transduction 100 million cells were collected, pelleted, and stored at -20°C as a T0 time point for gDNA harvesting. After media containing 1 $\mu\text{g/mL}$ puromycin was added, cells were passed at confluency maintaining 50 million cells per replicate. At each time point of 8, 15 and 25 days the replicates were counted, pooled and sampled, pelleted and stored at -20°C .

The HP8 screen was analysed as follows. First a Stouffer-weighted meta-analysis was performed to combine *p*-values, weighted in $1/\text{S.D.}$ of nontargeting sgRNA LFCs on the 2 replicates at $\text{FDR} < 0.05$ at day 25. Next, those with a median LFC < -1 were selected. Then the known human essential genes (Hart *et al*, 2015) were filtered out and the essential chicken genes as found in the DF1 ALV screen and previous work on chicken Cord Lining Epithelial Cells (CLEC) via the same meta-analysis as above, with a permissive threshold of unadjusted *p-value* < 0.05 were filtered out. Using HP8 transcriptomic data produced previously in the lab non-expressed genes and those with no matching annotations in the RNA sequence data were removed.

2.2.5 DF1 Cell screen

In total, 8×10^7 DF1-Cas9 cells were transduced in $6 \times >80\%$ confluent T-150 flasks at an MOI of ~ 0.3 with 8 $\mu\text{g/mL}$ polybrene. After 48 h fresh DMEM was added and at 72 h 2.5 mg/mL puromycin was added. Cells were passed every 2-3 days until all cells expressed the BFP lentivirus marker, monitored using flow cytometry. Subsequently, a sample was then taken for the T0 time point. RCAS-A-GFP and RCAS-J-GFP viruses were prepared by transfection of plasmids RCAS-A-GFP and RCAS-J-GFP into DF1 cells which were then cultured until the majority of the cells were infected, determined by fluorescent microscopy. The supernatant was harvested when DF1 cells were $>80\%$ confluent and spun at $300 \times g$ to remove excess cells and was added to the puromycin-selected DF1-Cas9^{Blast} cells. Once most of the cells were GFP positive the cells were subjected to FACS for negative GFP cells. In the 2 subsequent sorts, RCAS infection was repeated 4 times between 10-14 days. After the 3rd sort, the endpoint cell sample was taken. Pairwise comparisons of guide copy numbers between samples were done using the test sub-command from MAGeCK. Candidate genes with significantly depleted or enriched guides were selected based on a cut-off of < 0.05 .

Several possible hit genes were verified using top-performing sgRNAs as determined by screen count data. These sgRNAs were cloned into a px459 vector, transfected using lipofectamine 2000 and selected with puromycin. The resulting cells were then exposed to RCAS viruses and fluorescence was measured 72 h later to determine the infection state.

2.3.6 CVI988 Screen

2.3.6.1 sgRNA design

sgRNAs were designed using CRISPOR sgRNA designer. 1-3 guides were selected for each gene depending on the length of each specific gene and whether it overlapped with others. Guides were chosen based on predicted efficiency so that there was a C terminal guide and N terminal guide and a middle guide wherever possible. All guides were synthesised as Alt-R sg crRNA (2nM) along with tracrRNA from Integrated DNA Technologies (IDT) and both were subsequently diluted in duplex water to 10 μ M.

2.3.6.2 CVI988 Screen

24 h before transfection 225,000 DF1-Cas9 cells were seeded into a 12-well plate with 10% FBS DMEM media (Thermo Fisher Scientific) and incubated at 38.5 °C with 5 % CO₂. 24 h after seeding cells the media was replaced with 800 μ L fresh warmed 10% FBS DMEM media. The crRNA/tracrRNA complex was prepared by mixing 0.67 μ L, 1 μ L or 2 μ L synthetic guide RNA with 2 μ L tracrRNA and heating at 95 °C for 5 mins and allowing to cool to room temperature for a least 30 mins. To transfect, 3 μ L of Lipofectamine 2000 (Thermo Fisher Scientific) and 47 μ L of warmed opti-MEM media (Thermo Fisher Scientific) were mixed. The resulting 50 μ L of the Lipofectamine 2000/Opti-MEM was added to the crRNA/tracrRNA complex, vortexed, spun down and incubated at room temperature for 20 mins.

Subsequently, each transfection solution was added dropwise to a single well of a 12-well plate. Trypsinized CVI988 heavily infected DF1-cas9 cells were added dropwise simultaneously to each well, equating to 2.25×10^{11} pCVI988-mCherry genomes. After 48 h the growth media was changed to 2% FBS DMEM media and cells were cultured for another 72 h at 38.5 °C with 5 % CO₂ before IncuCyte S3 (Sartorius) scanning for red fluorescence area. Each experiment was independently repeated 3 times and the mean was plotted.

2.3.6.3 PCR

Cells were harvested and DNA extracted using a proteinase K based DNA extraction buffer containing 10 mM Tris-HCl, pH 8, 1 mM EDTA, 25 mM NaCl, and 200 μ g/mL Proteinase K incubated at 65 °C for 30 min followed by 95 °C 5 mins. 5 μ L of extracted DNA was used for PCR with primers located outside the sgRNA-targeting sites to identify successful editing. All primers were synthesised by IDT. 25 μ L GoTaq (Promega) reactions were used with

thermocycler conditions as 95 °C 2 mins then 35 cycles of 95 °C 30 secs, 58 °C 30 secs, 72 °C 2 mins and final extension of 72 °C 5 mins.

2.3.6.4 Sequencing

From the PCR gel bands were excised and gel purified (Sigma-Aldrich) and sent for sanger sequencing (Source Bioscience) to confirm the deletion. Alignments were made using DNASTAR or MEGA11 to CVI988 Rispens sequence.

2.3.6.5 HRM

For high-resolution melt analysis (HRM) MeltDoctor™ HRM Master Mix (Thermo Fisher Scientific) was used following the manufacturer's recommendations.

2.3.6.6 qPCR for MDV-1 and MDV-2 genome copy number

For the real-time qPCR to determine MDV-1 and MDV-2 genome copy numbers previously published methods were used (Baigent *et al.*, 2016). Real-time qPCR carried out to detect the CVI988 strain of MDV and the chicken ovotransferrin gene enabled calculation of CVI988 copies per 10,000 cells using a dilution series of pCVI988 BAC DNA and pGEMT-ovo to produce standard curves respectively. The primers used were pp38-CVI (5* - CCCACCGTGACAGCC-3*, T_m = 57.7) and pp38-Vir(1) (5* - CCCACTGTGACAGCC-3* , T_m = 54.1). PCR amplification was performed in triplicates using ABI 7500 Fast real-time PCR system (Thermo Fisher Scientific). PCR amplifications were completed in 20 µL reactions containing 10 µL ABsolute Blue® q-PCR Lo Rox master-mix (Thermofisher UK), 0.5 µM forward and reverse primers, 0.2 µM probes, and 4 µL extracted DNA. The PCR thermocycler conditions used were 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

2.3.6.7 Generation and characterisation of MDV gene deleted cell lines MSB-1

1.5x10⁶ MSB-1 cells were resuspended in 96 µL Opti-MEM medium. Gene-specific MDV crRNAs were mixed with an equimolar amount of tracrRNA to a final duplex concentration of 10µM in 10 µL and incubated at 95 °C for 5 mins and cooled to room temperature. The mixture was mixed with cell suspension and electroporated at 225 volts and 1.0 pulse length. 48 h after electroporation cells were single-cell sorted into 96 well plates. After 10-days incubation, cells were collected and lysed using a Proteinase K based DNA extraction buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 25 mM NaCl, and 200 µg/mL Proteinase K) at 65 °C for 30 min and subsequently analysed using PCR, with primers outside the target site to identify correct MDV gene edit (Table 1).

2.3.6.8 Virus reactivation following co-culture

Following a 2-day incubation with Sodium Butyrate at 2.5 mM, 1x10⁶ MSB-1 WT cells or ΔMDV gene cells were cultivated with CEF monolayers in a 12-

well plate for 5 days (Zhang *et al.*, 2022b). The CEF monolayers were fixed with an ice-cold 4% acetone/methanol solution. The presence of reactivated MDV-1 and MDV-2 was determined using an immunofluorescence assay with fluorescent microscopy. For MDV-1 identification the cells were stained with anti-pp38 MAb BD1 (1:200) (generated at Pirbright Institute, Woking, UK) as the primary antibody followed by secondary Alexa Fluor 488 goat anti-mouse IgG1 (1:200) antibody. For MDV-2 identification the cells were stained with anti-MDV-2-gB antibody MAb Y-5 (1:500) (provided by Dr L.F. Lee, USDA-ADOL, MI) as the primary antibody followed by secondary Alexa Fluor 568 goat anti-mouse IgG2a (1:200) antibody. The plates were visualized using an IncuCyte Scanner (Sartorius).

2.2.7 gga-mir-21/Meq Interaction

2.2.7.1 gga-miR-21 editing

Within the gga-miR-21 promoter, all 5 of the AP-1 transcriptional binding sites were mutated following previously published work (Stik *et al.*, 2013), restriction enzyme sites were added and the 593-bp sequence was synthesised by GeneArt (Thermo scientific). The supplied mutated pMA plasmid was digested with HindIII and KpnI. This fragment was ligated into similarly digested and purified pGL3-firefly-gga-miR-21 promoter plasmid, made previously in the group and subsequently amplified in *E. coli* DH5- α to create mutant pGL3-firefly-gga-miR-21promoter.

GGTACCCTCTGTGCAGCGTTAACACCTTTGTGCTTTGGGAGAGCGTTTGC
AGCTCCGAAAACCTCATCAATCTTTATAATGTGAACATAGGAACAAGGTGT
TCAGTAACGTATTGCAGTTTTTGTGCAAGTCATCTGCCATAAAGCATGAA
TTTTTTTGAAACAGGGTTCATAGTTCTCTCTTTGTTCCCTTTGTTAATTTTTT
CCGATGGGAGGAGCCTGCAGAATTTGCTAATGCATTCTTTCTGGATAAGG
ATAACACCCAGATTGTCCTAATAAGGACTTAGTTTGAGAAAGGGGCCGTC
TCCTTTGAGGAGATAGGGGCAAGTCATAGGGCGAGCAGTTTCTTTTTTAA
CTGAGGGATTTTTTTAGCACAAACCCCTTTCCTTATTAATCGGTTCAAACCA
GTTCTTACAGGAAGTCTGGTGATAAATGTGGGACTTCTGAGAAGTCATT
CATTTCAATTCTCAGCACCGCATCAGCACACAGCCCTGGCTGCCCTCTCC
CAGGCTTTAAGGAACTTTGCTCACTGCTTCTTTCTGTGTTACCAACAGT
GTATTATCACTGATTCTCTCCGAAATATTTTTCTTTTAAAGCTT

gga-miR-21 promoter sequence mutated at AP-1 response element bases are shown in bold & underlined, sourced from Stik *et al.* (2013). The grey highlighting represents the AP-1 response elements. Red highlights the KpnI (5') and HindIII (3') restriction sites which were added respectively.

2.2.7.2 Electrophoresis Mobility Shift Assay

Electrophoresis Mobility Shift Assay (EMSA) was performed using reagents from the DIG Gel Shift Kit, 2nd Generation Kit and chemiluminescent

detection (Roche), according to manufactures instructions. DNA fragments were prepared using gga-miR-21 amplification Fwd/Rev primers (table 19) which were subsequently DIG-labelled and incubated with MSB-1 cells nuclear extract which was extracted using NE-PER Nuclear Extraction Reagents (Thermo Fisher).

2.2.7.3 *In vivo* Dual Luciferase Assay

96 well plates were seeded with 2.7×10^4 DF1 cells per well 24 h prior to transfection(s) or to 80% confluence. 0.5 μ L Lipofectamine 2000 (Invitrogen) was diluted with 24.5 μ L Opti-MEM (ThermoFisher) per well. Immediately thereafter 100 μ L of Opti-MEM was used to dilute the purified plasmid DNA for 4 replicates. pcDNA 3.1/myc-His B-MEQ plasmids were added at 100 ng per well whereas pGL3-Renilla and pGL3-Firefly plasmids were added at 20 ng per well. The diluted Lipofectamine 2000 was added to the diluted DNA and incubated for 20 min at room temperature. 100 μ L of fresh warm media was added and 35 μ L of diluted DNA/Opti-MEM solution was added to the corresponding well. Cells were incubated for 48 h and luciferase activities were determined using Dual-Glo® Luciferase Assay System (Promega) according to the manufacturer's protocol. The Firefly/Renilla ratio was calculated, and all assays were performed in quadruplicate.

2.2.7.4 Western blot analysis

Expression of Meq in DF1 and HP8 cells was determined using western blot analysis with the monoclonal antibody against MDV Meq (FD7, generated at the Pirbright Institute) and $\alpha\beta$ -actin or α -tubulin were used as a control (Sigma Aldrich). Approximately 1×10^6 cells were collected and boiled with TruPAGE LDS sample buffer (Sigma) and DTT (Merck) for 10 min. Next, samples were run on 4-12 % TruPAGE (Sigma) precast gel at 140 volts for 2.5 h, resolved proteins were blocked with skimmed milk and then incubated overnight at 4 °C with FD7 anti-meq antibody. After, blots were incubated at room temperature for 1.5 h with secondary antibody IRDye® 680RD goat anti-mouse IgG (LI-COR). Blots were visualised using Odyssey Clx (LI-COR).

2.2.7.5 Taqman RT-qPCR for gga-miR-21

The reverse transcription-qPCR was to detect the change in gga-miR-21 levels from various cell treatments. RNA was extracted from 1×10^6 cells using the RNeasy 96 Blood and Tissue kit (Qiagen). The High-Capacity RNA-to-cDNA Kit (ThermoFisher) was used to reverse transcribe 50 ng of RNA to cDNA, using 7 μ L Multiscribe reverse transcriptase buffer including Multiscribe reverse transcriptase, 3 μ L of TaqMan hsa-miR-21 primer (ThermoFisher), brought up to 15 μ L with RNase free water. The RT reaction was used as a template for qPCR using 1 μ L the hsa-miR-21 TaqMan probe (ThermoFisher, assay ID: 4427975), 10 μ L ABsolute Blue qPCR Low ROX mix made up to 20 μ L with RNase free water. The PCR conditions used were 95 °C for 15 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All qPCR tests were run in triplicate on the ABI 7500 Fast Real-time PCR System (Fisher Scientific). All values were normalised to those of

endogenous small nuclear U6 RNA and values were calculated using the arithmetic comparative $2^{-\Delta\Delta C_t}$ method.

2.2.7.6 Meq deletion

The Meq gene was deleted in Cas9 nuclease-expressing MSB-1 cells, which were transfected with two lenti U6-sgRNA/EF1a-mCherry plasmids each expressing a single sgRNA sequence targeting the N and C terminal respectively. 1.5×10^6 MSB-1-Cas9 cells were electroporated with 7 μ g of each plasmid using an optimised 275 V and a pulse time of 1.5 ms. At 48 h post electroporation cells were sorted using fluorescence-activated cell sorting (FACS) using FACSaria II (BD bioscience), and cultured for 3 further days, at which point DNA and RNA were extracted. PCR of extracted DNA using GoTaq (Promega) following the manufacturer's direction was performed to confirm the deletion. Primers flanked the Meq gene can be seen in Table 18.

Target	Sequence 5'-3' Target
Meq-C-F DNA	<u>CACCGT</u> CTCGGGTCGACTTCGAGA
Meq-N-F DNA	<u>CACCGC</u> GTAAACCGTCCCCGGCGA
Meq-N-F	ATGTCTCAGGAGCCAGAG
Meq-C-R	TCAGGGTCTCCCGTCA
pp38-gN-F DNA	<u>CACCGG</u> GTATGTTAGTCGGTAGAA
pp38-gC-F DNA	<u>CACCGC</u> TCTCGTCGGCGACCCCTGCG
Fwd pp38 primer	GATTCCACCTCCCCAGAATCC
Rev pp38 primer	TTCGAAGCAGAACACGAAGGG
*Underlined bases represent BbsI cloning sites	

Table 18. Meq deletion Primers and DNA oligos for sgDNA cloning.

Target	Sequence 5'-3' Target
Sg1 gga-miR-21- promoter	<u>CACCG</u> TTAATCACTGACCGATGGG
gga-miR-21 promoter ^{mut} only PP2-FWD	CCTGTCTACGTGTGGCTCAG
gga-miR-21 promoter ^{mut} only PP2-REV	GGGTTTGTGCT AAAAA ATCCCTC
gga-miR-21 amplification Fwd	TGCAGCGTTAACACCTTTGTG
gga-miR-21 amplification Rev	GACTACGCCACGACTCTTACT
*Underlined bases represent the BbsI cloning site. Bold bases represent those that are muted concerning the WT gga-miR-21 promoter sequence.	

Table 19. gga-miR-21 promoter editing primers and DNA oligos for sgDNA cloning.

2.7.7 HP8 gga-miR-21 promoter editing

Cas9 nuclease expressing HP8 cells were transfected with lenti U6-sgRNA/EF1a-mCherry plasmid with cloned single sgRNA targeting gga-miR-21 promoter. 1.5×10^6 HP8 Cas9 cells were electroporated with 5 µg gga-miR-21-promoter sgRNA expression plasmid and 2 µg donor DNA template using an optimised 275 V and a pulse time of 1.5 ms. The donor DNA was amplified using gga-miR-21 amplification Fwd/Rev primers (table 19). At 48 h post electroporation cells were sorted into U-shaped 96-well culture plates using fluorescence-activated cell sorting (FACS) using FACS Aria II (BD bioscience). 2 weeks later single cell colonies were screened using junction PCR targeting the edited DNA using primers gga-miR-21 promoter^{mut} only PP2 FWD/REV (table 19), using GoTaq (Promega) and the manufacturer's instructions. The mutated PCR products were then purified (Sigma-Aldrich) for Sanger DNA sequencing (Eurofins).

2.2.7.8 RCAS-Meq virus infection

The Replication-Competent ALV (avian leukosis virus) long terminal repeat (LTR) with a splice acceptor (RCAS) vector was used as a viral vector to deliver Meq oncogene to HP8 cells. Confluent T-25 flasks of DF1 cells were transfected using Lipofectamine 2000 (Fisher Scientific) with 1 µg RCAS plasmid, T-25 flasks were passed until all cells were infected with RCAS-Meq-GFP or RCAS-GFP observed using a fluorescent microscope. Subsequently, a confluent T-25 flask of RCAS-infected DF1 cells was co-cultured with 2 million HP8 cells for 5 days and HP8 cells were then sorted by fluorescence-activated cell sorting (FACS) using FACS Aria II (BD bioscience). Sorted cells were then used for RNA and protein extraction.

Chapter 3: CVI988 essential and non-essential genes screen

3.1 Summary

The MDV genome has been extensively analysed, particularly those genes that are associated with oncogenicity. However, not all genes have been studied so thoroughly and few studies have compared all genes across the MDV genome. The decrease in the expression of a gene can provide insights into its function. With CRISPR-Cas9 technology enabling rapid easy editing of many genes, gene knockout can be achieved, and the resulting phenotypic changes can be measured. In this chapter, the expression of all MDV-predicted protein-coding genes was knocked down individually and the effect on infection and spread of vaccinal MDV strain CVI988 was quantified.

3.2 Introduction

Many MDV genes critical to pathogenesis have been rigorously investigated but many genes have not been studied thus, inferences are made from what is known about Herpes simplex virus among other herpes viruses. Most genes of MDV contain significant homology to HSV. However, there are several genes which are unique to MDV, named LORFs (UL, ORFs), R-LORF (Repeat-Long ORF) and SORF (US, ORF). Twelve LORFs, 16 R-LORFs and 4 SORFs have been identified thus far (Davison *et al.*, 2004). R-LORF7 otherwise known as MEQ is perhaps the most studied of all.

Demonstrating homology to the Jun/Fos family of transcription factors displaying transactivation and repression activities, MEQ is highly associated with the neoplastic nature of MD (Qian *et al.*, 1995). R-LORF2 is otherwise known as vIL-8 and possesses significant homology to mammalian IL-8 and is involved in the recruitment of lymphocytes to infected cells, aiding in the transmission of viral particles to new host lymphocytes (Boodhoo *et al.*, 2016). The promoter of R-LORF14a, also known as pp38, spans across the origin of replication and has been linked to lytic viral replication (Boodhoo *et al.*, 2016). See section 1.2.6 for more on MDV pathogenic genes.

Despite the similarities to HSV, including the ability to establish latent infection in host cells, MDV possesses some striking differences. HSV produces cell-free virions whereas MDV is strictly cell-associated, only producing virus-free particles in feather epithelial cells. MDV infects the bursa of Fabricius and the thymus by a distinctly separate route from that of HSV. Additionally, MDV-1 consistently forms lymphomas (Davison *et al.*, 2004). Thus, unique features of MDV-1 including its oncogenicity are attributed to its unique genes and inferences can be made to their function depending on expression between strains which possess different virulence. For example,

the non-oncogenic viruses MDV-2 and HVT do not produce MEQ, leading to the early conclusion MEQ is the primary oncogene in MDV (Shamblin *et al.*, 2004). However, avirulent MDV-1 vaccine strain CVI988 containing MEQ is not oncogenic. Furthermore, not all MDV genes have been studied to date and we are not aware of any study comparing the essentiality of all protein-coding genes for viral infection and spread *in vitro*.

In this study, we utilised a BAC construct that had been engineered to express mCherry fluorescence which enabled easy virus identification and offered a method for quantitative measurements. Furthermore, CVI988 is currently the most effective commercial vaccine against vv+ strains of MDV and is widely used globally either alone or in combination with other vaccines to protect against symptomatic MDV. Therefore, it would be interesting to uncover essential or non-essential genes in CVI988. Such a systematic approach will help confirm current genes known to be essential, whilst providing insights on lesser-studied genes. Furthermore, non-essential genes maybe of interest in recombinant virus production, since currently, CVI988 has proven difficult to engineer for recombinant virus technology compared to its counterpart HVT.

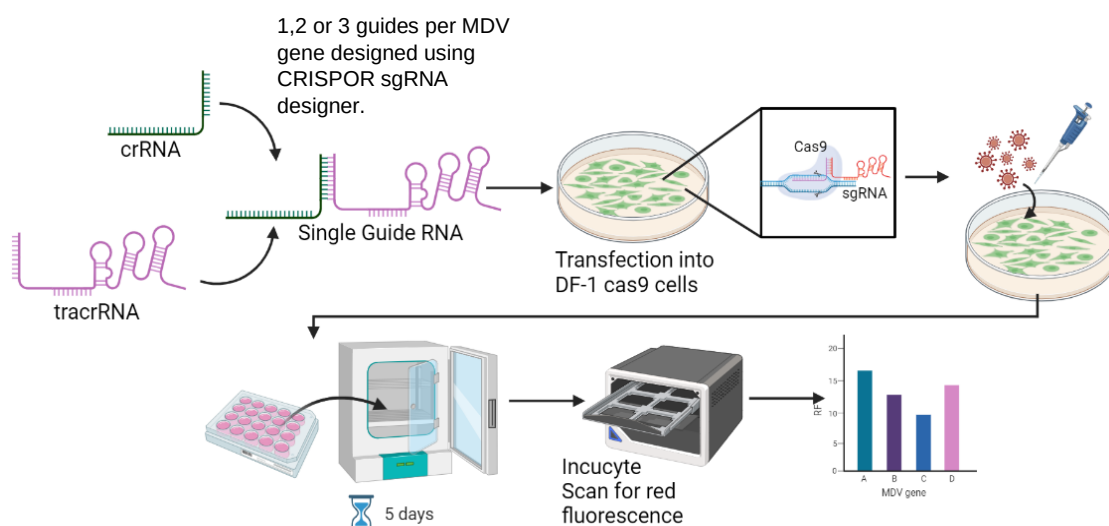


Figure 7. Diagram illustrating the procedure of the Marek's Disease virus essential/non-essential gene screen. First 1, 2 or 3 guides per MDV gene were designed using CRISPOR sgRNA designer. Guides were synthesised as synthetic crRNAs and complexed with tracrRNA. crRNA/tracrRNA guides were transfected into DF1-Cas9^{Blast} cells. Immediately after CVI988-mCherry virally infected CEF cells were added to the culture medium. Cells were incubated for 5 days after which IncuCyte S3 scanning for red fluorescence was carried out. Created with BioRender.

In the last decade, CRISPR technologies have revolutionised genome manipulation, making once quixotic projects now possible. Here we applied CRISPR-Cas9 editing to systematically analyse the 142 ORFs of MDV to

assess their implication in viral replication and spread in cell culture. Figure 7 summarises the experiment.

3.3 Aim of chapter

The major goal of this project was to systematically analyse the MDV genome for genes that are essential and non-essential to MDV replication and spread *in vivo*. To achieve this, I planned to complete an arrayed CRISPR-Cas9 screen to systematically analyse the 142 ORFs of MDV to assess their implication in viral replication and spread in DF1 cell culture. The longer-term goal was to validate some of the essential and non-essential genes for virus replication and spread to provide evidence of an efficacious method that could be used for other viruses and direct further study of genes required for replication or non-essential genes that could be targeted in recombinant vaccine developments.

3.4 Results:

3.4.1 DF1-Cas9^{Blast} construction

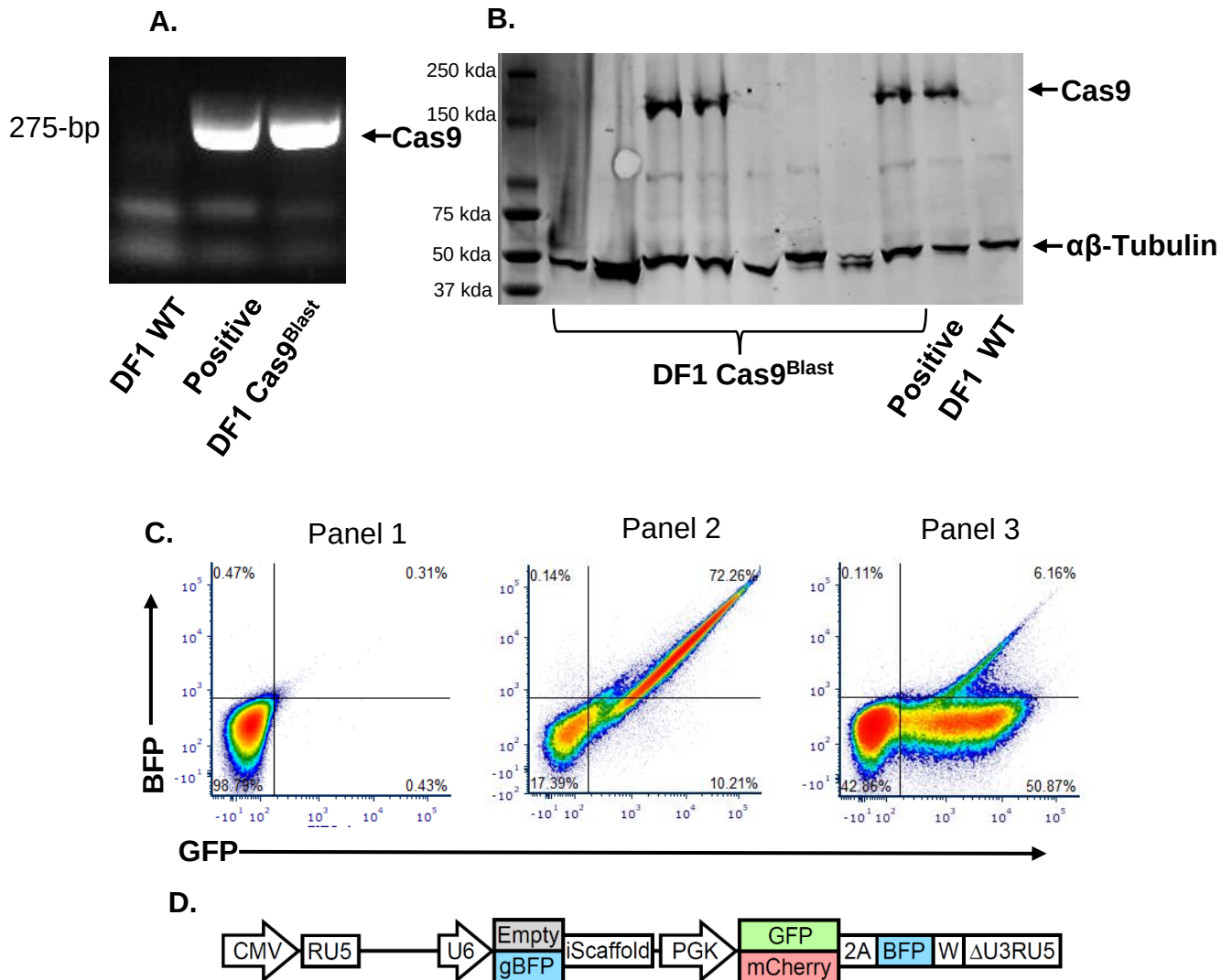


Figure 8. Construction of DF1-Cas9^{Blast} cell line and validating Cas9 activity. (A) Cas9 PCR, using primers located in the Cas9 protein coding region. (B) Cas9 Western Blot showing 8 DF1 Cas9^{Blast} clones from the Cas9 transfection, (C) Cas9 reporter assay, whereby GFP expression only indicated the presence of Cas9. Cells were transduced with the reporter construct shown in 'D'. Panel 1 shows the untransduced cells, panel 2 shows transduced WT DF1 cells and panel 3 shows DF1-Cas9 expressing clone. (D) Shows the reporter construct that was used, the construct has BFP and GFP expression, with a sgRNA against the BFP, therefore in the presence of Cas9 protein the expression of BFP will be diminished leaving only GFP.

Having a Cas9 cell line with effective Cas9 activity is critical to most CRISPR screens and is favourable to delivery Cas9 via plasmid due to higher constitutive expression levels. To construct the DF1-Cas9^{Blast} cell line, Lenti-

Cas9-Blast plasmid was transfected into DF1-WT cells and selected with Blasticidin, after which PCR was used to confirm the integration of Cas9 into the genome of the DF1 cells (figure 8A), since the Cas9 construct after selection for 2 weeks will only be present if integrated into the genome. A positive band shown at 275-bp. The selected mixed population was then single-cell sorted into 96 wells and clones expanded, which were then tested for protein expression using Western Blot (figure 8B). Cas9 is 163 kDa protein, several clones showed no expression of Cas9 whilst others showed expression equal to the control cells DF1-Cas9-puromycin. Cas9 activity was evaluated using the BFP/GFP reporter construct, the reporter expresses both GFP and BFP in WT cells but when Cas9 introduced the sgRNA against BFP within the construct targets BFP, reducing BFP expression (figure 8C and 8D). The clone shown in figure 8C was used in downstream experiments.

3.4.2 CVI988-tk-mCherry infection in DF1-Cas9^{Blast} cells

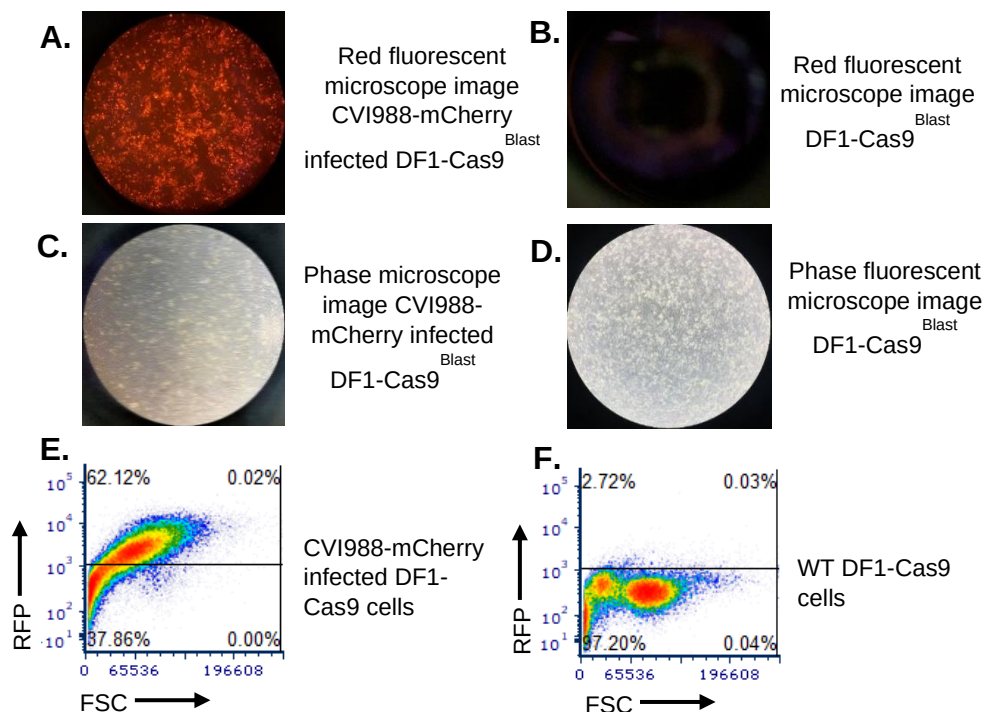


Figure 9. CVI988-tk-mCherry infection in DF1-Cas9^{Blast} cells. (A) Microscope image 10x magnification using red fluorescent lens CVI988-mCherry infected CEF cells co-cultured with DF1-Cas9^{Blast} cells. (B) Microscope image 10x magnification of DF1-Cas9^{Blast} cells using red fluorescent lens. C and D show the same wells of cells in A and B under 10x magnification using the white light lens. E and F show the FACS DF1-Cas9^{Blast} cells co-cultured with infected with CVI988-mCherry infected CEF (E) or uninfected (F).

Previously we had demonstrated that transfection of the BAC construct pCVI988-tk-mCherry was possible in DF1 cells, however, when cultured with SpCas9 protein a reduction in plaque number was observed. Additionally,

BAC pCVI988-tk-mCherry did not transfect well in DF1-Cas9^{Blast} cells. To overcome these hindrances an alternative method using CEF pCVI988-tk-mCherry infected cells and subsequent co-culture with DF1 cells was used to induce virus plaque formation in DF1 cells. CEF pCVI988-tk-mCherry infected cells were passed on both DF1 WT and DF1-Cas9^{Blast} cells and co-cultured to assess whether Cas9 expression would hinder viral expression (figure 9 and 10). Initially, it was shown that it was possible to gain CVI988-tk-mCherry infection in DF1-Cas9^{Blast} cells after co-culture with CVI988-tk-mCherry infected cells (figure 9). Next, a growth curve using IncuCyte Red fluorescent area measurement was used to quantify the growth of CVI988-mCherry overtime on CEF vs DF1 cells (figure 10A), which confirmed the findings of figure 9 that DF1 cells could be infected with CVI988, CEF cells were used as a positive control, as are well known to easily propagate CVI988 virus. It is plausible that the addition of Cas9 to the genome of cells could have off-target effects and effect virus infection therefore WT-DF1 and Cas9 DF1 cells were compared to observe if this would have any major implications on the assay. It was seen that DF1-Cas9^{Blast} cells maintained similar viral infection to WT DF1 cells. Subsequently, a CEF pCVI988-tk-mCherry stock was made and was used in all screening experiments.

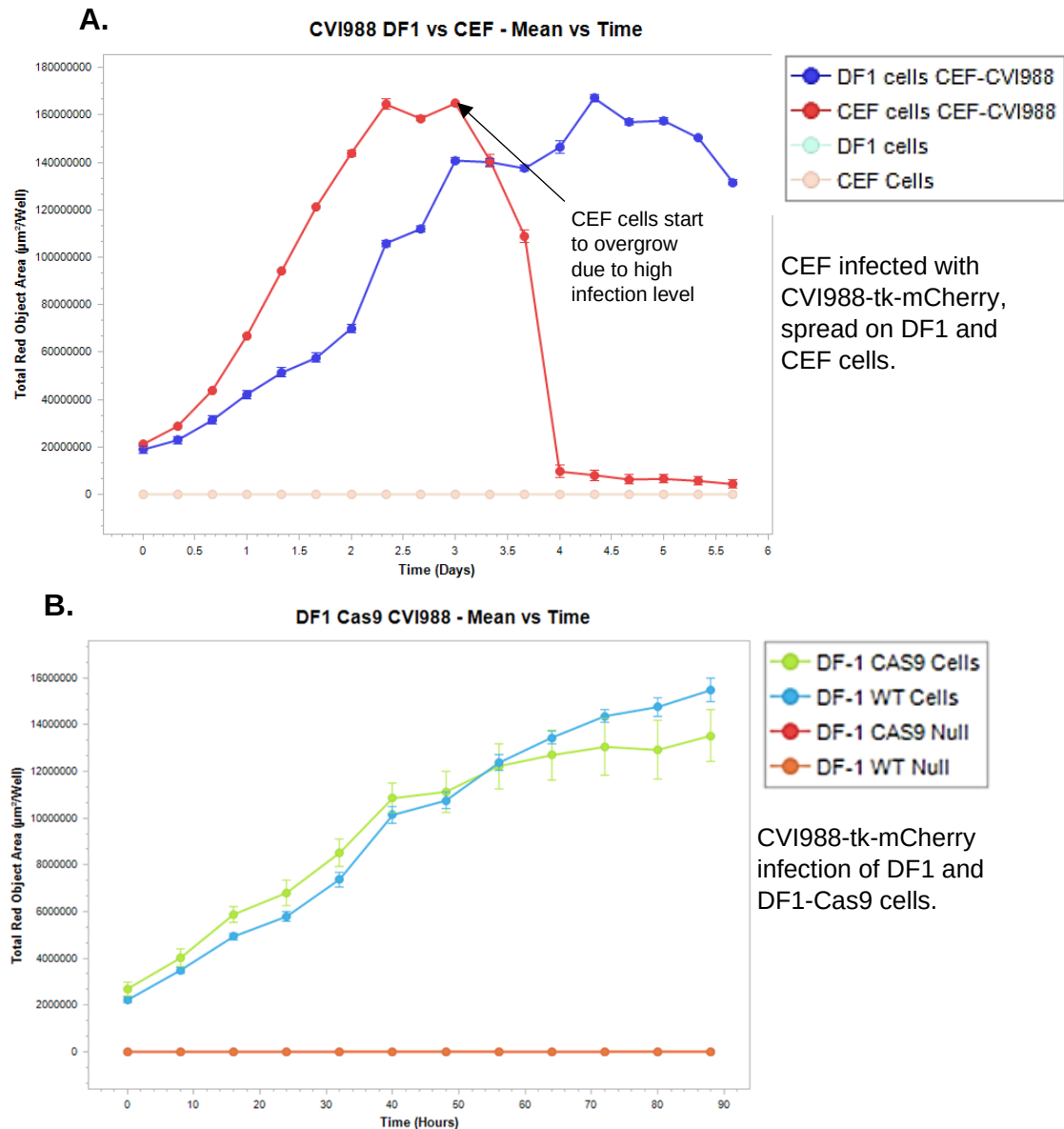


Figure 10. Comparison of the growth of CVI988-tk-mCherry virus in CEF, DF1 and DF1-Cas9^{Blast} cells. CVI988-tk-mCherry virus was propagated in CEF cells and co-cultured with several cell types to check the infection of the virus on these alternate cell types. Virus growth was measured using IncuCyte Red fluorescent area (A) DF1 WT cells and CEF cells co-cultured with CEF infected with CVI988-tk-mCherry. (B) DF1 WT cells and DF1-Cas9 cells both co-cultured with CEF cells infected with CVI988-tk-mCherry cells, DF1-Cas9 Null and DF1-WT Null represent co-culture with uninfected CEF cells.

3.4.3 Arrayed CRISPR screen optimisation

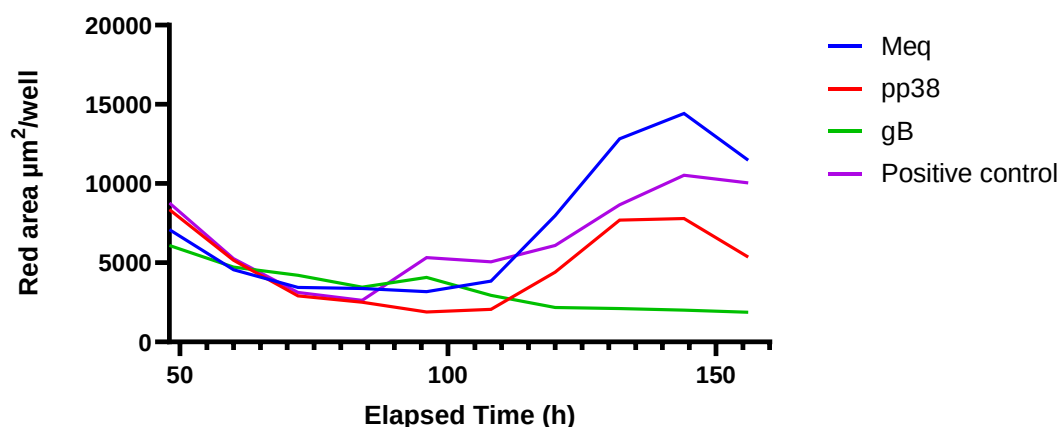


Figure 11. Incubation time optimisation post CVI988-tk-mCherry infection in DF1-Cas9^{Blast} cells. Meq, pp38 and gB 10 nm sgRNAs were transfected into a well of a 12-well plate, at the same time 40 μ L CEF infected with CVI988-tk-mCherry was added. This was incubated in the incuCyte for 6-days. Meq and pp38 are genes that are thought to be dispensable from viral replication but may influence the efficiency of viral replication. gB is an essential MDV glycoprotein for an effective MDV infection. The positive control is CVI988-tk-mCherry viral infection with no sgRNA. $n = 1$.

Once sufficient plaques could be formed on DF1-Cas9 cells, I optimised the protocol's stages. First, for a time course of pCVI-988-tk-mCherry infection, pCVI988-tk-mCherry infected CEF cells were added to the culture media of DF1-Cas9^{Blast} cells and incubated for 6.5 days and monitored by the IncuCyte S3 scanning for red fluorescence (figure 11). It was observed that 4-5 days post-infection saw the greatest divergence of red fluorescence when the essential genes (gB) and the non-essential genes (Meq and pp38) were targeted by previously validated sgRNAs, therefore giving the greatest range of fluorescent values in which gene essentiality could be observed within (data not shown, appendix 8.1.3) (Lupiani *et al.*, 2004a, Chi *et al.*, 2013, Zhang *et al.*, 2019a). The second optimisation was the volume of 10 μ M synthetic crRNA to transfect into a single well of a 12-well plate. 5.0, 7.5, 10.0, 15.0 and 20.0 nM were tested. A dose response to all non-essential and essential genes was seen; I thus chose 10 nM (1 μ L of 10 μ M per 1 mL) due to the fact all concentrations showed similar trends between the essential (gB) sgRNA and the non-essential (meq and pp38) sgRNAs and for simplicity, since 10 nM equalled 1 μ M (figure 12). Most genes in the screen were targeted by 3 sgRNAs therefore, as a redundancy measure as the efficiency of all guides were not pre-validated. Therefore, a total of 30 nm sgRNAs were used per well of a 12-well plate per gene.

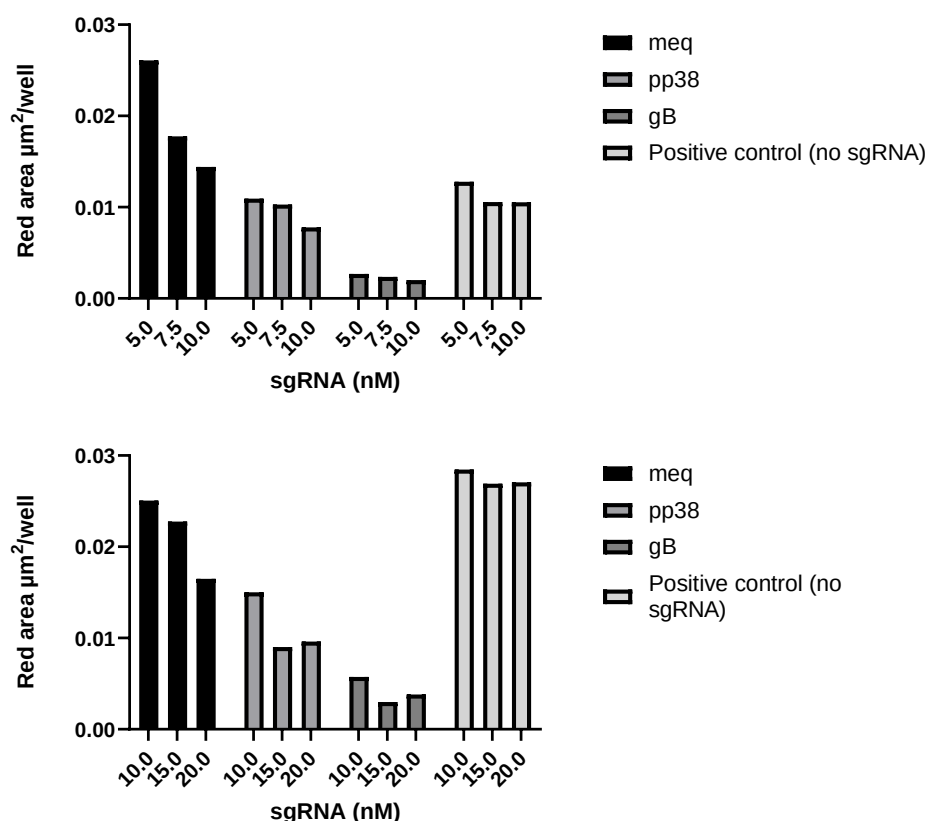


Figure 12. crRNA/tracrRNA volume optimisation per 1 mL media for 1 well of a 12-well plate. DF1-Ca9^{Blast} cells were transfected with 10 µM synthetic crRNA into a single well of a 12-well plate. 5.0, 7.5, 10.0, 15.0 and 20.0 nM crRNA were tested. n = 1.

3.4.5 MDV glycoprotein gene essentiality screen

Before significant effort was employed to screen all MDV genes a targeted screen of the MDV glycoproteins was performed. Since extensive research has been carried out on the essential nature of MDV glycoproteins within the literature, I would expect to see gC and gD as non-essential glycoproteins while all other would be expected to be essential in this assay. Therefore, I first used glycoproteins to validate the methodology. crRNA/tracrRNA complexes were transfected into DF1-Cas9^{Blast} cells, simultaneously co-cultured with CEF infected with pCVI-988-tk-mCherry and incubated for 5 days when the IncuCyte S3 scan was taken. Additionally, the controls were able to give reference points for gene essentiality. A positive control consisted of CVI988-tk-mCherry with no sgRNA, a negative control of no CVI988-tk-mCherry or sgRNA and the well studied MDV genes gB (MDV40), pp38 and Meq were included to allow comparisons to be made. Three independent biological replicates were completed. Figure 13A shows one of these IncuCyte scans and under close observation, a greater number of

plaques can be seen in MDV57 and MDV94 wells as well as many red fluorescent plaques in the positive control and no observable plaques in the negative control well. Figure 13B showed the mean of 3 biological replicates and although not significant (gC *p-value* = 0.0612 and gD *p-value* = 0.1817), the glycoprotein screen showed MDV 57 (gC) and MDV 94 (gD) to produce more plaques and red fluorescent area than essential glycoproteins (gB, gE, gH, gI, gK, gM and gL) (figure 13B). Published literature was able to corroborate our findings, with reports showing that gC and gD are not required in cell culture conditions of MDV, suggesting an efficacious assay (Anderson *et al.*, 1998, Tan *et al.*, 2001, Tischer *et al.*, 2005). After completion of the last scan, the genomic DNA was extracted from the infected cells and used for PCR analysis to check the editing efficiency of the guides (figure 14). This was important to give confidence that the phenotype differences shown as red fluorescent plaques were due to the sgRNA mediated genome editing of the virus. The glycoproteins were targeted with 3 sgRNAs as long as 2 or more sgRNAs mediated double strand breaks sections of the viral genome would be removed producing shorter gene sequences that could be observed by PCR when using primers targeting the upstream and downstream regions of a specific viral gene. It was observed that the majority of edits tested (7/8) glycoproteins showed shorter PCR bands than the WT, with the MDV-22 (gM) showing 2 bands, the shorter of which is expected to be the edited band (figure 14).

3.4.4 Arrayed CRISPR screen MDV glycoproteins

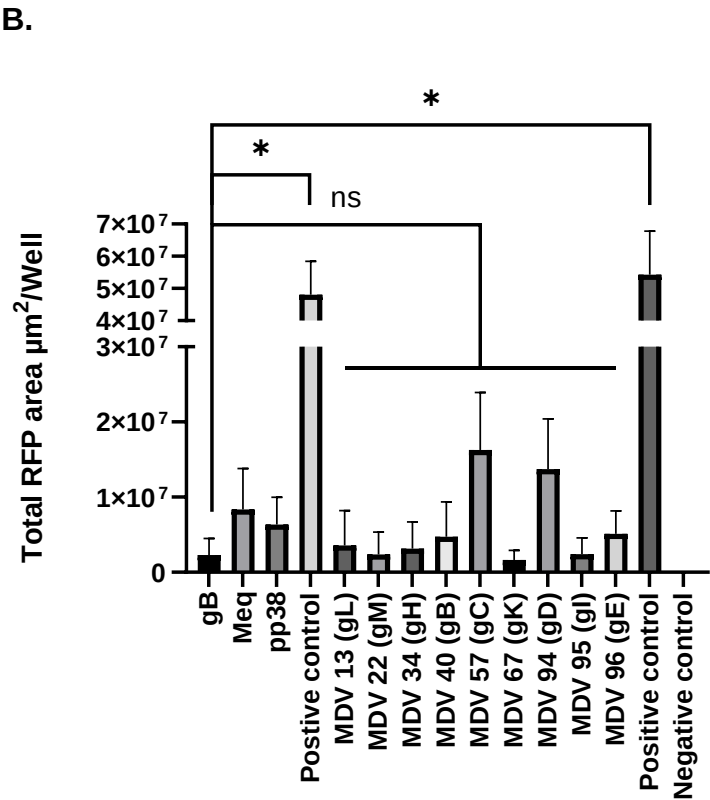
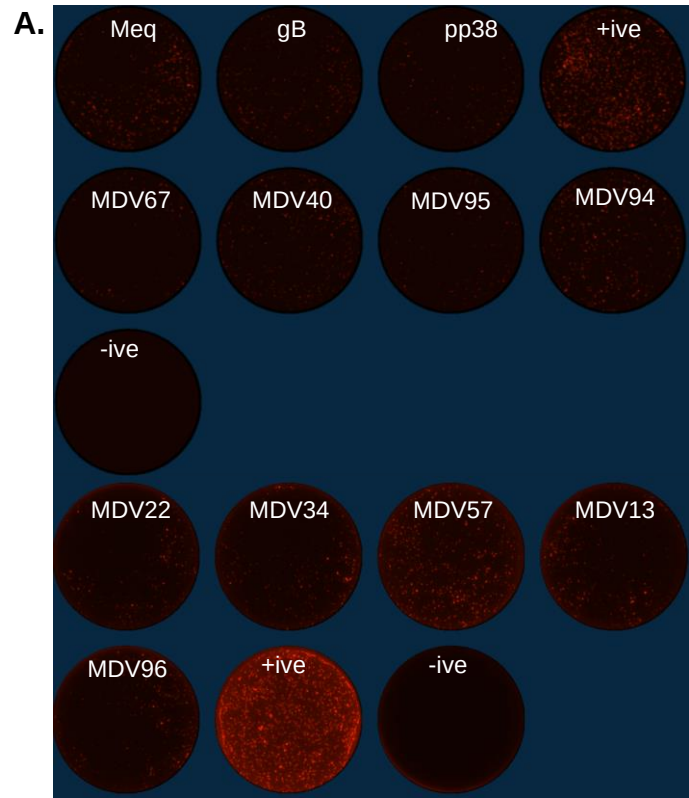


Figure 13. MDV glycoprotein gene essentiality screen. (A) IncuCyte scan images, (B) Total RFP area quantification from the IncuCyte scan. DF1-Cas9^{Blast} cells were transfected with a total of 30 nM sgRNA per well of a 12-well plate, at the same time 40 μ L of CEF cells infected with CVI988 were added to the same well. The plate was then incubated for 5 days when an IncuCyte S3 scan recorded the total RFP area. Positive control = CEF infected with CVI988-tk-mCherry virus only. Negative control = no virus, no sgRNA. gB was used as a comparison to all other glycoproteins as gB has been previously shown to be a critical essential gene for MDV replication in cell culture. Mean is plotted $\pm$ SD of the mean. Asterisk (*) indicates significance (unpaired t-test p -value < 0.05), n = 3.

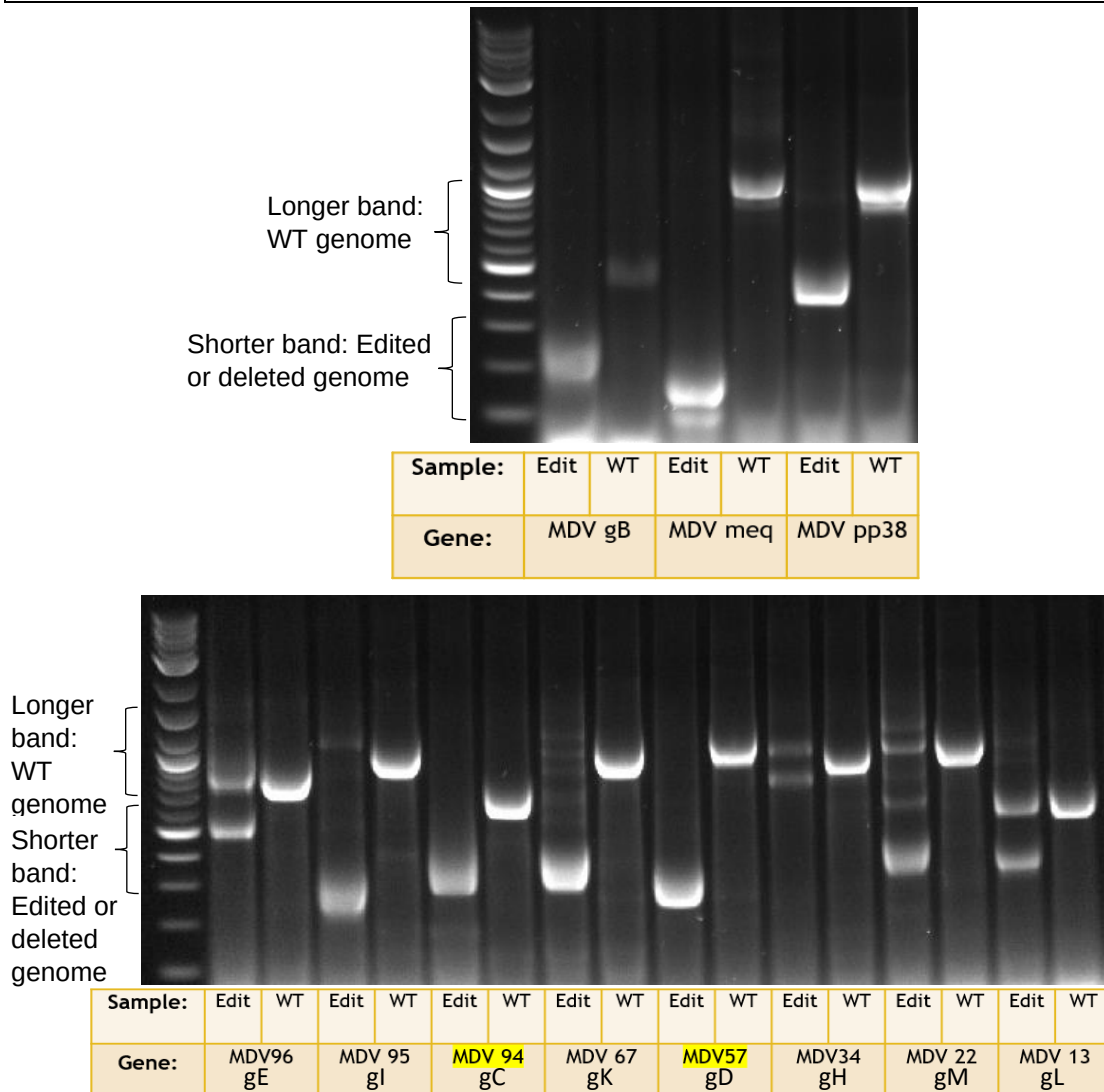


Figure 14. Gene editing efficiency assessed by PCR for MDV glycoprotein gene essentiality screen. DF1-Cas9^{Blast} cells were transfected with a total of 30 nM sgRNA per well of a 12-well plate, at the same time 40 µL of CEF cells infected with CVI988 were added to the same well. After 5 days of incubation the cells of the wells were harvested and gDNA extracted, this was used in PCR reaction using primers that flanked the edited region of each gene. The lower band shows a shift in the PCR amplicon indicating successful editing of two or more of the sgRNAs. Yellow highlighting indicates the non-essential MDV glycoproteins (gC and gD).

3.3.5 MDV-CVI988 gene essentiality screen

Following the successful demonstration of the glycoprotein gene essentiality screen (figure 13 and 14) concordant with the published data, I proceeded to the screening of the remaining MDV genes using the same approach. Whereby 30 nm crRNA/tracrRNA complexes were transfected into DF1-Cas9^{Blast} cells, simultaneously co-cultured with CEF infected with pCVI-988-tk-mCherry and incubated for 5 days when the IncuCyte S3 scan was taken. Three independent biological replicates were completed from which the gDNA was harvested. The screening results are shown in figure 15. The glycoprotein B (gB) was used as a reference control for genes required for viral replication (Schumacher *et al.*, 2000, Chi *et al.*, 2013). The HVT US2 non-targeting control sgRNA along with a positive control only infected with pCVI-988-tk-mCherry was used as a reference control for non-essential genes (Tang *et al.*, 2020) (figure 15). pp38 has also been shown to be non-essential for virus replication (Reddy *et al.*, 2002a) as well as meq (Lupiani *et al.*, 2004a); thus, both were included as reference point controls and were used to estimate the essentiality of all screened genes. Many genes indicated by the screen shown to be essential or non-essential to virus replication *in vitro* can be seen to be aligned with published literature (tables 20 and 21). The gDNA was used for PCR analysis for multiple sgRNAs targeted genes (figure 16) or High-Resolution Melt Curve analysis (HRM) for single sgRNA targeted genes (figure 17) to check if there was evidence of editing occurring of the guides in the assay for the top 10 most essential and non-essential genes as identified by the screen. It was observed that in the majority of edited genes saw strong deleted band compared to the WT, HRM showed that a single sgRNA was causing the editing of the gene.

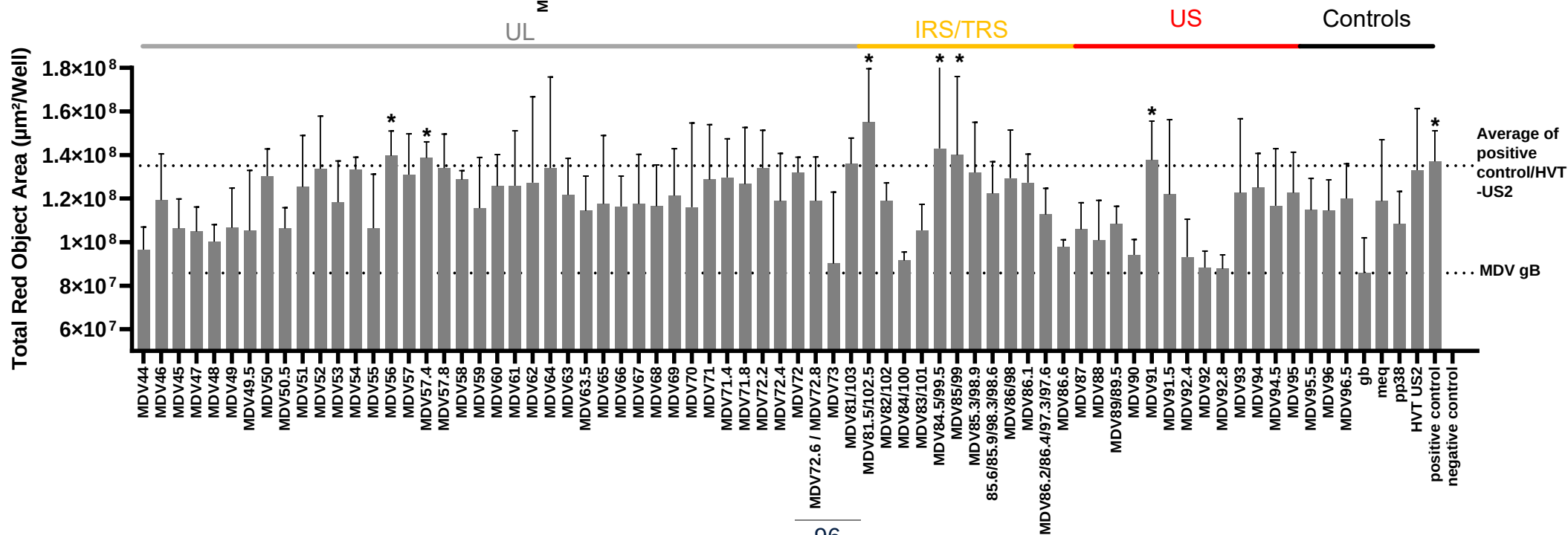
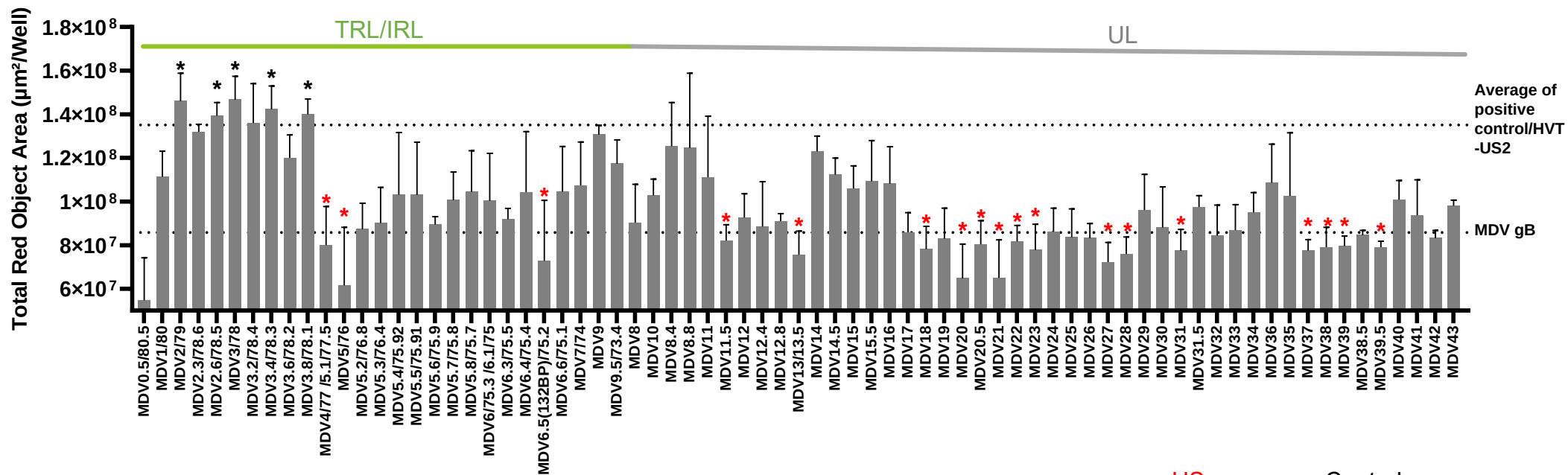


Figure 15. CVI988 screen for genes essential/non-essential in MDV replication and spread *in vitro*. DF1-Cas9^{Blast} cells were transfected with a total of 30 nM sgRNA per well of a 12-well plate, at the same time 40 µL of CEF cells infected with CVI988 were added to the same well. The plate was then incubated for 5 days when an IncuCyte S3 scan recorded the total RFP area. Positive control = CEF infected with CVI988-tk-mCherry virus only. Negative control = no virus, no sgRNA. HVT-US2 sgRNA is a non-targeting sgRNA to the MDV genome. gB was used to identify statistical significant (unpaired t-test *p-value* < 0.05) non-essential genes (black asterix (*)) as gB has previously been shown to be a critical essential gene for MDV replication in cell culture. The non-targeting HVT-US2 was used to identify statistical significant (unpaired t-test *p-value* < 0.05) essential genes (red asterix (*)). Mean is plotted +/- SD of the mean. n = 3. MDV genomic structure is shown above, TRL/IRL = terminal repeat long/internal repeat long, UL = unique long, IRS/TRS = internal repeat short/terminal repeat short and US = unique short.

Essential CVI988 Genes				
Smallest to largest	Rank	Other names/role	Conserved gene in α-,β- and γ-herpesviruses	Function/Additional details
MDV0.5/80.5	1*	N/A	N	Integration of the viral genome into chromosomes is facilitated by telomeric repeats (TMR) arrays, which reside at both ends of the viral genome, in addition to the IRL-IRS junction. These similar sequences are composed of a sort of telomeric repeat (sTMR) and multiple telomeric repeats (mTMR) (Bertzbach <i>et al.</i> , 2018). The sTMR possess 6 TTAGGG telomeric repeats and is present in MDV 0.5/80.5. sTMR is critical in the integration of the virus genome by acting as a spacer sequence between the packaging signal pac-1 and the DR-1 cleavage sites to enable packaging of the genome into the preformed capsid as deletion completely abolishes MDV replication (Greco <i>et al.</i> , 2014). In a second function, sTMR increases the efficiency of integration (Greco <i>et al.</i> , 2014).
MDV5/76	2*	R-LORF7, MEQ protein	N	Lupiani <i>et al.</i> 2004 reported meq is dispensable for viral replication (Lupiani <i>et al.</i> , 2004b). However, it is involved in the replication ability (V. Nair).

MDV20	3*	DNA helicase-primase associated protein, HSV-1 UL8-like protein	Y, essential	HSV-1 replication machinery includes a trimeric helicase primase complex of UL5, UL52 and UL8. UL8 influences the cellular location of the complex and may facilitate the loading of the complex onto the replication fork (Bermek <i>et al.</i> , 2017).
MDV21	4*	Origin binding protein, HSV-1 UL9-like protein	Y, essential	UL9 is thought to initiate DNA replication by binding the origin of replication. UL9 with UL5/UL52/UL8 are considered essential to HSV replication (Chattopadhyay <i>et al.</i> , 2006). Is conserved within the <i>alpha</i> herpesvirus and the Roseolovirus of betaherpesvirinae. (Inoue <i>et al.</i> , 1994).
MDV27	5*	DNA packaging protein (terminase), HSV-1 UL15-like protein	Y, essential	UL15 is believed to cleave the viral genome and package the viral genome into capsids (Baines <i>et al.</i> , 1994).
MDV6.5(132BP)/75.2	6*	N/A	N	Function unknown but an increased number of repeats is seen in serial cell passage. Additionally, when the 2 132-bp repeats in pathogenic MDV were deleted MDV remained pathogenic, suggesting MDV6.6/75.2 is not essential (Silva <i>et al.</i> , 2004). In our study CVI988 sequence showed 2 repeats present at MDV 6.5 (TRL) and 2 at MDV75.2 (IRL), The guide also targeted 2 sites.
MDV13/13.5	7*	Virion surface glycoprotein L, HSV-1 UL1-like protein, LORF4 binds MHC class II beta chain	Y, essential	LORF4 is unique to MDV-1 and not present in MDV-2 or HVT and produces a glycoprotein, gL (Liao <i>et al.</i> , 2021a). It has been identified to interact with the MHC class II beta chain, however, remains to be fully studied (Niikura <i>et al.</i> , 2004) (Liao <i>et al.</i> , 2021a). A study has also presented evidence that LORF4 is dispensable for virus growth <i>in vitro</i> (Iwai <i>et al.</i> , 2001). HSV-1 studies have shown no immunological response to gL (Ghiasi <i>et al.</i> , 1994). gL is thought to act as a chaperone for gH localisation to the cellular membrane (Kaye <i>et al.</i> , 1992). The formation of the gH:gL complex provides one route of entry into host cells and neutralising antibody has been observed for gH:gL complex (Reske <i>et al.</i> , 2007).
MDV28	8*	Tegument protein, HSV-1 UL16-like protein	Y, essential	UL16 has been reported to be involved in oxidative phosphorylation of glucose consequently increasing cellular ATP content, to provide additional energy for viral particle production (Li <i>et al.</i> , 2021a). The UL16 tegument protein accumulates on the cytoplasmic face of intracellular membranes and maybe involved in the nuclear egress of capsids (Gao <i>et al.</i> , 2017). Additionally, it has been reported UL16 is dispensable from HSV-1 in cell culture (Baines and Roizman, 1991).
MDV31	9*	Major capsid protein, HSV-1 UL19-like protein	Y, essential	UL19 forms proteins that form the capsid shell, and siRNA knockdown of UL19 <i>in vitro</i> reduced virus replication (Jin <i>et al.</i> , 2014).
MDV37	10*	DNA packaging, HSV-1 UL25-like protein	Y, essential	UL25 is thought to play a role in the capsid, most likely in the later stages to ensure DNA is retained before egress to the cytoplasm (Stow, 2001).
MDV23	11*	Myristylated tegument protein HSV-1, UL11-like protein	Y, non-essential	UL11 a tegument protein of HSV-1 and conserved among all herpes viruses, UL11 conducts myristylation and palmitoylation. UL11 accumulates on <i>trans</i> -golgi network membranes to assist in virion envelopment to form infectious virions (Baird <i>et al.</i> , 2010).

MDV18	12*	Minor capsid protein, DNA packaging, HSV-1 UL6-like protein	Y, essential	UL6 is necessary for the processing and packaging of replicated viral DNA and is a minor protein component of the capsid. UL6 mutants have been seen to fail to produce mature capsids or infectious viruses (Patel <i>et al.</i> , 1996).
MDV39.5	13*	LORF5 (miRNA-2), a hypothetical protein	N	The <i>alphaherpesvirus</i> duck plague virus (DPV) has 78 ORFs with LORF2,-3,-4,-5 and SORF3 genes unique to avian herpesviruses. A study has revealed that LORF5 aids in cell-cell transmission but is not relevant to replication (Shen <i>et al.</i> , 2021). MDV39.5 overlaps with the start of MDV 40 (gB) otherwise known as glycoprotein B which has been shown to be essential for virus entry (Chi <i>et al.</i> , 2013).
MDV38	14*	Protease, a minor capsid scaffold protein, HSV-1 UL26-like protein	Y, essential	In HSV-1 UL26 encodes a protease essential for the formation and maturation of the virus capsid (Register and Shafer, 1996).
MDV39	15*	Minor capsid scaffold protein, HSV-1 UL26.5-like protein	Y- essential	The substrate of the protease encoded by UL26 is involved in capsid formation (Register and Shafer, 1996) (Thomsen <i>et al.</i> , 1995).
MDV4/77/5.1/77.5	16*	R-LORF6, binds alpha-enolase, 23 kDa nuclear protein	N	The genomic sequence has three overlapping ORFs <i>meq</i> , 23 kD and RLORF6 and maybe involved in virulence (Spatz and Silva, 2007).
MDV20.5	17*	Origin binding protein, HSV-1 UL8.5-like protein	N	UL8.5 has been identified as the origin-binding protein C-terminal 2 (OBPC-2) and OBPC-2 can bind to the origin DNA. It has been proposed with OBPC-1 act to downregulate viral replication <i>in vitro</i> (Link and Schaffer, 2007). Overlaps with MDV-21, suggesting these guides maybe perturbing the function of that gene too.
MDV22	18*	Virion membrane glycoprotein M, HSV-1 UL10-like protein	Y, non-essential	UL10 encodes glycoprotein M and has been identified to be present in the virion and plasma membrane of infected cells (Baines and Roizman, 1993). Evidence shows that gM is non-essential for viral growth in many herpes viruses with only a few exceptions in <i>alphaherpesviruses</i> such as HSV-1 where deletion attenuates viral growth (Baines and Roizman, 1991, MacLean <i>et al.</i> , 1993, Dijkstra <i>et al.</i> , 1996, Striebinger <i>et al.</i> , 2016). gB, gH, gL, gM and gN are all conserved transmembrane proteins of herpesviruses suggesting their importance in viral growth (Striebinger <i>et al.</i> , 2016).
MDV11.5	19*	N/A		
MDV19	20	Capsid protein, DNA packaging/cleavage, HSV-1 UL7-like protein	Y, non-essential	The UL7 amino acid sequence is highly conserved and found in all <i>Herpesviridae</i> subfamilies (Johannsen <i>et al.</i> , 2004). Located between the essential UL6 and UL8 genes for virus replication in cell culture (Patel and Maclean, 1995). With the use of UL7 null HSV-1 mutant virus it was shown both plaque size and the number of progeny were reduced (Tanaka <i>et al.</i> , 2008).

Conserved genes in *alpha*-, *beta*- and *gamma*- *herpesviruses* are indicated by 'Y' for conserved and 'N' for non-conserved. *alpha*-, *beta*- and *gamma* *herpesviruses* genes are essential or non-essential for replication *in vitro* (Fields, 2007).

Rank in order of statistical significance, smallest to largest. Astrisk (*) in rank column indicates statistical significance compared to non-targeting sgRNA US2-HVT.

N/A = gene not defined in literature.

Table 20. Table of top 20 essential genes from the screen of CVI988 genes.

Non-Essential CVI988 Genes				
Largest to smallest	Rank	Other names/role	Conserved gene in α -, β - and γ -herpesviruses	Function/Additional details
MDV81.5/102.5	1*	N/A	N	
MDV3/78	2*	R-LORF2; chemokine, vIL8	N	vIL8 is an IL-8 homologue, functionally vIL-8 aids in the recruitment of immune cells to the site of infection in which MDV preferentially binds (Parcells <i>et al.</i> , 2001).
MDV2/79	3*	R-LORF1; HSV-1 ICP0-like protein	N	MDV-2 encodes the RNA component of the telomerase enzyme, vTR this telomerase RNA component interacts with the protein component TERT, and the collaboration of these components determines catalytic activity and telomere-binding ability of telomerase (Fragnet <i>et al.</i> , 2003). However, it has been seen to be dispensable for replication <i>in vitro</i> and lytic replication <i>in vivo</i> (Trapp <i>et al.</i> , 2006). HSV-1 ICP0 is believed to regulate the viral lifecycle between the lytic and the latent state. Additionally, it has been reported ICP0 mutants possess viral growth inhibition (Smith <i>et al.</i> , 2011).
MDV84.5/99.5	4*	N/A	N	

MDV3.4/78 .3	5*	R-LORF4 (miRNA-6)	N	R-LORF4 is found within the 4.2 k.b fragments of the IRL/TRL region. Northern blotting indicates this region is transcriptionally active during latency. Therefore, may have a role in gene regulation (Yao <i>et al.</i> , 2007). R-LORF-4 is conserved to MDV-2 only (Yao <i>et al.</i> , 2007). When using GFP and mRFP insertion into RLORF4 showed no reduction in pathogenicity (Jarosinski <i>et al.</i> , 2015). RLORF4 is likely to mediate MDV virulence by inhibiting the interferon- β responses (Liu <i>et al.</i> , 2019).
MDV3.8/78 .1	6*	Transcript identified in MD lymphoblastoid chicken cells	N	
MDV85/99	7*	N/A	N	
MDV56	8*	Probable membrane protein, HSV-1 UL43-like protein	N	UL-43 HSV-1 mutants have been observed to have reduced plaque sizes and lower entry kinetics (Liu <i>et al.</i> , 2016).
MDV2.6/78 .5	9*	N/A	N	
MDV57.4	10*	Hypothetical protein		
MDV91	11*	HSV-1 US2-like protein	N	It has been seen that US2 in MDV, HSV-1 and -2 is non-essential and virulence is not altered in cell culture (Cantello <i>et al.</i> , 1991, Jiang <i>et al.</i> , 1998).
MDV3.2/78 .4	12	R-LORF3 (miRNA-10)	N	R-LORF3 is found within the 4.2 k.b fragment of the IRL/TRL region. Northern blotting indicates this region is transcriptionally active during latency. Therefore, may have a role in gene regulation. R-LORF-3 is conserved to MDV-2 only (Yao <i>et al.</i> , 2007).
MDV81/10 3	13	N/A		
MDV64	14	Envelope/tegu ment protein,	Y, essential	Has been shown that deletion of either the UL49.5 homologous gene or its complex partner UL10 (gM) resulted in only single cells positive for viral infection. Concluding that either of these genes is critical in viral growth and spread in cell culture (Tischer <i>et al.</i> , 2002).

		HSV-1 UL49.5-like protein		
MDV57.8	15	LORF8, 23 kDa protein	N	R-LORF8 contains a miRNA cluster including the homologue to miR-155, and miR-M4 is thought to play a role in lymphomagenesis (Zhao <i>et al.</i> , 2009a).
MDV72.2	16	N/A		
MDV52	17	Ribonucleotide reductase, large subunit, HSV-1 UL39-like protein	Y, non-essential	UL39 encodes a ribonucleotide reductase (RR), HSV-1 RR is not essential for viral growth in cultured cells when cellular RR is active (Goldstein and Weller, 1988). However, in non-diving cells, the knockdown of UL39 was sufficient to inhibit HSV-1 replication (Zhe <i>et al.</i> , 2008).
MDV54	18	Tegument protein, virion shutoff protein, HSV-1 UL41-like protein	N	UL41 is an endoribonuclease tegument protein, which has been shown to decrease anti-viral IFIT3, thus providing immune evasion (Jiang <i>et al.</i> , 2016). Additionally, UL41 shortens the half-life of host mRNAs redirecting protein synthesis to viral proteins (Su <i>et al.</i> , 2015).
MDV2.3/78.6	19	N/A	N	
MDV72	20	LORF11	N	Is found in all 3 serotypes of MDV. Has been deleted using cosmid technology, the <i>in vitro</i> growth remains comparable to WT suggesting LORF11 is non-essential to virus replication in cell culture. However, <i>in vivo</i> deletion of LORF11 produced an attenuated virus (Lee <i>et al.</i> , 2007).
Conserved genes in <i>alpha</i>-, <i>beta</i>- and <i>gamma</i>- herpesviruses are indicated by 'Y' for conserved and 'N' for non-conserved. <i>alpha</i>-, <i>beta</i>- and <i>gamma</i> herpesviruses genes are essential or non-essential for replication <i>in vitro</i> (Fields, 2007). Rank in order of statistical significance, smallest to largest. Astrisk (*) in rank column indicates statistical significance compared to essential gene glycoprotein g sgRNA. N/A = gene not defined in literature.				

Table 21. Table of top 20 non-essential genes from the screen of CVI988 genes

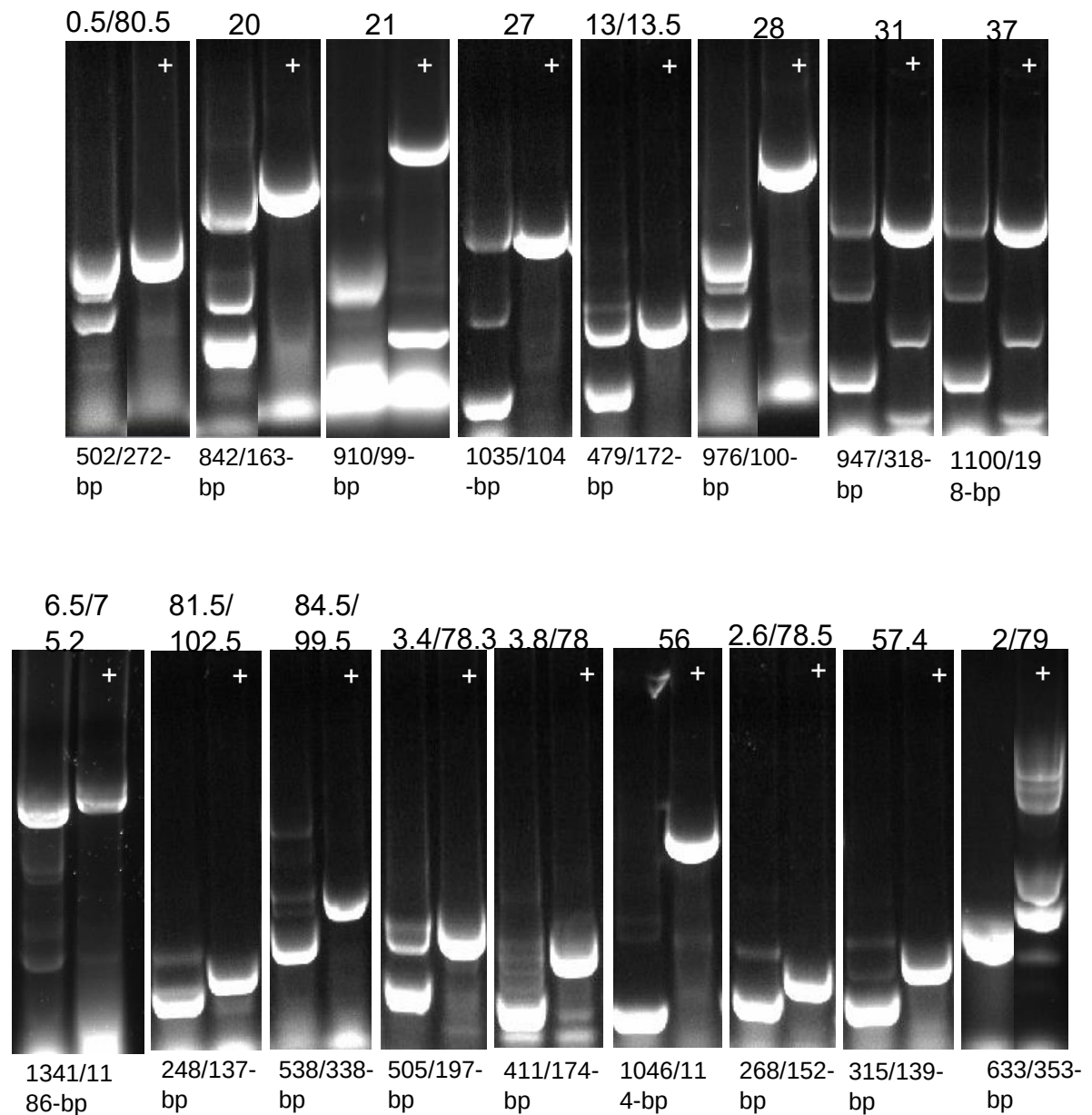


Figure 16. PCR identification of gene editing in the top 10 essential and non-essential genes from CVI988 screen for genes essential/non-essential in MDV replication and spread *in vitro*. DF1-Cas9^{Blast} cells were transfected with a total of 30 nM sgRNA per well of a 12-well plate, at the same time 40 μ L of CEF cells infected with CVI988-tk-mCherry were added to the same well. After 5 days of incubation the cells of the wells were harvested and gDNA extracted, this was used in PCR reaction using primers that flanked the edited region of each gene. The lower band shows a shift in the PCR amplicon indicating successful editing by two or more of the sgRNAs. A shorter amplicon in each case indicates deletion of the nucleotide sequence of the specified gene. Below indicates the expected size of each PCR amplification band.

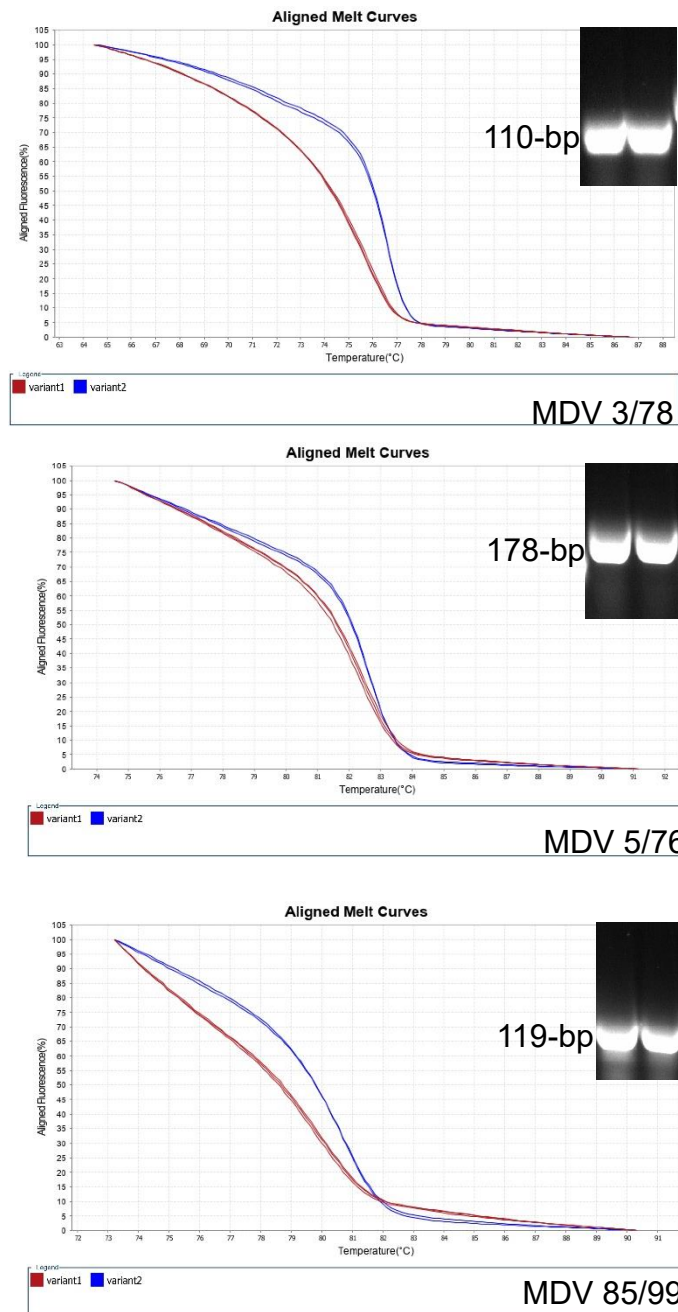


Figure 17. High-Resolution Melt Curve analysis to test sgRNA editing in genes too short to be targeted by deletion. Most genes can be targeted by 3 sgRNAs allowing PCR amplification to identify deleted sections of the genome to confirm editing. However, due to the length of some genes and that genes overlap across the genome this is not always possible. To gain an indication to whether a single sgRNA has made an edit in the MDV genome HRM can be used which detects the small edits made by a single sgRNA. Curve separation from the WT indicates sgRNA-mediated gene editing at the amplified site.

3.3.6 MDV gene deletion in MSB-1 cell line and reactivation assays

The CVI988 gene screening approach had been seen to be repeatable among biological replicates (figure 15). I wanted to confirm the gene essentiality of some high-ranking essential genes to confirm the phenotype was correct using a different experimental approach. I first picked 3 genes that were of interest, MDV11.5 had little literature explaining its role in MDV infection, MD20 was well characterised as an essential gene in MDV replication and spread in cell culture and MDV19 was also seen to have little corresponding literature and was not significant in the screen, therefore was of interest; to see if insignificant genes could still affect replication in an alternative assay (table 20).

The MDV transformed lymphoblastoid cell line MSB-1 (Akiyama and Kato, 1974, Nazerian and Witter, 1975) has been previously reported to be latently infected with both MDV-1 strain BC-1 and MDV-2 strain HPRS-24 (Hirai *et al.*, 1990, Yao *et al.*, 2007). Treatment of MSB-1 cells with sodium butyrate and subsequent co-culture on CEF monolayers causes MDV reactivation in MSB-1 cells and subsequently forming plaques on the CEF monolayers. I hypothesised that deletion of essential MDV genes would produce zero/fewer plaques compared to WT MSB-1 co-cultured CEF cells. To test this, I assessed the infectivity of the gene-deleted MDV MSB-1 cells by co-culturing with CEF monolayers after treatment with histone deacetylase inhibitor sodium butyrate to increase the latent to lytic switch and therefore, virus reactivation and plaque formation.

Gene-deleted cell lines were constructed using sgRNAs cloned into the px330a vector. Two sgRNA expressing px330a were electroporated into MSB-1 cells. Subsequently, these cells were single-cell sorted into 96 well plates and screened for gene-deleted cell clones using primers targeting DNA outside the deleted region. PCR confirmed pure edited cells were obtained, where the smaller band represents the edited cell clones in all cases (figure 18, table 10 for expected sizes), which was also corroborated by sequencing (appendix 8.1.1).

Immunofluorescence assay (IFA) was used to identify the virus plaques formed on CEF, using the anti-pp38 MAb BD1 as the primary antibody for MDV-1 and anti-MDV-2-gB MAb Y-5 antibody for MDV-2. I observed that edited MDV-19 and 20 genes produced far fewer plaques than WT cells ($p\text{-value} = \leq 0.05$), confirming these genes are required for effective virus reactivation *in vitro* (figure 19).

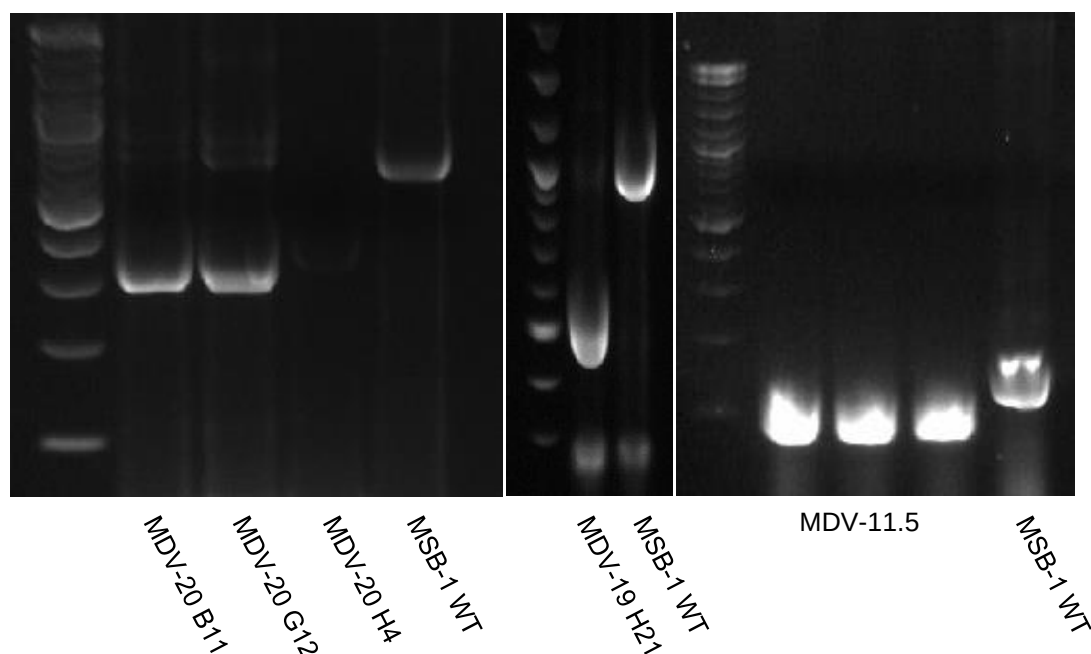


Figure 18. PCR confirmation of deleted viral genes in MSB-1 cells. sgRNAs targeting the 5' and 3' ends of the targeted gene were cloned into lenti U6-sgRNA/EF1a-mCherry expression vector and transfected into MSB-1-cas9 cells, FACS was used to isolate single cells with sgRNA expression. These clones were then screened using PCR for deletion of the respective genes. The lower amplicon shows successful deletion of target MDV gene.

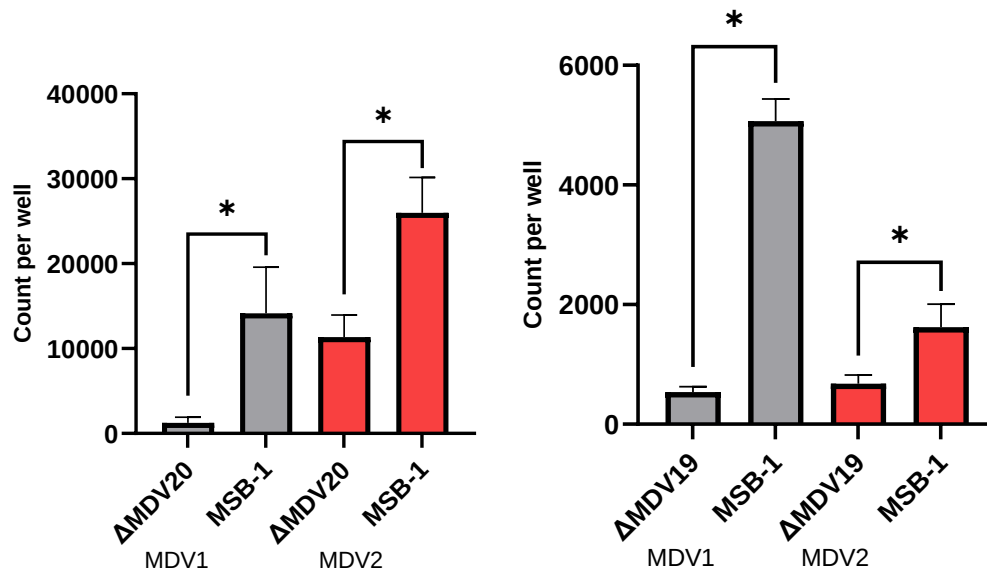


Figure 19. Reactivation of MDV in MSB-1 cells compared to reactivation in edited MSB-1 cells. sgRNAs targeting the 5' and 3' ends of the targeted gene were cloned into lenti U6-sgRNA/EF1a-mCherry expression vector and transfected into MSB-1-cas9 cells, FACS was used to isolate single cells with sgRNA expression. These clones were then screened using PCR for deletion of the respective genes. The WT and edited clones were treated with sodium butyrate for 48 h, to trigger MDV expression. These cells were then plated on CEF monolayers and incubated for 5 days. The cells were then fixed and stained for MDV-1 and MDV-2. Mean $\pm$ SD is shown. Asterisk (*) indicates unpaired t-test p -value < 0.05 $n = 3$.

3.5 Discussion

CRISPR-Cas9 gene-editing techniques have rapidly become widespread in microbiology to interrogate genotype-phenotype associations due to their low cost, specificity, flexibility, and ease of use. Manipulating viral genomes has become a desirable technique for functional genomics including non-coding RNAs, the development of recombinant vaccines and applications in gene therapy. Bacterial artificial chromosomes (BACs) have made the propagation of strictly cell-associated viruses like MDV significantly easier. Previously, BAC cloning has allowed the insertion of avirulent vaccine strain CVI988 (Rispen) in a BAC cassette (Petherbridge *et al.*, 2003), this BAC-CVI988 product was subsequently modified to contain mCherry at the Thymidine kinase (TK) region of the BAC construct. These fluorescent viruses have enabled easy quantification of virus replication and spread in cell culture. Combining this with CRISPR-Cas9 gene editing has enabled easy quantification of virus replication when specific CRISPR-Cas9 viral genomic alterations are made. Viral gene deletion has previously been observed for MDV-1 gene pp38 and MDV-1-miR-M4 gene deletions in lymphoblast cell lines (Zhang *et al.*, 2019a, Zhang *et al.*, 2019b). Here we have re-purposed this approach to make gene deletions in cell culture to subsequently quantify the effects of viral infection and spread.

Transfection of CEF cells with pCVI988-tk-mCherry results in a many plaques. However, when CRISPR components (crRNA/tracrRNA and spCas9) were added to the transfection solution only a few plaques were formed. It was presumed this was due to free spCas9 cutting pCVI988-tk-mCherry outside the cell, where DNA repair mechanisms are not present to re-establish a full-length BAC clone. To combat this, we trialled plasmid-expressed Cas9 so that expression would only occur intracellularly. However, only a few plaques would form and plaque numbers were inconsistent. A solution was to use Cas9-expressing cells, without *in ovo* gene editing of chickens to form Cas9-expressing chickens and then harvesting CEF cells from their eggs, it was decided to use DF1-Cas9^{Blast} cells. However, DF1-Cas9^{Blast} cells were not conducive to plaque formation when compared to CEF cells, not producing enough to be able to quantify significant change. Therefore, rather than transfection of the BAC-CVI988 construct, I trialled co-culture of DF1 with CVI988-tk-mCherry infected CEF cells. Using this method, significantly more cells would become infected. A predetermined number of CVI988 infected CEF cells was co-cultured with DF1-Cas9^{Blast} cells. After 5 days significant viral infection was seen and differences in the permissiveness of cells edited for known essential and non-essential genes were observed (figure 12).

Several free online tools are available for sgRNA design, and ranking guides based on on-target efficiency and off-target activity. However, not all guides that rank highly work well, possibly a consequence of local sequence effects

or epigenetic factors (Isaac *et al.*, 2016). Our lab has successfully used two sgRNA located at either end of a gene or miRNA to knockout MDV genes and MDV miRNAs (Zhang *et al.*, 2019a, Zhang *et al.*, 2019b). Furthermore, it has been shown that very virulent RB-1B MDV replication could be reduced by 50 % when using a single sgRNA targeting essential genes and completely blocked when several are used (Hagag *et al.*, 2020). This study aimed to target each gene with three synthetic crRNAs. Due to the nature of MDV genomes several ORFs overlap or are very short; for this reason, some ORFs could only be targeted by 1 or 2 sgRNAs. Nevertheless, the same volume and concentration of sgRNA were used for each ORF. Due to time considerations, sgRNAs were not validated before their use, a second reason for using multiple sgRNAs.

Some of the most studied genes within MDV and herpes viruses are their glycoproteins since these often determine the tropism of these viruses. Therefore, the investigation of these genes would provide a good estimation of the quality of our screening methodology. All but two glycoproteins (MDV57 (gC) and 94 (gD)) are known to be essential for virus growth in cell culture, which was verified by the screening assay developed here (Tan *et al.*, 2001, Tischer *et al.*, 2005). PCR was performed to check the MDV gene deletions mediated by sgRNAs. All but one showed strong deletion bands and one showed several bands in the edited cells (figure 14). This was again seen in the PCR of the top 10 essential and non-essential genes (figure 16). This maybe explained by the targeting of a gene with several guides, therefore introducing the possibility of being 3 PCR bands depending on the sgRNAs that mediate a cut in the genome, since the well contains many viruses, these may all be edited in unique ways producing several band amplicons (figure 14 and 16).

As the glycoprotein screen produced the expected results, a complete screen of all ORFs was completed using three biological replicates. For the top non-essential and essential genes (table 20 and 21), I employed PCR, HRM and sequencing to assess if these results were artefacts of successful deletion. This is particularly important for non-essential genes to prevent false positives from being selected for further investigation. All ORFs were verified by one or more of the stated methods (figure 14,16 and 17).

The results indicate a clear variability in plaque formation (figure 15). For reference controls, gB (essential gene), pp38, meq and HVT-US2 (non-targeting control guide) were used that had been previously verified in the lab (data not shown) and by others (Lupiani *et al.*, 2004b, Chi *et al.*, 2013, Zhang *et al.*, 2019a). 11 non-essential genes and 19 essential genes were found to be statistically significant ($p\text{-value} < 0.05$). However, it is likely other genes beyond the < 0.05 $p\text{-value}$ threshold are likely to elicit a noticeable effect on MDV replication and spread in cell culture. This is evidenced by MDV19 gene in the MDV reactivation assay (figure 19) which was shown in the screen to

not be significant but produced significantly fewer plaques in the MSB-1 reactivation assay (*p-value* 0.05). It may require more biological repeats to reduce the standard deviation between replicates to increase the number of genes considered statistically significant. Another protocol alternation that may have reduced variation among biological repeats was to increase the number of CVI988-tk-mCherry genomes delivered by CEF co-culture to the well of DF1-Cas9^{Blast} cells. This may have the impact of creating more plaques thus reducing the impact of variation among repeats.

The MDV genes meq and pp38 have both been seen to be dispensable for efficient viral replication and spread in cell culture (Reddy *et al.*, 2002b, Khedkar *et al.*, 2018). Therefore, inferences could be made on gene essentiality based on these controls, any gene inline or below gB could be considered essential and any genes inline or above positive control/HVT-US2 were considered non-essential (figure 15). Additionally, any genes above or in line with meq/pp38 are also likely to be non-essential to viral replication but the reliability of inferences made from meq/pp38 should be made with care.

A literature search demonstrates essential genes are predictably more studied than non-essential genes, supporting the efficacy of the screen, with many of the essential genes identified in this study having been previously validated (tables 20 and 21). For the top 20 genes on the essential end of the spectrum, many (8/20) were involved in the viral nucleocapsid and tegument formation and function (table 20). The nucleocapsid is critical in herpesvirus biology, they ensure the pressurised double-stranded-DNA genomes, they ensure the delivery of the contents to defined locations and they have a key role in the replication cycle (Draganova *et al.*, 2021). The tegument is a matrix layer positioned between the nucleocapsid and the viral envelope, which contains several tegument proteins. These tegument proteins have been observed to be indispensable for the construction of mature virions, playing key roles in virion envelopment and virion trafficking (Guo *et al.*, 2010). Therefore, considering this evidence and the evidence presented in table 20 it was not surprising to have many essential genes related to capsid and tegument function. Two further genes of the essential 'top 20' were directly related to DNA replication, being involved in DNA helicase and origin of replication binding, furthermore demonstrating the screen had identified key factors involved in CVI988 replication and spread *in vitro*. There are 8 MDV glycoproteins known with two glycoproteins in the list of top 20 essential genes glycoprotein L (gL/MDV13) and glycoprotein M (gM/MDV22). Glycoprotein L (gL) is the only glycoprotein seen to have sequence variation depending on the virulence of the strain and is essential for the surface expression of glycoprotein H (which was ranked as the 46th most essential gene). gH is a highly conserved glycoprotein associated with cell-cell spread in MDV (Wu *et al.*, 2001, Santin *et al.*, 2006). gM has been seen along with its putative partner UL49.5 (MDV64) to be essential for MDV-1 growth in cultured cells (Tischer *et al.*, 2002). Although gM was a statistically significant

essential gene in the screen, UL49.5/MDV64 was seen to be one of the least essential genes (table 21), which was one of the contradictory results of the study, as previously it has been suggested that UL49.5 is essential for alpha-, beta- and gamma-herpesviruses replication, thus further investigation is required to determine the validity of this result (Fields, 2007). It has been reported that glycoproteins B, E and I are indispensable for MDV-1 spread from cell to cell (Schumacher *et al.*, 2000, Schumacher *et al.*, 2001). However, in our screen both gE and gI ranked low in terms of essentiality, ranking at 86 and 113 respectively. Additionally, in our study gB ranked at position 27 (control gB sgRNAs) and 56 (screen-designed sgRNAs) in terms of essentiality. This highlights large variations between sgRNAs targeting the same gene and results between studies. Highlighting the requirement for validation of genes once shortlisted as essential or non-essential, the need for more repeats to increase confidence and the requirement for gene KO to be checked before inferences are made.

Perhaps a surprising result of the essential gene shortlist is Meq since it had previously been described as dispensable for virus replication by Lupiani *et al.* (2004), although the viral quantification method was crude and only showed selected plaques. However, in our screen, Meq was seen to be the 2nd most 'essential' gene for MDV replication and spread in cell culture. This maybe a result of Meq's role as a major transcription factor and thus may influence MDV's ability to form an effective replication cycle. Finally, the top essential gene was MDV0.5/80.5 which encodes the viral sTMR an essential component for MDV to integrate into the host cell genome and, therefore expected to rank highly among the essential genes (Greco *et al.*, 2014).

Some of the genes identified as non-essential genes may possess important functions *in vivo*; these include MDV3/78 (vIL-8) and MDV57.8 (miRNA-M4) which are involved in B/T-cell recruitment to the infection site and lymphomagenesis respectively (Parcells *et al.*, 2001, Zhao *et al.*, 2009a). A vIL-8 deletion study has shown vIL-8 to be involved in the early stages of pathogenesis, presumably due to the chemoattraction of T and B-cells, since chicken B-cells and activated CD4⁺ T-cells are the main targets for MDV. Furthermore, it has been indicated that vIL-8 impairs viral replication in lymphoid organs by impairing pp38 (lytic protein) expression (Cui *et al.*, 2005). *In vivo*, it has also been observed that deletion vIL-8 attenuated the virus (Parcells *et al.*, 2001, Cui *et al.*, 2005). Although in cell culture vIL-8 is shown to be non-essential, it would be expected to somewhat differ in an *in vivo* experiment.

One consideration worth thought for future screens is the use of controls in the assay, although we used non-targeting sgRNA in the CVI988 screen perhaps an improvement to this would be to design sgRNAs targeting a non-coding region within the CVI988 genome. The principle behind this is that the BAC-CVI988-tk-mCherry construct is then cut by targeted Cas9 protein,

allowing for subsequent endogenous cellular repair mechanisms to repair the complete genome. Thus, this would represent a more faithful control to the sgRNAs targeting coding regions, perhaps adding in better downstream statistical analysis.

Using MSB-1 cell co-culture assays the validity of the screen could be corroborated. Edited cells would be expected to show a statistically significant reduction in MDV-1 infected CEF cells and this was observed in all edited cell lines with most showing no viral plaques, confirming the functional effects of MDV-1 gene deletions. MSB-1 cells are also latently infected with the avirulent MDV-2. During the reactivation assay, MDV-2 CEF infection was observed to decrease in all edited cell lines compared to WT, however, this was not nearly as pronounced as MDV-1. This suggests MDV-1 edited genes may also reduce MDV-2 replication capability and spread, but it is possible that homologous genes in MDV-2 may compensate.

3.6 Conclusion

In this study, we presented a CRISPR-Cas9 screen to assess the essentiality of viral genes. Such a method may apply to other viruses, especially where a fluorescent virus has been engineered, efficient viral antibodies are available, or CPE can easily be quantified as a measure of viral infectivity. Revealing essential genes in viruses may highlight understudied genes that may have a significant role in viral replication and spread. Here we have shown proof that an arrayed screen can be used to identify viral genes essential to the replication and spread in cell culture. Some genes were expected such as sTMR and gL but others such as Meq and UL49.5 were less expected results. Including the understudied genes MDV11.5 and MDV6.5(132-bp)/75.2) that were shown to be essential. Future work should look at validating these prementioned genes, to reveal whether the genotype-phenotype correlation suggested here is repeatable in cell culture.

These data could be used to inform new avenues of research, perhaps leading to improved vaccines. Additionally, the identification of non-essential genes may allow improvements in vaccine viruses. For example, deleting non-essential genes would reduce the size of the virus genome perhaps making viral replication more efficient or allowing other immunogenic sequences to be substituted without affecting the viral replication.

Chapter 4: HP8 essential gene screen

4.1 Summary

The functional role of a gene can be investigated through the manipulation of a gene's expression, either by overexpression or by reducing its expression. The reduction of gene expression can be used to reveal phenotypic traits associated with a gene, such as a role in cell proliferation. MDV-transformed cell lines, derived from MD lymphomas, have been studied extensively to explore gene functions associated with oncogenicity and MDV latency. Many studies have investigated the requirements for cell proliferation in cancer cell lines using Genome-Scale CRISPR Knock-Out (GeCKO) approaches. However, this method has not yet been employed to study MDV-infected T-lymphocyte cell lines. In this chapter, I perform a GeCKO screen of host genes on a latently infected oncogenic MDV-transformed cell line HP8 with the aim of revealing the genetic determinants of the transformed phenotype. The screen revealed a strong dependency on mitochondrial-associated genes as well as IRF4, a gene also shown to be essential in EBV-transformed cell lines.

4.2 Introduction

The deregulated proliferation of lymphoblastoid cells results from abnormal regulation of oncogenes and/or tumour suppressor genes. Given that the primary virulence outcome of MDV is lymphoma involving multiple organs, cancer-vulnerable genes are of interest. The MDV genome has been well studied to date, particularly the MDV virulence genes involved in all aspects of the life cycle. However, less is known about how the host genes contribute to the phenomenon of MDV reactivation from the latent phase and the maintenance of the transformed phenotype. Several studies have looked to uncover genes of interest in the oncogenesis of MDV, using assays such as the microarray analyses of host cell gene expression in CEF cells infected with MDV-1 strain RB1B (Morgan *et al.*, 2001). This study showed that macrophage inflammatory protein, interferon response factor 1, interferon-inducible protein, quiescence-specific protein, thymic shared antigen 1, major histocompatibility complex (MHC) class I, MHC class II, b2-microglobulin, clusterin, interleukin-13 receptor alpha chain, ovotransferrin and a serine/threonine kinase were up-regulated by an increase of over 2-fold compared to uninfected CEF cells (Morgan *et al.*, 2001). More recently, a second study using microarray analysis representing 32,773 chicken transcripts identified 269 genes which were differentially regulated in the tissues of MDV-infected chickens in the neoplastic stage of infection compared to uninfected control chickens (Chen *et al.*, 2011). This included the upregulation of genes such as extracellular matrix proteins MMP2 and

the collagen proteins COL1A2, COL6A3 and COL12A1; their increased expression is important for the invasion of tissues and metastasis. AURKA and NEK2 mitotic kinases were significantly upregulated, potentially resulting in unregulated proliferation. Additionally, PTTG1 is involved in the mitotic checkpoint and cell cycle and STAT1 upregulation has been seen to promote transformation (Chen *et al.*, 2011). However, no study to date has systematically analysed the influence of gene knockouts across the whole chicken genome in latently MDV-infected transformed T-cell lines.

CRISPR-Cas9 relies on the Cas9 enzyme guided by sgRNA to mediate loss-of-function mutations and gene inactivation by inducing frameshift mutations in the coding region of DNA. Cancer cell lines are inherently effective for pooled CRISPR screens because they grow well *in vitro* and can easily be expanded to large numbers. Also, the Cas9 enzyme can be stably expressed in immortalised cancer cell lines. Therefore, there is only the requirement for delivery of the much smaller sgRNA, increasing mutation efficiencies. Using survival as the phenotypic readout, such methods provide hits of high confidence. However, these stringent phenotypic selection methods have been seen to reveal far fewer hit genes than comparable RNAi technology due to their intrinsic differences in methodologies (Park *et al.* 2017). Other studies have overcome this problem in the identification of only a few hits by sampling the selected sgRNA population of cells for longer and at multiple time points, thereby increasing the number of hit genes.

Epstein-Barr virus (EBV) is a virus associated with lymphoma. A CRISPR-Cas9 dropout study in EBV lymphoma cells reported the depletion of 87 genes from Burkitt's lymphoma cell line and 57 from the lymphoblastoid cell line related to cell growth and survival. This comparative study demonstrates the effectiveness of genome-wide loss of function studies to identify host dependency factors for cancer cell growth in a related virus. Some key genes identified included MDM2 and MDM4, proteins involved in p53 regulation and thus tumour suppression. CD19 and CD81 were identified as critical for LMP21-mediated AKT pathway activation which protected against TNF-alpha programmed cell death and IRF4 was seen to be critical for lymphoblastoid cell line MYC induction (Ma *et al.*, 2017b). Yet again a similar approach has been applied to HHV8 transformed cell lines (Mesenchymal stem cells), the major finding being XPO1 being important in cell proliferation and survival through modulating p53 (Gruffaz *et al.*, 2019). A second CRISPR-Cas9 screen study on HHV8-infected test-immortalized microvascular endothelial (TIME) cells, reports many genes localising to the mitochondria being critical in the HHV8-induced tumour cells. The same study shows how direct inhibition of mitochondrial respiration increases death in HHV8 latently infected cells (Holmes *et al.*, 2020). An example in a livestock species was presented by Tan *et al.* (2020) who used the Bovine Herpes Virus Type 1 (BoHV-1) to infect an adherent kidney cell line (MDBK), identifying two membrane tethering complexes involved in viral entry and egress. EBV, HHV8 and BoHV-1 are all members of the *Herpesviridae* family, but each study represents a different cell type. EBV is associated with B-cells, HHV8

Mesenchymal stem cells and BoHV-1 MDBK cells (an adherent kidney cell line) which contrasts with the T-cells used in MDV infection.

Several studies have used dropout CRISPR/Cas9 screens to identify genes and specific pathways which facilitate oncogenesis including virally induced cancer and cell fitness (Hart *et al.*, 2015, Tzelepis *et al.*, 2016, Ma *et al.*, 2017b, Gruffaz *et al.*, 2019, Tan *et al.*, 2020a, Tian *et al.*, 2023). Each of these studies has used a CRISPR-Cas9 loss-of-function method, showing clearly that CRISPR-Cas9 loss-of-function studies have produced validated results while studying cancers, including herpesvirus-induced cancers (Ma *et al.*, 2017b, Gruffaz *et al.*, 2019, Tan *et al.*, 2020a, Tian *et al.*, 2023). Additionally, CRISPR-Cas9 editing of Marek's disease transformed cell lines MDCC-HP8 and MDCC-MSB-1 has been seen to be successful (Zhang *et al.*, 2019a). The aforementioned evidence suggests a CRISPR-Cas9 screen could be performed on HP8 cells. We hypothesised that applying a genome-wide loss-of-function screen to HP8 cells would reveal genes essential in maintaining the transformed phenotype and latency in HP8 cells.

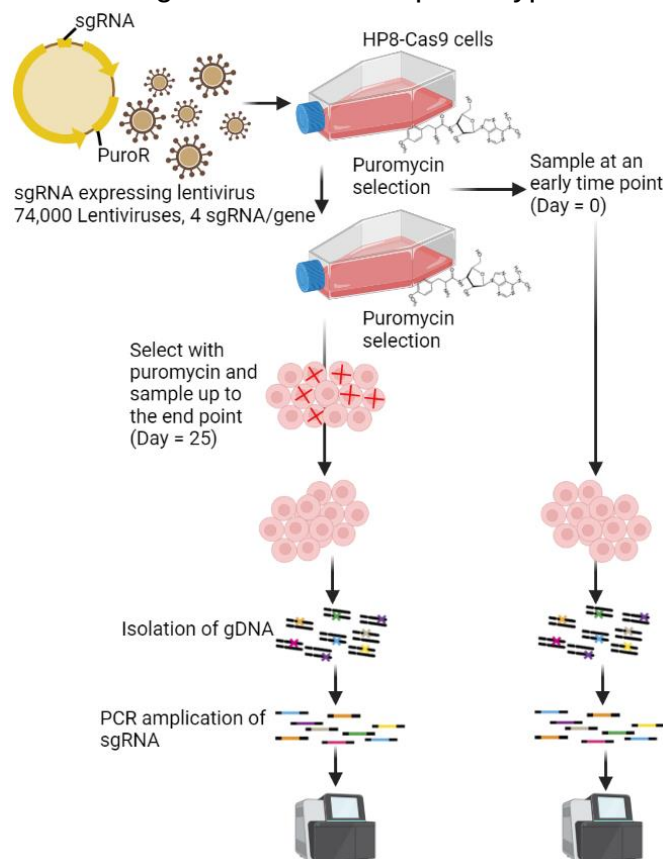


Figure 20. HP8 GeCKO screen workflow and screening strategy. The screen used a pKLV2 chicken CRISPR library of sgRNAs that were used to transduce HP8 cells. This cell population was then selected with the pKLV2 selection marker puromycin to remove untransduced cells. The cells were then selected for 25-days. 4 samples were collected post selection at 3, 8, 14 and 25 days. gDNA was extracted and PCR was then used to amplify the

CRISPR DNA which was sent for sequencing to identify genes depleted in the population. Created with BioRender.

4.3 Aim of Chapter

One major goal of this project was to investigate host cell factors that contribute to the transformed phenotype of latently transformed MDV-infected cell line HP8. To achieve this, I employed the use of a Genome-Scale CRISPR Knock-Out (GeCKO) screen. The GeCKO screen is predicted to reveal genes specific to MDV latently infected cells that are required for the maintenance of the transformed phenotype in these cell lines. A long-term outcome was to perform gene ontology analysis to investigate the pathways specific to MDV-infected cells and to validate hit genes involved in the maintenance of the transformed phenotype. Figure 20 summarises the principle of the experiment.

4.4 Results:

When MDV infects a susceptible chicken, its favoured target is CD4⁺ T-cells. MDV can switch between two replication states: lytic and latent, and the latter triggers a series of cellular changes that culminate in no virus replication and oncogenic cellular division. These transformed lymphocytes latently infected with MDV have been clonally isolated and used in Marek's disease research. HP8 cells are one such cell line, which is infected by *Gallid herpesvirus 2* (MDV-1 strain GA) and were used throughout this study.

4.4.1 HP8-Cas9^{Blast} cell line construction

Selection of the optimal delivery system for Cas9 and the library is integral to achieving high editing efficiencies and accuracy (Ford *et al.* 2019). Cas9 protein at 163 kDa is a large protein combining its delivery with sgRNA lentiviral vector leads to low lentiviral titre due to its size. Thus, commonly Cas9 is delivered before the sgRNA library, which as a result can be delivered at a higher titre (Sanjana *et al.* 2014). Expression of Cas9 in this manner requires quantification of Cas9 activity as it is possible that differences in expression, stability and localisation could remove the selectable marker adjacent to the Cas9 protein. Therefore, Cas9 expression should be periodically tested before introducing the sgRNA library (Doench *et al.* 2014). Nevertheless, one of the first genome-wide loss of function studies successfully used the lenti CRISPR-Cas9 construct approach (Shalem *et al.* 2014). Alternatively, a stably expressing Cas9 cell line of your cells of interest can be produced which benefits from higher Cas9 expression and are less likely to drift in its performance over time. However, the integration of Cas9 must not affect the phenotype of interest, for example, proliferation. The success of this form of Cas9 delivery was first demonstrated by Wang *et al.* 2014. Due to the benefits of Cas9 cell lines, this was the chosen method to pursue all CRISPR-Cas9 editing techniques.

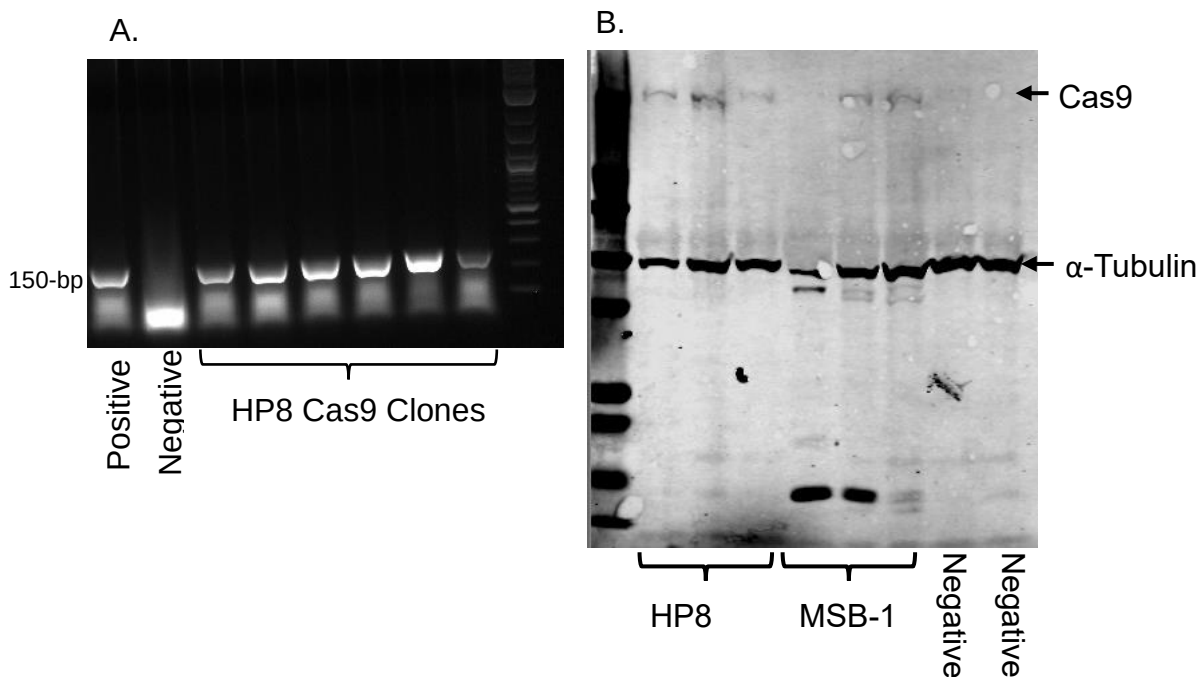


Figure 21. PCR to identify Cas9 sequence in HP8 cells. PCR amplification band at ~150-bp indicates the presence of the Cas9 sequence in those HP8 cells (A). Detection of Cas9 expression by western blotting in HP8-Cas9^{Blast} cells. From single-cell clones, cells were cultured and prepared for Western Blot (B). The blot was probed with an anti-Cas9 monoclonal antibody and as a loading control anti- α -tubulin. Some clones show Cas9 expression.

To generate the HP8-Cas9^{Blast} cells, HP8 cells were electroporated with lentiCas9-Blast, after 48 h cells were selected with 1 mg/mL puromycin for 1 week, and these cells were sorted into 96 well plates. After culturing HP8-Cas9^{Blast} cells were harvested and from 6 clones the genomic DNA was isolated, which was subsequently used in a PCR using primers to target Cas9. A PCR amplification band at ~150-bp indicates the presence of the Cas9 sequence in those HP8 cells (figure 21A). The single-cell clones were subsequently tested by WB to test for Cas9 protein expression. The first 3 positive PCR clones were used in WB. Not all clones showed detectable levels of Cas9 protein despite being positive for Cas9 DNA (figure 21B). To test Cas9 activity within cells the electroporation of 2 sgRNAs targeting viral gB in HP8-Cas9^{Blast} cells was performed and PCR using primers flanking the deleted sequence (figure 22A) was used to show successful editing. A shorter band indicates that the sgRNAs have been directed to make precise cuts of the target gene, proving the Cas9 activity of the HP8-Cas9^{Blast} cells. A second approach was used to validate Cas9 activity using a BFP/GFP reporter construct which consisted of GFP and BFP markers, with a sgRNA targeting BFP. Therefore, Cas9-expressing cells would cause a reduction in

legitimate BFP transcripts and thus a reduction in BFP expression. Several clones were tested using this reporter construct, and the best-performing clone was then used for subsequent assays (figure 22B). Cas9 integration into a cell's genome has the potential to change cellular phenotypes. Therefore, since the phenotype of interest was proliferation, the growth curves of HP8-Cas9^{Blast} were analysed (figure 23). This showed no significant growth defect at 5000, and 7500 cell starting number (p -value > 0.05) however 10,000 cells showed a lag phase. Also, the rate of growth was consistent across all three cell starting numbers, therefore this HP8-Cas9^{Blast} clone was used for subsequent experiments.

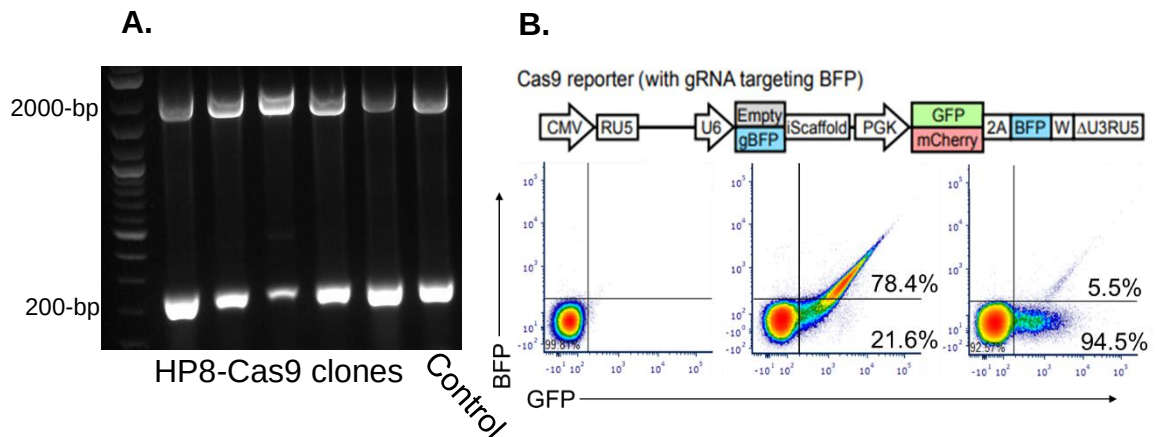


Figure 22. Cas9 activity tested in HP8-Cas9^{Blast} cells. HP8-Cas9^{Blast} cells were electroporated with synthetic sgRNAs gB-C and gB-N. 24 h later the cells were harvested and PCR amplification of gB was performed (A). The presence of active Cas9 in the cells would result in a smaller gB edited band compared to a larger WT band. All clones tested showed significant gB editing comparable to the control lane which used a previously made Cas9 cell line. Analysis of Cas9 activity using Cas9-reporter construct (B). Cas9-reporter lentivirus was prepared and HP8-Cas9^{Blast} cells were transduced. 4 days after transduction the cells were analysed using flow cytometry. The presence of green cells only indicates effective Cas9 activity in those cells. Panel 1 shows untransduced HP8-Cas9 cells, panel 2 shows WT HP8 cells transduced with the GFP/BFP reporter construct and panel 3 shows HP8-Cas9 cells transduced with the GFP/BFP reporter construct. The percentages give indication of the number of cells showing Cas9 activity.

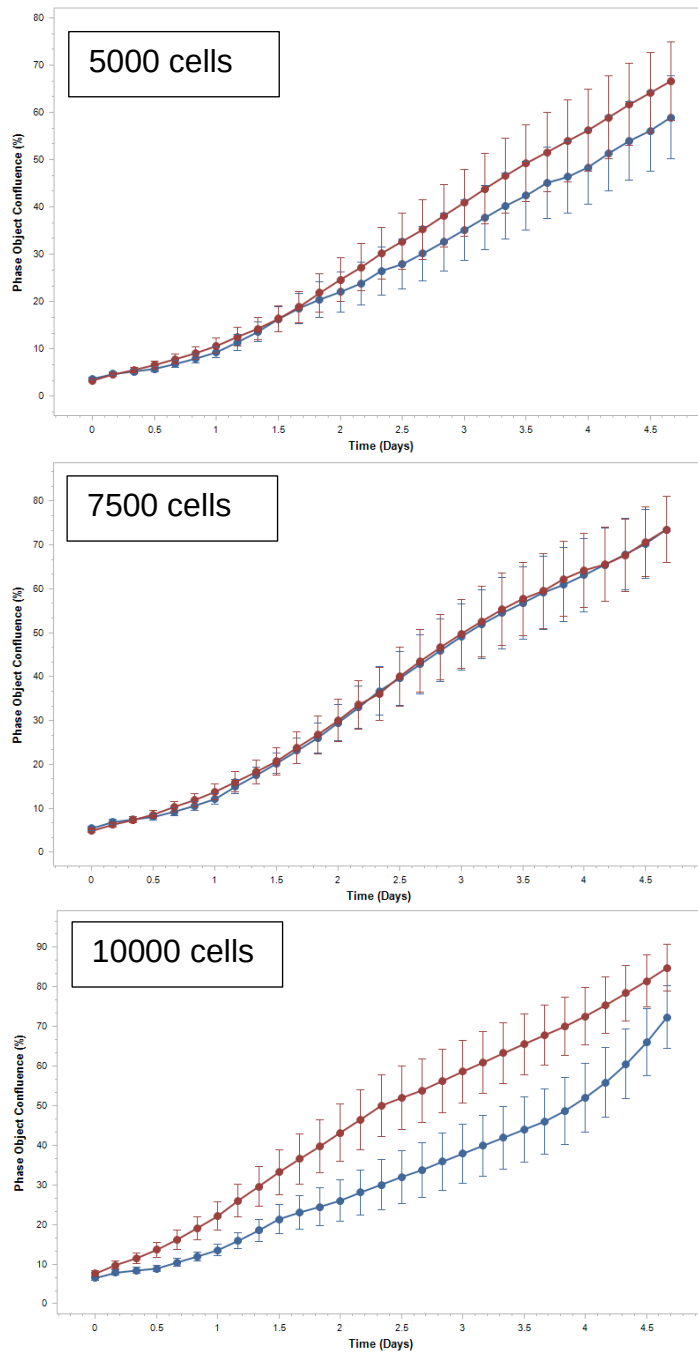


Figure 23. HP8-Cas9 cell growth curve. The growth of HP8-Cas9^{Blast} clone activity was compared HP8 WT cells. Cells were plated in 96-well plate and proliferation tracked in IncuCyte S3 for 4.5 days. Error bars show +/- SD of 5 technical replicates.

4.4.2 HP8 transduction optimisation

Once lentivirus was produced in 293FT cells, 293FT cell media supernatant containing lentivirus particles was used to perform a titration of lentivirus infection on HP8 cells which is identified through the expression of BFP marker, expressed in cells where the lentivirus had successfully integrated. In chicken cells (DF1 cells), we saw reduced transduction efficiency compared to 293FT cells, which would be expected since lentivirus usual target in human cells, and HP8 cells saw very poor transduction efficiency (~2.5%) (figure 24). Considering this I performed several optimisation experiments intending to increase the transduction efficiency:

- Spinfection (figure 25).
- Cell number (figure 26).
- Transduction reagent (figure 27).
- Supernatant harvest time (figure 28).
- 293FT cell passage number (figure 29).
- Fresh or frozen lentivirus (figure 30).
- The concentration of lentivirus (figure 30).
- Transfection reagent (figure 31).

Each of these assays (figures 25-31), used pKLV2 chicken lentivirus, which was transduced into HP8 cells for 48 h before media was changed and incubated for a further 4 days before viral transduction was quantified using flow cytometry to detect the blue fluorescent marker of the pKLV2 plasmids.

The cell type used during this experiment, HP8 cells are suspension cells that remain suspended even after prolonged periods in a cell culture dish. Therefore, suspension cells can often be more difficult to transduce, one common methodology to improve transduction is the use of spinfection. Thus, spinfection was trailed and compared to only using incubation with the infectious lentivirus from dilutions of lentivirus supernatant to HP8 culture media of 1/1, 1/5, 1/10, 1/20, 1/40. A clear observable increase in transduction was seen when using spinfection (figure 25) and therefore it was chosen to continue using spinfection for lentivirus transduction.

Using spinfection requires the use of 6-well cell culture plates in a large tabletop centrifuge. Along with the requirement for large amounts of lentivirus supernatant for transduction and a large number of cells required to infected in a pooled CRISPR screen. For these logistic reasons, it was investigated if using 2 million cells per well of a 6-well plate compared to the standard protocol of 1 million cells per well of a 6-well plate well would negatively affect the transduction of lentivirus into HP8 cells. It was observed that similar levels of transduction were achieved using the 2 million cells, therefore it was chosen to use 2 million cells per 6-well plate well during the transduction of the lentivirus into HP8 cells (figure 26).

For the transduction of lentivirus, there are two reagents commonly used to enhance the transduction of target cells, Dextran and polybrene. These were added to the cell culture along with the lentivirus and incubated for 48 h. Dextran was trialed at concentrations from 0-25 µg/mL and Polybrene 0-14 µg/mL (figure 27). It was observed that there was little difference between the reagents but polybrene at 12 µg/mL was seen to induce the highest lentivirus infection and thus was chosen to be used in the following experiments.

During the lentivirus production process, the lentivirus can be harvested at several time points for Lipofectamine 2000 this was 72 h and 96 h. In order to see at which time point the highest lentivirus titre was achieved, 72 h lentivirus harvest was compared to 96 h lentivirus harvest across 1/1, 1/5, 1/10, 1/20, 1/40. Of the 5 dilutions trialed four showed higher lentivirus transduction rates at a 72 h harvest, only 1/1 dilution was contrary and was suggested to be an anomalous result, probably due to the toxicity of pure lentivirus supernatant from the 293FT cell culture (figure 28). Therefore, to generate high titre lentivirus with Lipofectamine 2000 a 72 h harvest time was selected.

The cells which produce lentivirus particles, 293FT cells, their condition can influence the titre of lentivirus produced by them. One of the main influencing factors involved in 293FT cell condition is the number of passages they have been through. Therefore, it was trialed to see whether this impacted lentiviral titres. Cells that were new were defined as <5 passages were compared to cells that were old which had been passed >10 times. Across the 1/5, 1/10, 1/20, 1/40 dilutions of lentivirus supernatant, it was seen that lower passage cells produced higher lentivirus titres and thus were used in subsequent experiments (figure 29). Again the 1/1 dilution was considered an anomalous result since the toxicity of the pure lentivirus cell medium.

To store lentivirus after it is produced it is often frozen at -75-80 °C, freezing virus is associated with its degradation, particularly over time or if multiple freeze-thaw cycles occur. Therefore, it was trialed whether the effect of freezing the lentivirus or its direct use after harvesting (fresh) would have any implication on the viral titre. It was observed that frozen lentivirus had no negative effect on the viral titre obtained across all dilutions and therefore frozen lentivirus was used subsequently (figure 30). Additionally, ultracentrifugation of the lentivirus supernatant was trialed as a method to concentrate the lentivirus, so that more concentrated lentivirus could be added to the HP8 cells. It was observed that ultracentrifugation increased the transduction of HP8 cells by around 2 times across all dilutions (figure 30).

Several different reagents were available to produce lentivirus, Lipofectamine 2000, Lipofectamine 3000 and Transit lenti. Each of these was used to produce lentivirus according to the manufacturer's instructions and harvested at 48 h and 72 h since these were the suggested optimal harvesting times of each of the reagents. It was observed that the Transit Lenti reagent produced

the highest overall titre over these time points and thus was selected for use thereafter.

The results of these experiments (figure 25-31) informed my future methodology for lentivirus generation at high concentrations. Briefly, this involves the use of Transit Lenti transfection reagent, harvesting of lentivirus at 48 h and 72 h to maximise the amount of lentivirus gained from a single experiment, the use of 2 million cells per well of a 6-well plate, use of frozen lentivirus, use of polybrene at 12 $\mu\text{g}/\text{mL}$ and concentrating lentivirus through ultracentrifugation and then later using Lenti-x concentrator solution as an alternative due to its use at standard centrifuge speeds. The outcome of these adaptations improved the transduction percentage from $< 4\%$ to $\sim 15\%$.

The lentivirus produced would be pooled and titrated on HP8-Cas9^{Blast} cells. Titration allowed for the estimation of the MOI of the target cell. For the HP8 screen, I aimed for $> 100\times$ representation per guide.

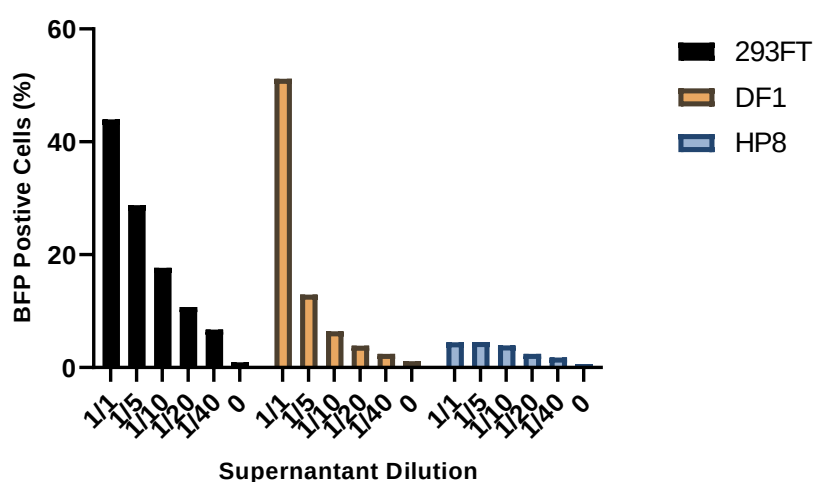


Figure 24. pKLV2 library lentivirus preparation titrated on different cell types. Lentivirus was prepared using 293FT cells. The supernatant was harvested and added to the 3 cell types for 48 h with 8 $\mu\text{g}/\text{mL}$ polybrene. 4 days later the cells were analysed for BFP using flow cytometry.

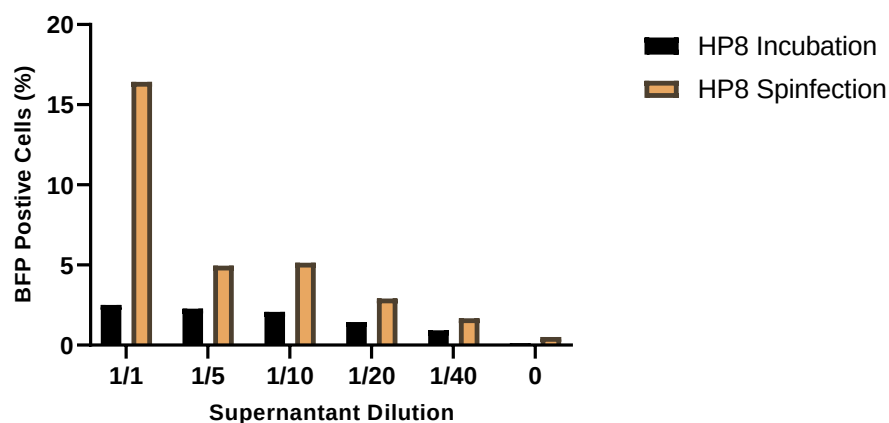


Figure 25. HP8 transduction with and without spinfection. pKLV2 lentivirus library supernatant was added to HP8 cells with 8 $\mu\text{g}/\text{mL}$ polybrene. Half the cells were incubated for 48 h and half were spininfected at 800 x g for 2 h at 32 °C and incubated for a further 46 h. 4 days later transduction efficiency was measured using flow cytometry.

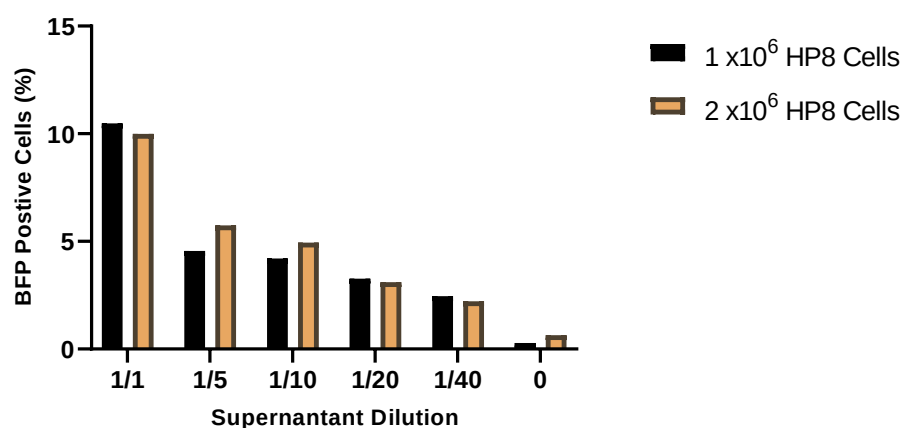


Figure 26. The effect of cell number on transduction efficiency. Using pKLV2 lentivirus supernatant and 8 $\mu\text{g}/\text{mL}$ polybrene 1 or 2 x 10⁶ HP8 cells were transduced for 48 h via incubation with lentivirus supernatant. 4 days later transduction efficiency was measured using flow cytometry.

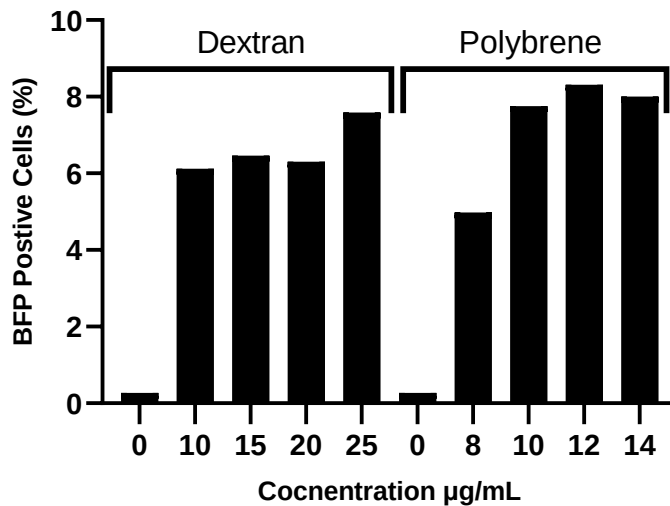


Figure 27. The effect of using different transducing enhancers on transduction efficiency. Transducing agents can increase lentivirus transduction efficiency, with two major agents used Dextran and Polybrene. Using pKLV2 lentivirus supernatant and transduction of HP8 cells using varying concentrations of dextran and polybrene for 48 h. 4 days later transduction efficiency was measured using flow cytometry.

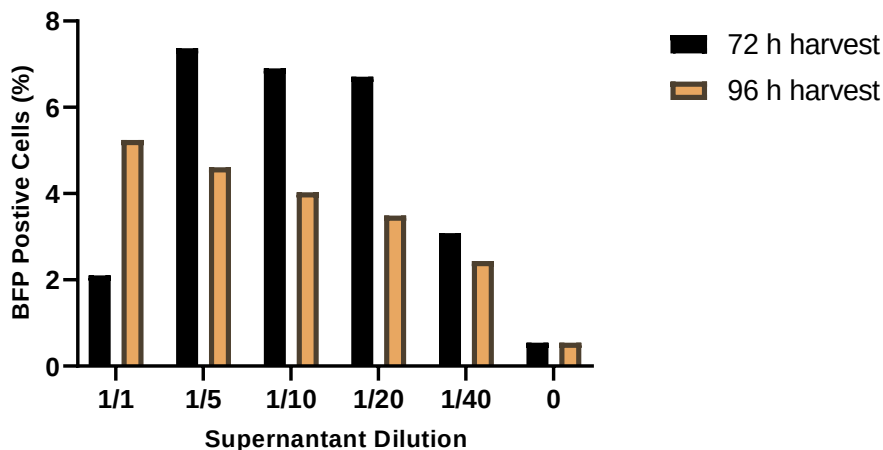


Figure 28. The harvest time of lentivirus affects transduction efficiency. After transfection of the lentivirus reagents using Lipofectamine 2000, the supernatant was harvested at 72 h and 96 h post transfection. Transduction of HP8 cells was then performed at dilutions from 1/1-1/40. 4 days after transduction the lentivirus transduction efficiency was analysed using flow cytometry.

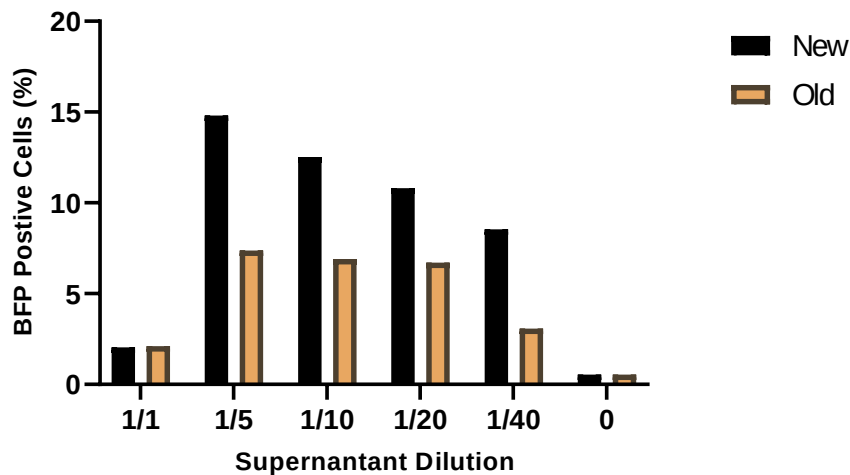


Figure 29. The effect of 293FT passage number on lentivirus transduction efficiency. Lentivirus was produced using low passage number (< 5 passages) (new 293FT cell) and high passage number cells (> 10 passages) (old 293FT cells). The pKL2 lentivirus library supernatant was used to transduce HP8 cells. Transduction was then measured 4 days post transduction using flow cytometry.

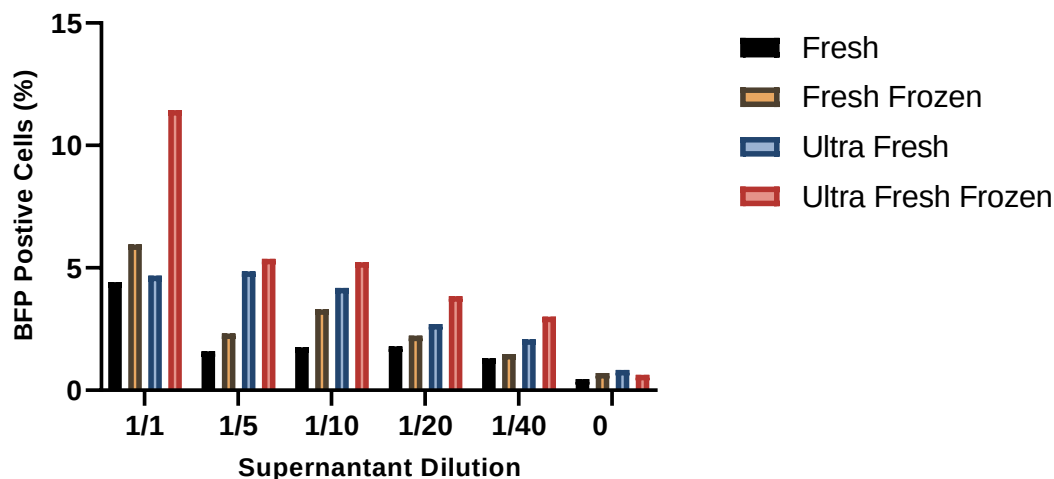


Figure 30. The effect of ultracentrifugation and the effect of the freeze-thaw cycle on transduction efficiency. Lentivirus was produced in 293FT cells and either used to transduce HP8 immediately or after freezing at -80 °C. Additionally, the virus supernatant was used unconcentrated or concentrated via ultracentrifugation.

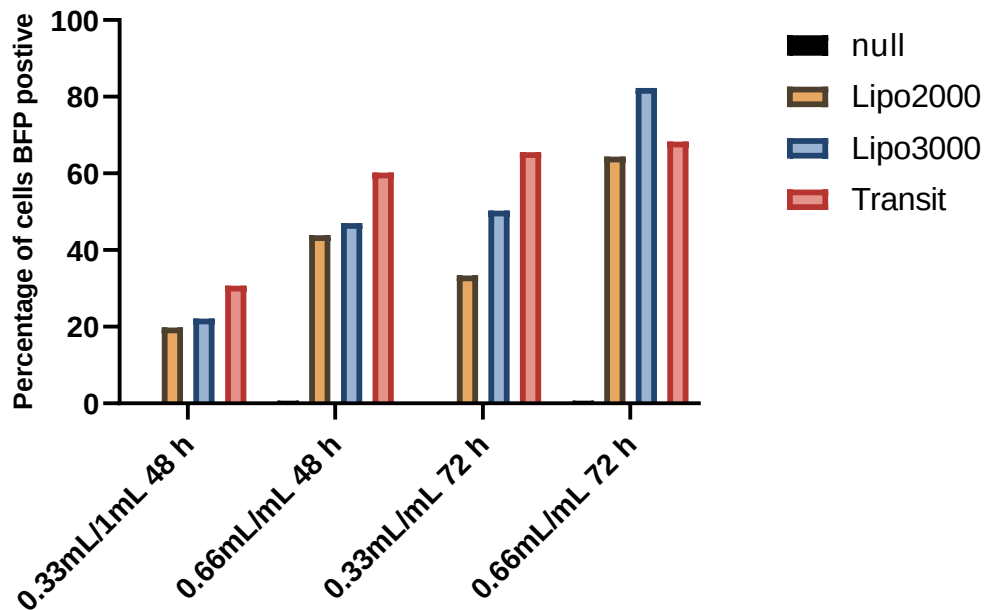


Figure 31. Lentivirus titre depends on the transfection reagent used.

Three transfection reagents were used following the manufacturer's recommendations to make lentivirus in 293FT cells, with harvesting at 48 h and 72 h post-transfection. Overall Transit Lenti produced higher titres across the dilutions and harvest time tested therefore was used in subsequent experiments.

4.4.3 HP8 screen for essential genes

To identify essential genes for the HP8 cell line I needed to identify guides that targeted genes which were essential to the continued proliferation of HP8 cells. Therefore, we mutagenized a pool of HP8 cells with a sgRNA library. Genes that are essential to the proliferation of the cell will result in the death of those cells over time, therefore comparing the genetic profile of the cell population at the early time points to later time points and to other non-MDV infected chicken cell lines (to remove essential chicken genes), genes which are essential to and specific to HP8 cells could be inferred. The HP8 screen used a library of 71,000 sgRNAs with 4 sgRNA per chicken gene. Titration on the cells of interest (HP8 cells) was performed so that the number of cells that needed to be transduced could be estimated (figure 32). For the HP8 screen, 25 μ L was selected, since this produced ~15% transduction with little improvement in transduction percentage above 25 μ L, thus being the most efficient use of the pKLV2 lentivirus stocks that were produced. The target was an sgRNA coverage of >100 x which would require 50 million cells. The titrated lentivirus was then used to transduce 50 million HP8 cells this was performed at an MOI of 0.15. In both repeats >100x representation was achieved. After spinfection and a 48 h incubation, the

cells were passed and puromycin selection started 1 day later. Flow cytometry was used to check the progression of antibiotic selection (figure 33). Figure 33 shows that by day 7 >80% of cells were positive for the BFP marker, showing that puromycin selection was killing untransduced cells.

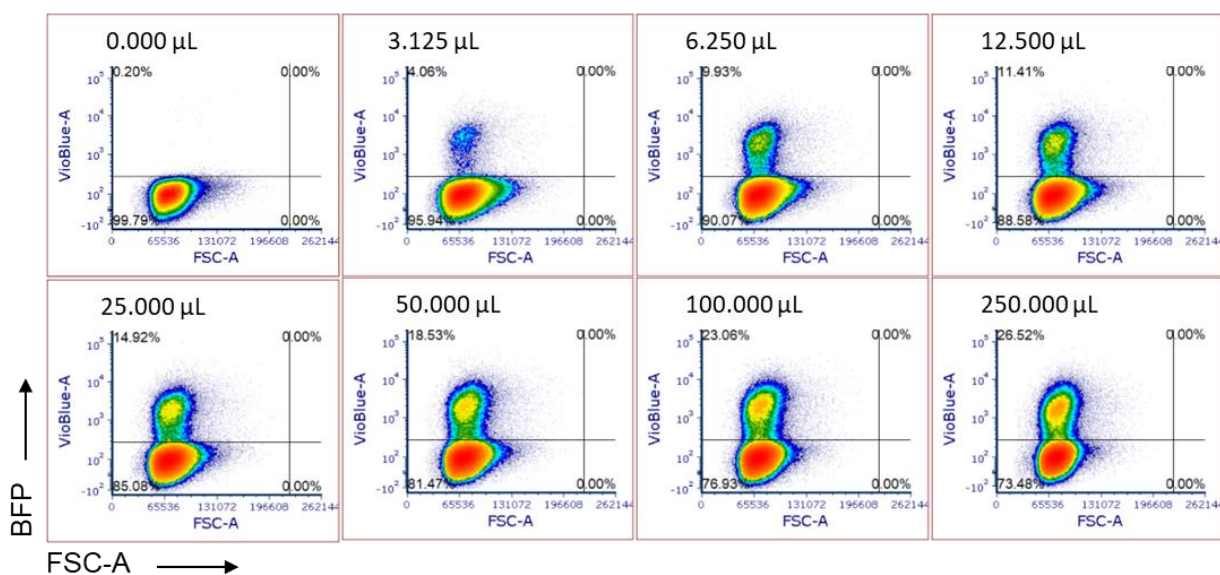
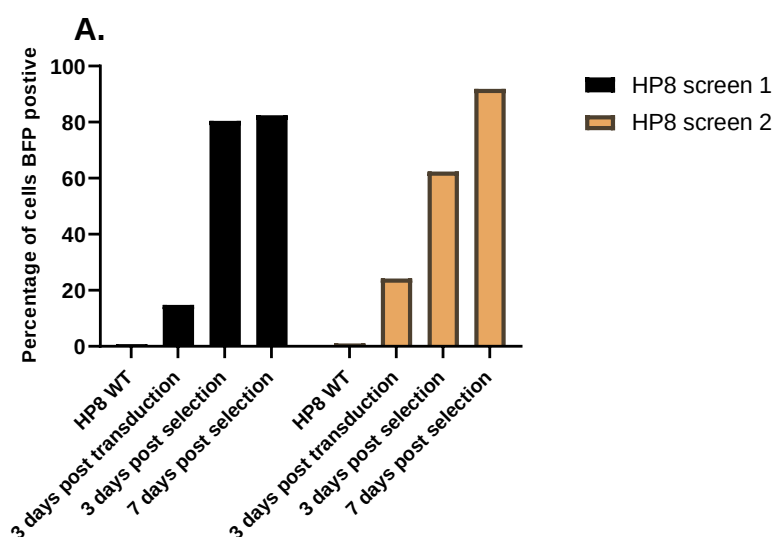


Figure 32. Lentivirus titration on HP8 cells. The concentrated lentivirus supernatant was titrated on HP8-Cas9^{Blast} cells using 3.13 μL, 6.25 μL, 12.50 μL, 25.00 μL, 50.00 μL, 100.00 μL, 250.00 μL /1 mL media. After 5 days of incubation flow cytometry was used to analyse the BFP expression from lentivirus infected cells. BFP (blue fluorescent protein), FSC-A (forward scatter-area).



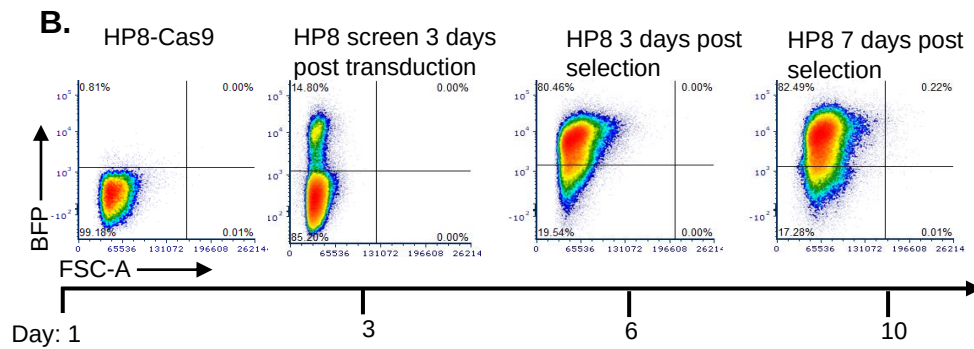


Figure 33. HP8-Cas9^{Blast} dropout screen transduction percentage throughout puromycin selection. From day 1 to day 10 several samples of cells were taken and analysed by flow cytometry. Bar chart (A) and FACS data (B) showing the lentivirus infection and selection progression. BFP (blue fluorescent protein), FSC-A (forward scatter-area).

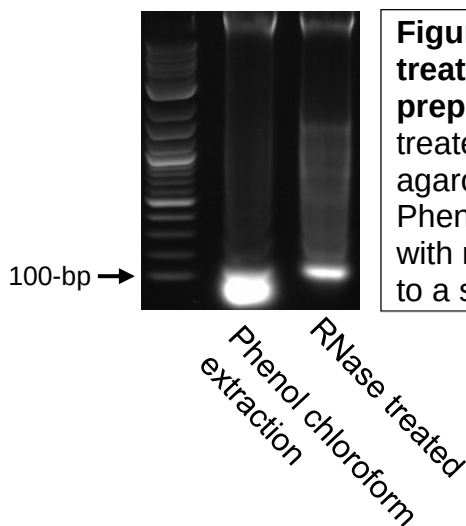
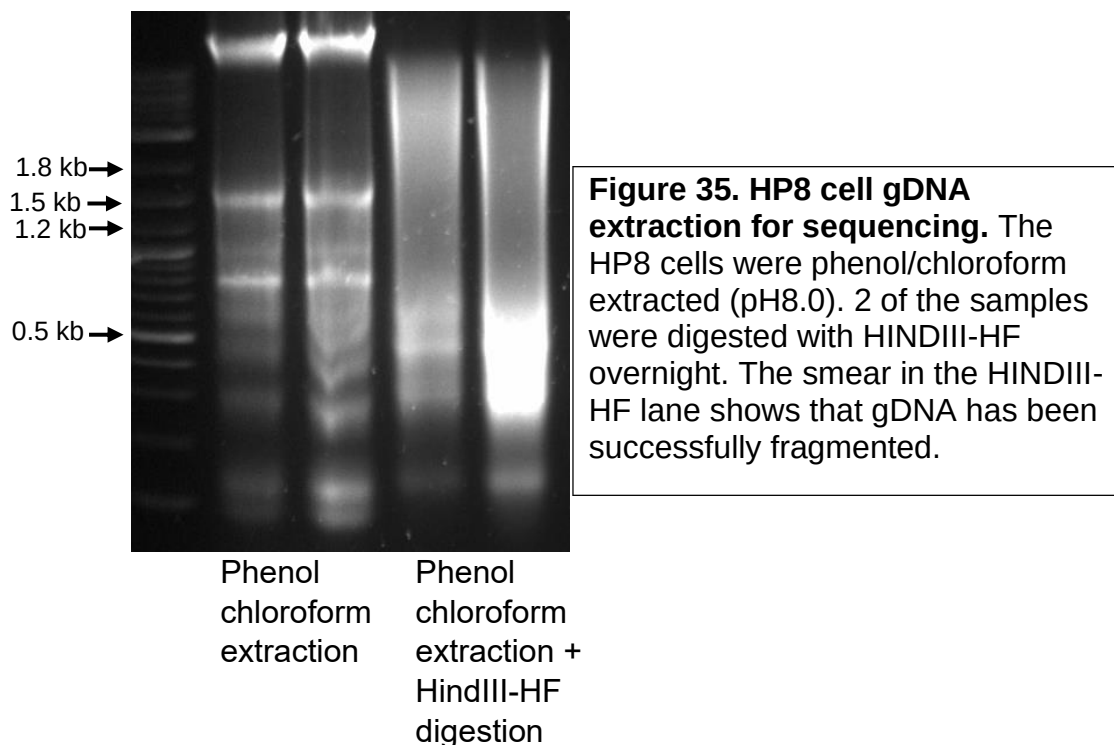


Figure 34. The effect of RNase treatment on the sample preparation. Extracted gDNA was treated with RNase and run on an agarose gel. The gel image shows Phenol-chloroform extracted sample with no RNase treatment compared to a sample that was RNase-treated.

Cells were passed up to 25 days maintaining sufficient representation at each passage (>500 cells per guide). Cells were sampled at 72 h post-transduction and 3-, 5-, 15- and 25-days post-selection. gDNA was extracted using phenol-chloroform extraction, and treated with RNase to remove RNA, which could comprise a large portion of the nucleic acids collected. RNase treatment was confirmed to reduce background, as shown by the reduced size of the band below the 100-bp marker in the RNase-treated sample compared to the Phenol-chloroform extracted sample with no RNase treatment (figure 34). To increase the concentration of target DNA in our sample and to reduce the number of PCR reactions required the gDNA was HindIII-HF restriction digested, the resulting gDNA ran on a gel (figure 35), and the samples were run on the agarose gel in duplicates. As expected, the digested DNA showed a 'streak' on the gel image, with no clear banding

indicating digestion had been completed (figure 35). RNase-treated and HindIII-treated gDNA samples were checked to determine whether the target sample was affected using Sybr green qPCR (figure 36A), this showed a difference in Ct value of 1.8 roughly corresponding to a 3.5 increase in the targeted template after HINDIII-HF digestion and gel extraction and purification.



This was further confirmed by the standard PCR, which showed a much more intense band in the HindIII-HF treated gel extracted and purified sample (figure 36B). The results were as expected showing less RNase contamination and increased concentration of the target DNA fragments, allowing for fewer PCR reactions to be performed, thus making the assay more manageable in relation to both time and reagent costs (figure 36). Figure 37 shows a typical gel after phenol/chloroform DNA extraction, RNase treatment and HindIII-HF digestion. The gDNA HindIII-HF digested fragments of interest containing the lentivirus CRISPR regions are between 1.2-1.8 kb.

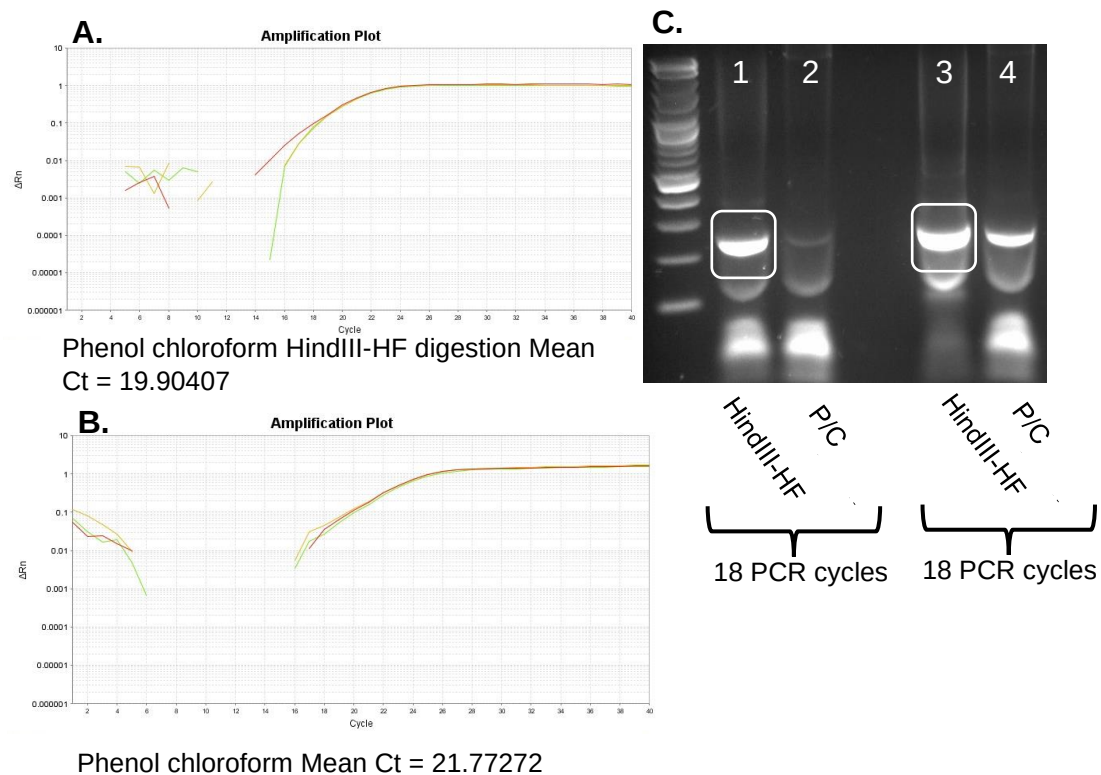


Figure 36. HindIII-HF treatment of extracted untreated gDNA. The extracted cells were used in qPCR using primers targeting the conserved barcode regions of DNA (A and B), (A) HindIII-HF digestion and gel purification, (B) no HindIII-HF digestion or purification. The gel picture show PCR using the same primers, in both lanes (lane 1 and 3) the HindIII-HF-treated samples show a larger band (C) (P/C = phenol chloroform extraction).

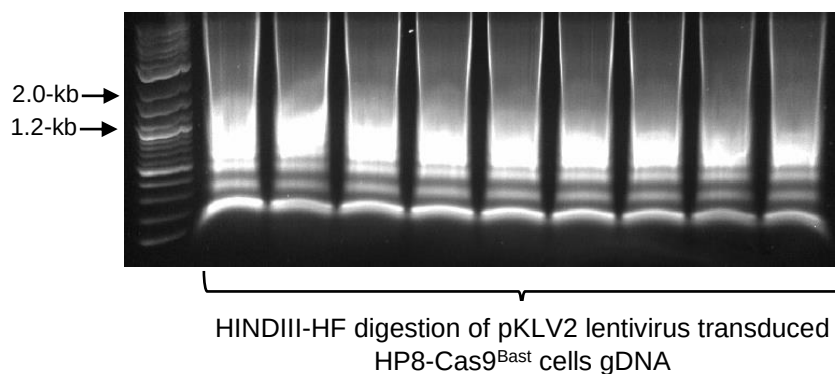


Figure 37. gDNA HindIII-HF digestion. pKLV2 lentivirus transduced HP8-Cas9^{Bast} cells gDNA extraction by phenol chloroform (pH 8.0), followed by gel extraction and run on an agarose gel where the DNA between 1.2 kb-1.8 kb was extracted.

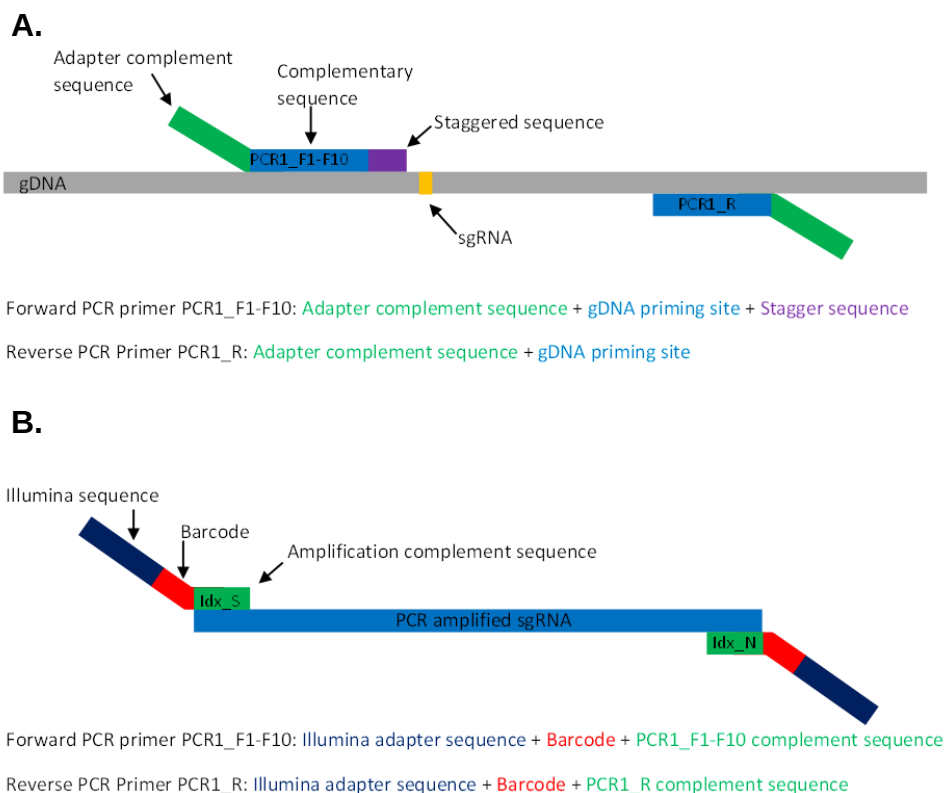


Figure 38. Schematics of PCR primers used for sgRNA amplification and sequencing. (A) PCR primers used for PCR₁ reaction, the staggered sequence is used to ensure there is a varied length of amplicons which is required for an optimal library of sequences for NGS reactions. The complementary sequence of the primer binds to the conserved CRISPR regions of the integrated pKLV2 CRISPR plasmid. The adapter complement sequences are complementary to the adapter primers seen in B. (B) PCR primers used for PCR₂ reaction, the amplification complement sequence binds the complementary region on the PCR₁ primers, and the barcode is used to identify samples when they are pooled for NGS sequencing and the Illumina sequences are to enable the sequences to anneal to the oligos of the Illumina flow cell.

Collected gDNA can be used as a template to PCR amplify the CRISPR regions within the integrated lentivirus genomes, which are subsequently used for NGS. Copy numbers of all CRISPR guides are collated by matching reads between samples and relevant controls to identify genes with significantly enriched or depleted guides. As a result of the disruptive nature of guides to a target gene, guides that target non-essential genes will be enhanced over time. Therefore, the depletion of guides to a specific gene can be used to identify genes involved in the proliferation of HP8 cells. After gDNA was extracted HindIII-HF digestion was performed for 20 h. The products of approximately 1.2-1.8 kb were extracted for gel purification which was subsequently used as a PCR template.

Before the preparation of the samples, several optimisation experiments were performed to optimise the two PCRs required to prepare samples for sequencing (figure 38 illustrates the primers used). PCR_1 amplified the CRISPR regions within the integrated lentiviral particles and PCR_2 added the indexing adapters for NGS sequencing. The first and second PCR were optimised for annealing temperature to gain the most efficient PCR amplification of the target regions, (figures 39 and 41 show the temperatures trialled for each PCR). 62 and 65 °C were chosen for PCR_1 and PCR_2 respectively. PCR_1 was also optimised for the number of cycles as the number of cycles increases the possibility of bias in amplicons, and it has been recommended to use between 18 and 22 cycles (figure 41). Therefore, the lowest number of cycles was selected (18) that still showed PCR amplification bands (figure 41). PCR_2 was also optimised for the amount of template DNA per 25 µL reaction as using a high volume of DNA template produced a significant amount of background amplification, therefore, I investigated the use of lower template inputs, selecting 25 ng per 25 µL PCR reaction (figure 42). Using these optimisation steps the extracted gDNA was amplified in the two-step PCR (PCR_1 and PCR_2), purified, and sent for sequencing, figure 43 and figure 44 show the PCR amplification results for HP8 screen repeat 1 and 2 respectively.

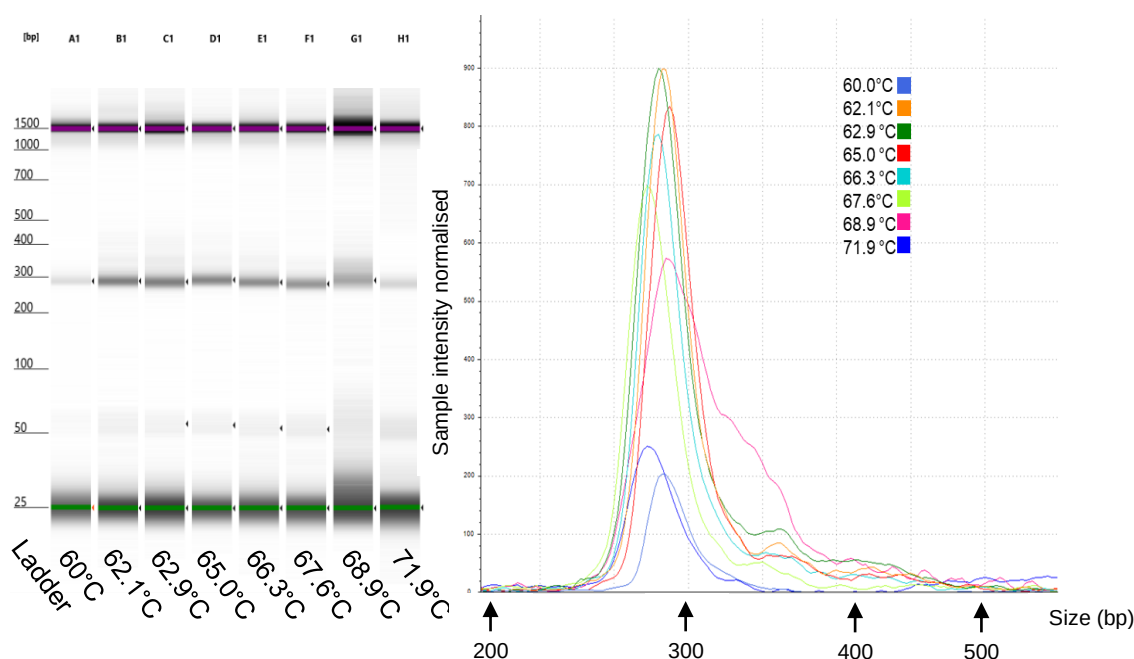


Figure 39. PCR_1: Annealing temperature optimisation. After gDNA extraction the annealing temperature for PCR_1 was tested between 60.0 and 71.9 °C. Right shows the tapestation gel image and left shows the electrogram image of the same result. The PCR_1 target amplicon was ~280-bp.

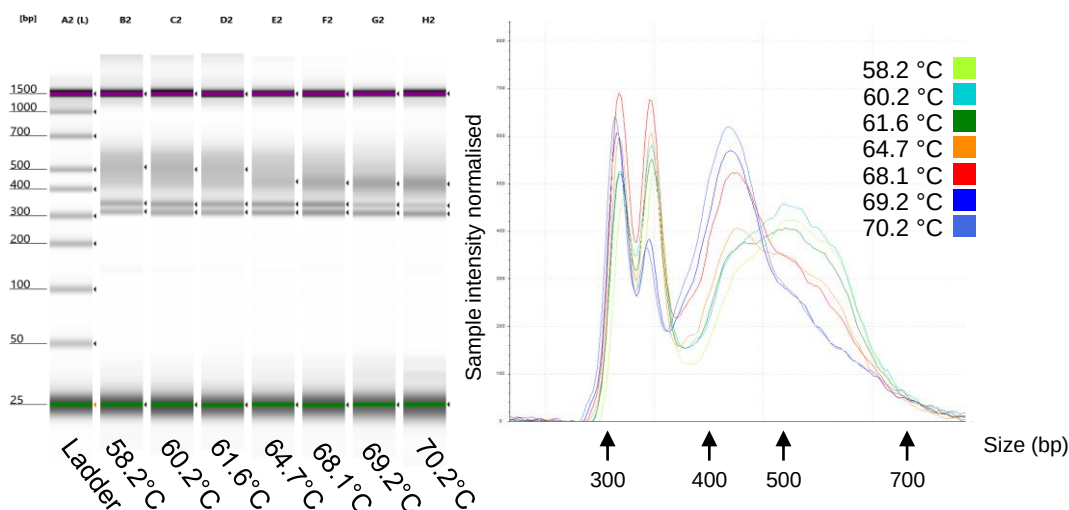


Figure 40. PCR_2: Annealing temperature optimisation. After gDNA extraction the annealing temperature for PCR_2 was tested between 58.2 and 70.2 °C. Right shows the tapestation gel image and left shows the electrogram image of the same result. The target amplicon was ~350-bp.

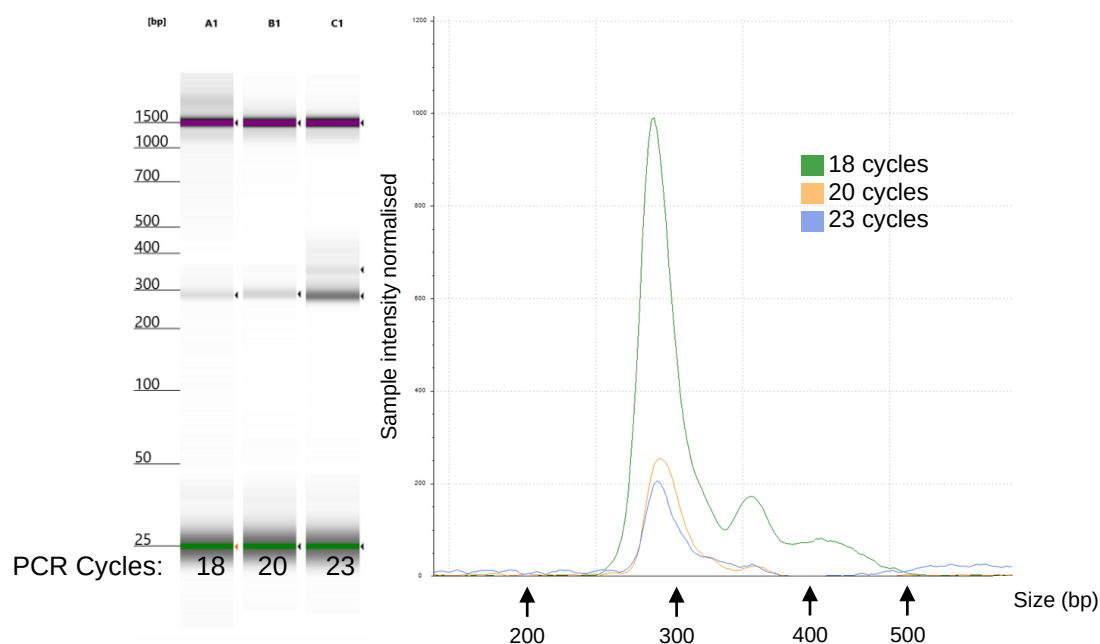


Figure 41. PCR_1: Number of cycles optimisation. After gDNA extraction the number of cycles of PCR_1 was tested at 18, 20 and 23. Right shows the tapestation gel image and left shows the electrogram image of the same result. The target amplicon was ~280-bp.

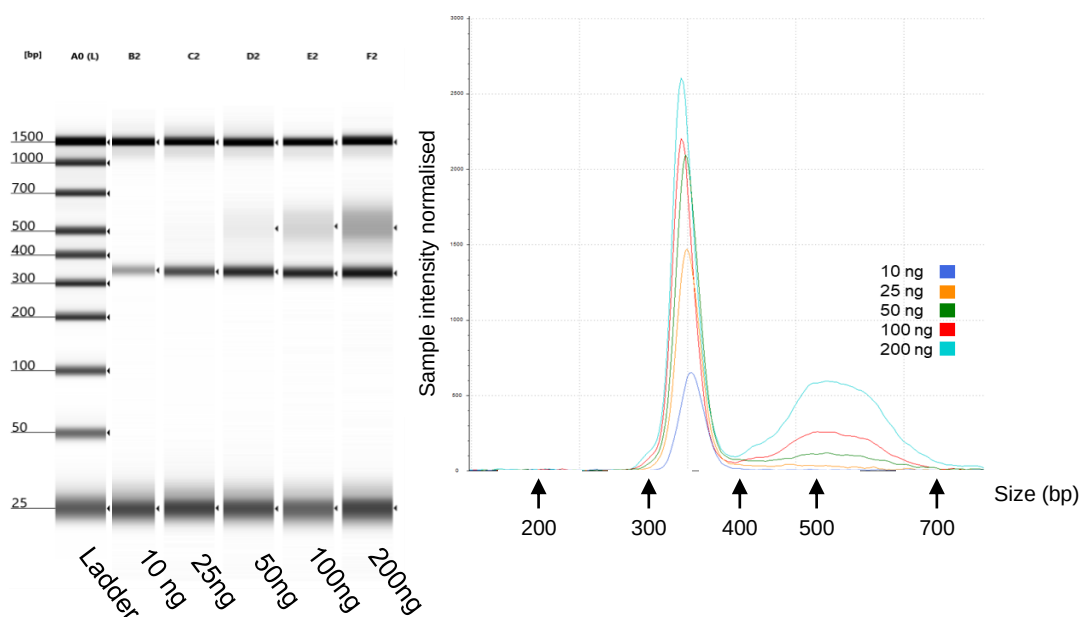


Figure 42. PCR_2: Amount of DNA template added optimisation. Due to the non-specific bands seen in figure 41, the weight of DNA was reduced to reduce off-target amplification. After gDNA extraction PCR_1 was tested carried out at 62 °C, PCR purified and used as template for PCR_2. Right shows the tapestaion gel image and left shows the electrogram image of the same result. The target amplicon was ~350-bp.

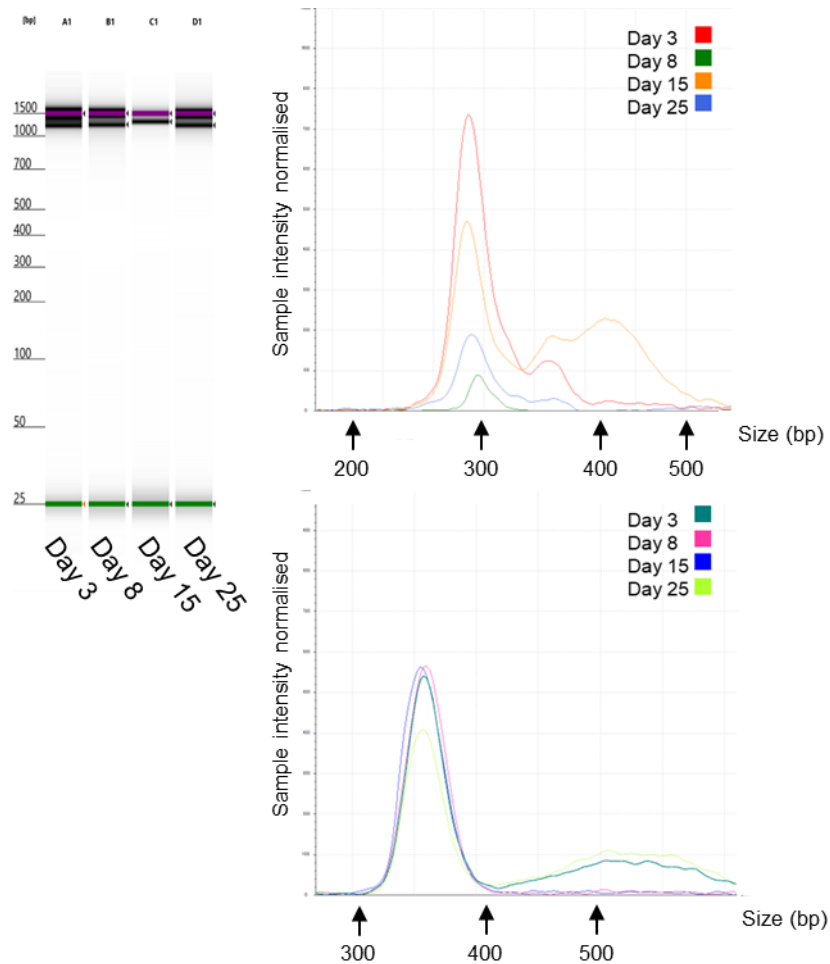


Figure 43. HP8 screen repeat 1 PCR amplification of CRISPR gDNA.

gDNA was extracted from the 4 samples and HindIII-HF digestion carried out and PCR_1 and PCR_2 amplification of the gDNA. Tape station gel image shows HindIII-HF digestion and gel purification result. Top electrogram shows PCR_1 amplification. Bottom electrogram shows PCR_2 amplification. HindIII-HF target 1.2-1.5 kb, PCR target 270-290-bp and PCR_2 target 340-360-bp.

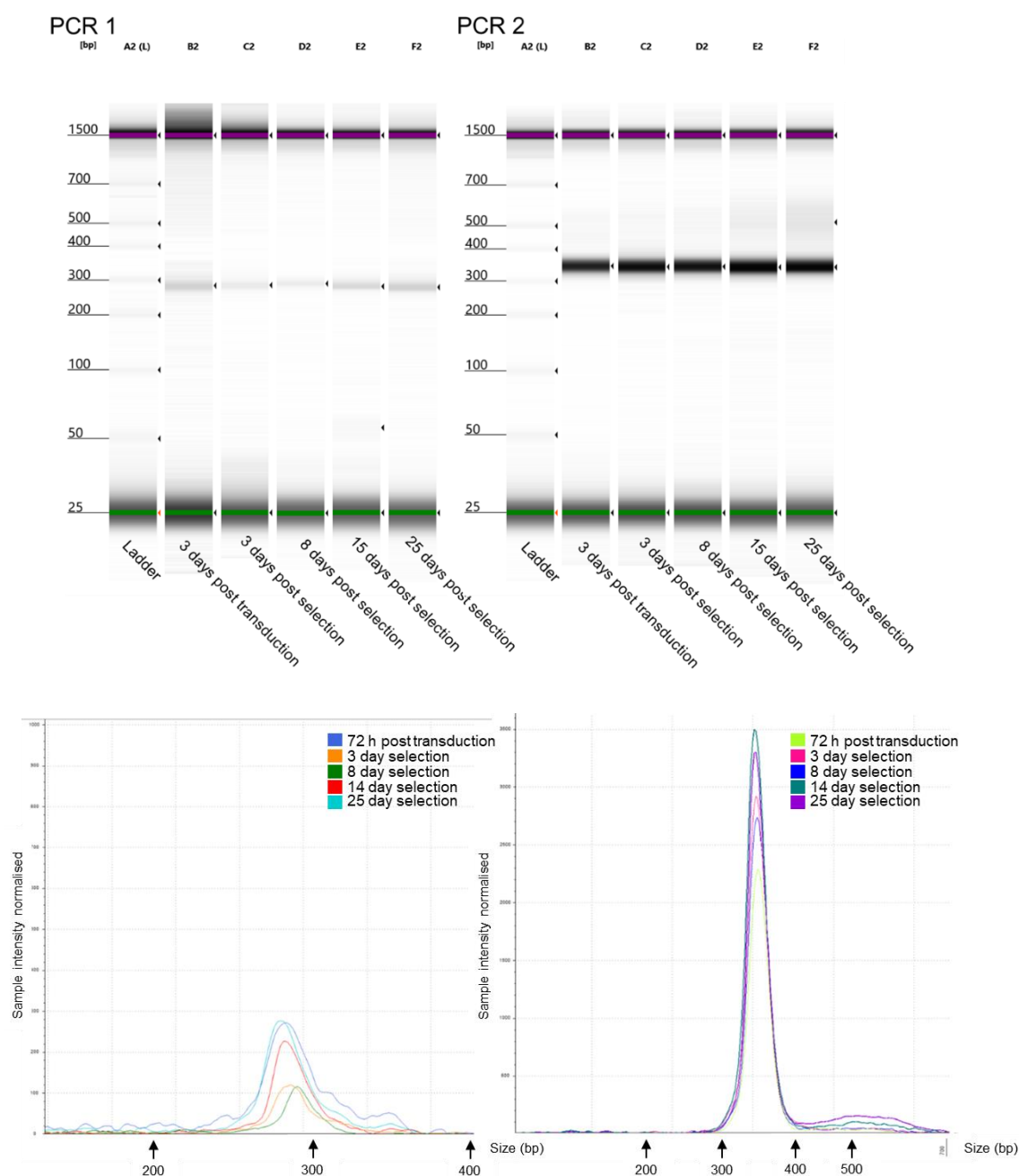


Figure 44. HP8 screen repeat 2 PCR amplification of CRISPR gDNA. gDNA was extracted from the 5 samples and HindIII-HF digestion carried out and PCR_1 and PCR_2 amplification of the gDNA. Tapestation gel image shows HINDIII-HF digestion and gel purification result (top left). Gel image shows DNA amplicons from PCR_2 (top right). Electograms show PCR_1 (left) and PCR_2 (right) amplification. HindIII-HF target 1.2-1.5 kb, PCR target 270-290-bp and PCR_2 target 340-360-bp.

For the HP8-screen there were 2 biological repeats each sampled at 4 or more time points. As cells harbouring mutations to genes essential for HP8 proliferation strongly essential genes would drop out earlier hence day 3 was chosen for analysis, to reveal a larger list of genes related to proliferation day 25 was also chosen, giving a stringent and more relaxed list of host genes required for the proliferation of HP8 cells. After NGS sequencing the read data was processed by a Stouffer-weighted meta-analysis, and two replicates were performed to improve the accuracy of the cell populations studied. This resulted in 2027 negatively enriched genes at $FDR < 0.05$ at day 25. This was further filtered by selecting genes with a log fold change (LFC) < -1 , giving 1,941 gene hits. Human essential genes were then filtered out giving 977 gene hits (Hart *et al.*, 2017a). Next, the DF1-RCAS screen (chapter 5) and screen data of chicken cord epithelial cells (CLEC-213) (unpublished, Finn Grey) were used to remove chicken essential genes (genes negatively selected against with an unadjusted $p\text{-value} < 0.05$) giving 467 genes. Finally, using transcriptomic data of HP8 cells previously produced in the lab any unexpressed genes and genes without matching annotation in the HP8 transcriptomic data were removed. This ensured only genes that are known to be expressed are present in our shortlist. At the day 25-time point, this gave 339 genes, and 155 genes at day 3 (appendix 8.1.2). Figure 45 illustrates the hits $p\text{-value}$ scores for both day 3 (red) and day 25 (grey) samples, and all genes (black). Many genes (highlighted in black) show small $p\text{-values}$ however these represent the portion of genes removed through processing due to their general essentiality.

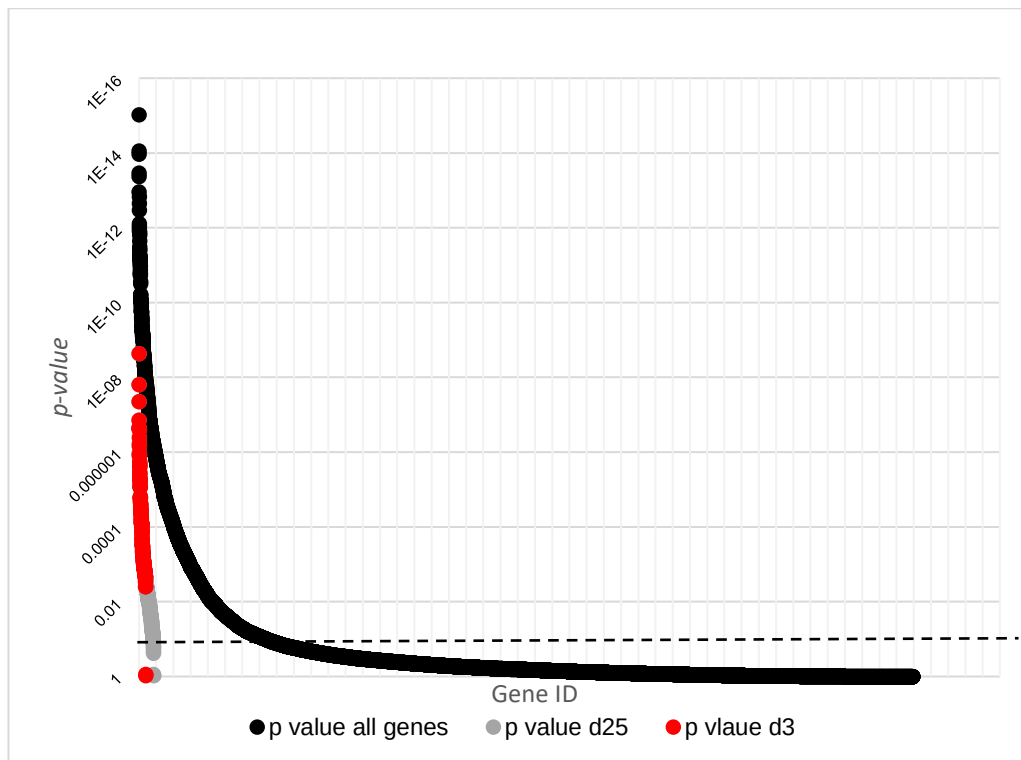


Figure 45. HP8 screen RRA (*p-value*) of all genes (black), day 3 (red) and day 25 (grey). The smaller the value the more significant the association of the gene to HP8 cells continued proliferation. Dotted line represents *p-value* of 0.05).

LatI	LatIII
ARF6	CEPT1
CHM	GNPNAT1
DMXL1	IRF4
EFR3A	OXSRI
IPMK	RPRD1B
ITPK1	SUPT7L
NDUFS1	ZFX
PDHB	
PYROXD1	
RHOA	
SLC16A1	

Table 22. Common hit genes of host dependency factors of HP8 cells and the EBV cells lines P3HR1 and GM12878 which correspond to latency I and III state.

The most comparable study to the one carried out here was the study by Ma *et al.* (2017), who investigated the host dependency factors resulting from the

EBV-transformed cell states. They identified 57 hits in an EBV LatI Burkitt's Lymphoma cell line and 87 hits in an EBV LatIII lymphoblastoid cell line. Table 22 shows gene similarities between our study and the study by Ma *et al.* (2017). In total 18 genes showed similarity when compared to the HP8 screen hit list at 25 days of selection. In addition to similarities, it was seen there were 7 annotated genes and 7 unannotated genes unique to HP8 cells as determined by our internal MDV negative chicken cell lines (DF1 and CLEC cells) and the DepMap portal database (Table 23 and appendix 8.1.2). These genes will require further downstream validation to confirm their requirement for the transformation of HP8 cells.

HP8 specific genes	Gene Summary (GeneCards)
ACSBG2	Enables acyl-CoA hydrolase activity and arachidonate-CoA ligase activity. Acts upstream of or within fatty acid metabolic process. Located in cytosol and mitochondrion.
CUBN	Endocytic receptor which plays a role in lipoprotein, vitamin and iron metabolism by facilitating their uptake. Among its related pathways are Cobalamin (Cbl, vitamin B12) transport and metabolism and Plasma lipoprotein assembly, remodeling, and clearance. Gene Ontology (GO) annotations related to this gene include calcium ion binding and signaling receptor activity.
ITFG2	Involved in cellular response to amino acid starvation; cellular response to glucose starvation; and negative regulation of TORC1 signaling. Located in the lysosomal membrane. Part of KICSTOR complex.
RAPGEF5	Members of the RAS subfamily of GTPases function in signal transduction as GTP/GDP-regulated switches that cycle between inactive GDP- and active GTP-bound states. Guanine nucleotide exchange factors (GEFs), such as RAPGEF5, serve as RAS activators by promoting acquisition of GTP to maintain the active GTP-bound state and are the key link between cell surface receptors and RAS activation.
SLC7A3	This gene encodes a member of the solute carrier family 7. The encoded protein is a sodium-independent cationic amino acid transporter. Alternate splicing results in multiple transcripts that encoded the same protein.
TNFRSF1A	Receptor for TNFSF2/TNF-alpha and homotrimeric TNFSF1/lymphotoxin-alpha. The adapter molecule FADD recruits caspase-8 to the activated receptor. The resulting death-inducing signalling complex (DISC) performs caspase-8 proteolytic activation which initiates the subsequent cascade of caspases (aspartate-specific cysteine proteases) mediating apoptosis. Contributes to the induction of non-cytocidal TNF effects including anti-viral state and activation of the acid sphingomyelinase.
TSPAN9	The proteins mediate signal transduction events that play a role in the regulation of cell development, activation, growth and motility.

Table 23. Annotated genes unique to HP8 cells as determined by a comparison between the HP8 screen hit genes and DepMap portal database for gene essentiality.

4.4.4 HP8 screen reveals mitochondria are essential in proliferation

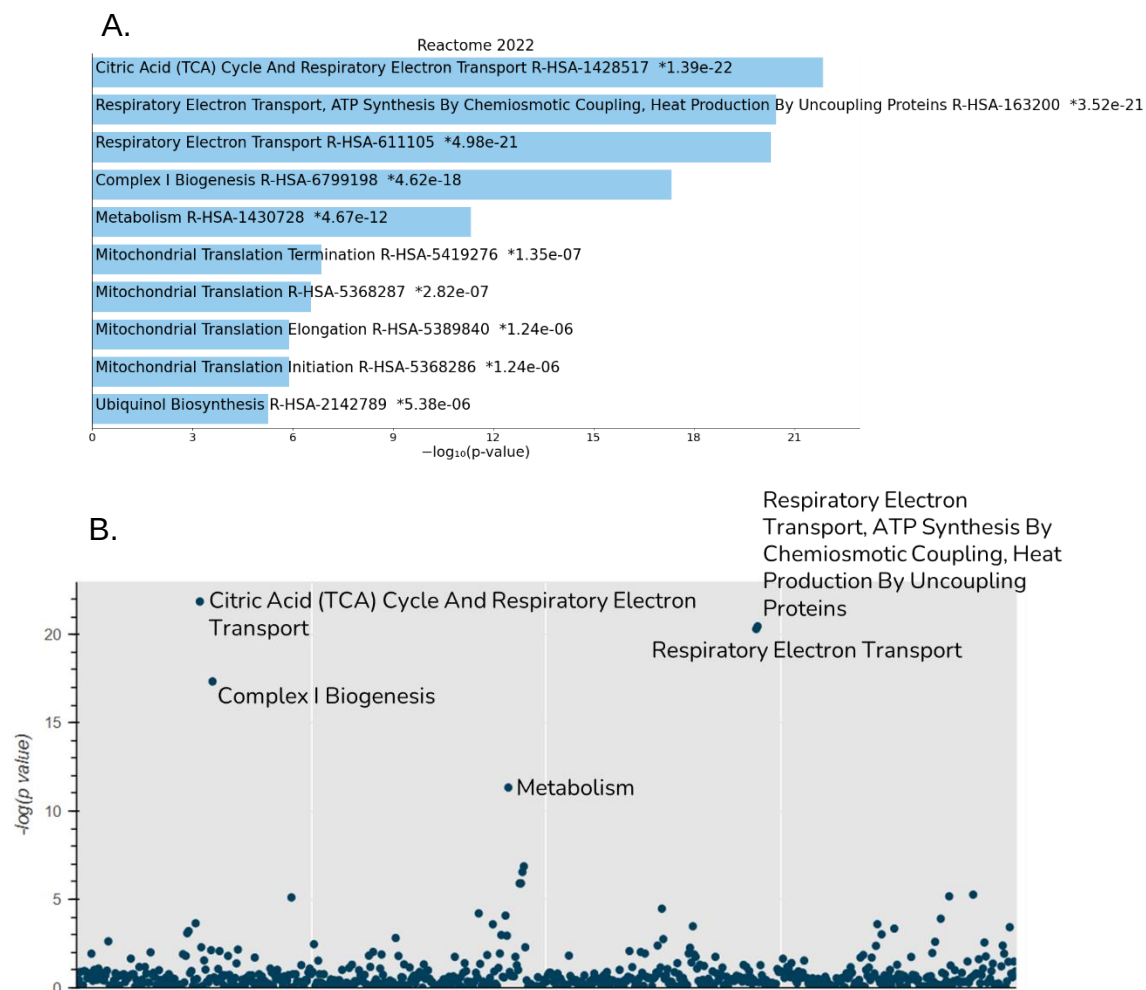


Figure 46. The most represented pathways from the shortlist of essential genes in HP8 cells. Both charts show terms from the Reactome_2022 gene set library with the top enriched terms labelled. The bar chart (A) of the top 10 enriched terms for the input gene set is displayed based on the $-\log_{10}(p\text{-value})$, with the actual $p\text{-value}$ shown next to each term. The term at the top has the most significant overlap with the input query gene set. Manhattan plot (B), each dot represents a single term along the x-axis. The y-values represent the $-\log_{10}(p\text{-value})$ corresponding to the enrichment of the input gene set.

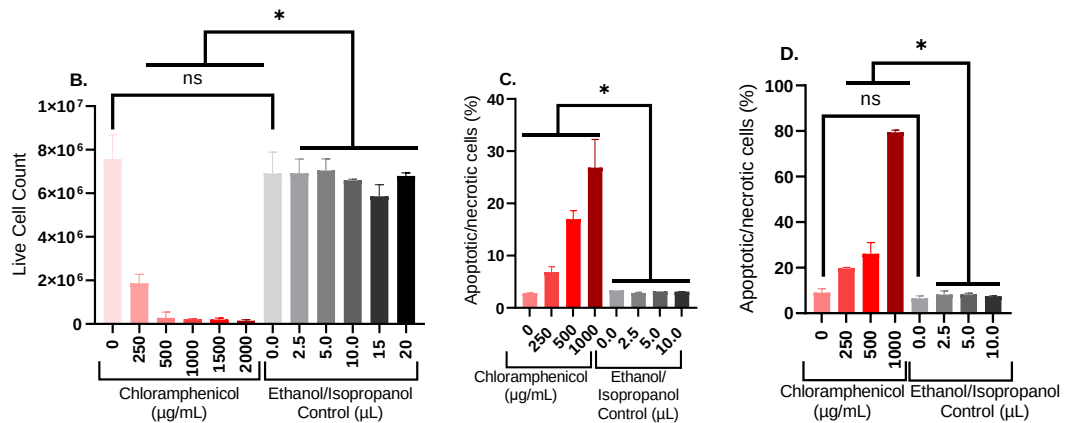
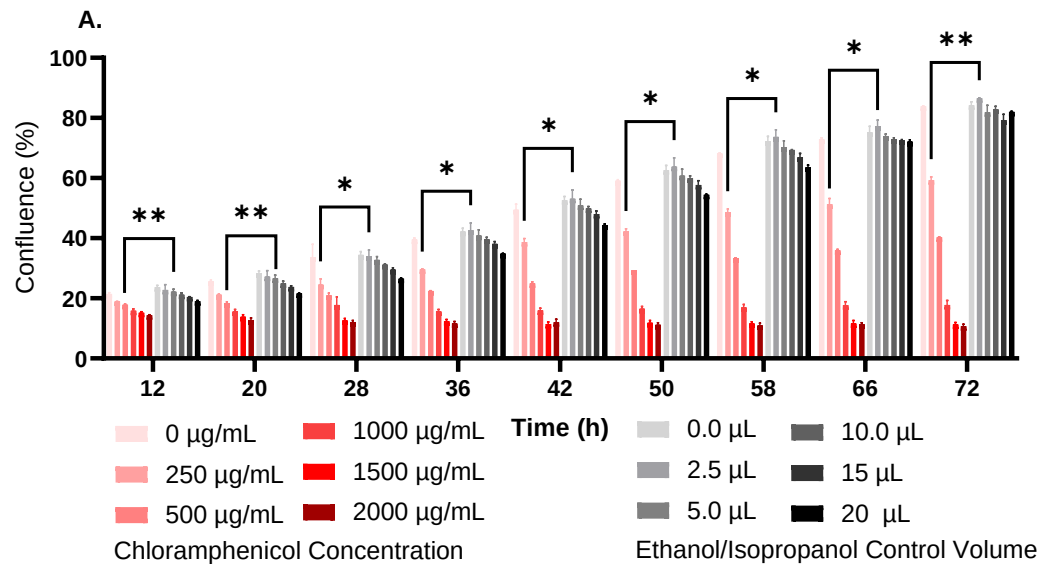


Figure 47. Chloramphenicol inhibits proliferation and increases apoptosis in HP8 cells and AVOL-1 cells. Proliferation assay HP8 (A) and AVOL-1 (B) and apoptotic assay HP8 (C) and AVOL1 (D) are shown. Mitochondrial function inhibitor chloramphenicol was added to the culture medium of HP8 cells for 72 h at concentrations of 0-2000 $\mu\text{g/mL}$ the growth was either recorded overtime using IncuCyte S3 scanner (A) or at 72 h timepoint using live cell counter (B). Also, at 72 h time point the percentage of apoptotic/necrotic cells were recorded using flow cytometry. (A) Significance calculated by paired t-test (first significant value shown only) compared to the control indicated on the graph. *p-value* indication: * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001, (B, C, D) significance calculated by unpaired t-test compared to the control indicated on the graph. Mean $\pm$ SD of two biological replicates.

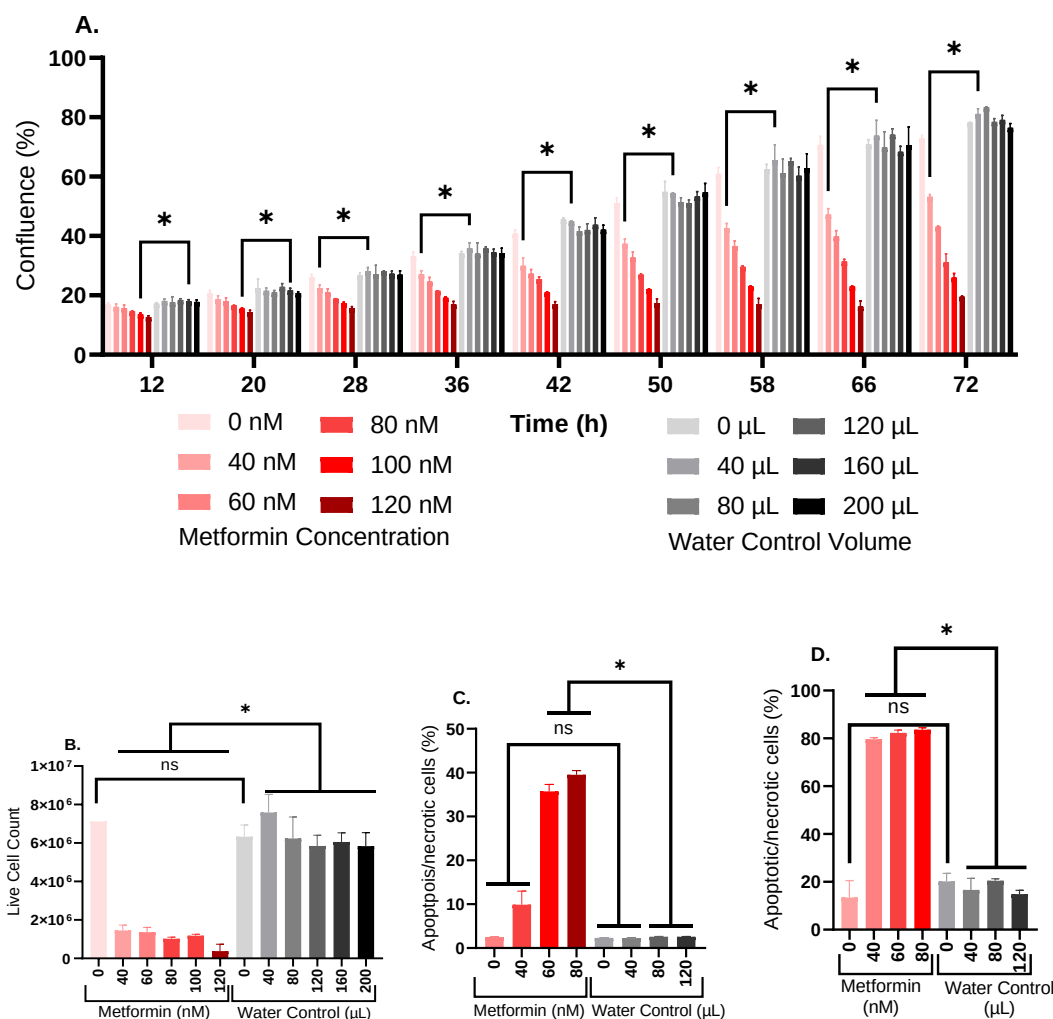


Figure 48. Metformin inhibits proliferation and increases apoptosis in HP8 cells and AVOL-1 cells. Proliferation assay HP8 (A) and AVOL-1 (B) and apoptotic assay HP8 (C) and AVOL1 (D) are shown. Mitochondrial function inhibitor Metformin was added to the culture medium of HP8 cells for 72 h at concentrations of 0-120 nM the growth was either recorded overtime using IncuCyte S3 scanner (A) or at 72 h timepoint using live cell counter (B). Also, at 72 h time point the percentage of apoptotic/necrotic cells were recorded using flow cytometry. (A) Significance calculated by paired t-test (first significant value shown only) compared to the control indicated on the graph. *p*-value indication: * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001, (B, C, D) significance calculated by unpaired t-test compared to the control indicated on the graph. Mean +/- SD of two biological replicates.

From the 339 genes shortlisted at day 25, gene pathway analysis was performed (Reactome 2022, enrichr) which showed a large number of genes associated with the mitochondria (13% Appendix 8.1.2) (figure 46). Therefore, to validate the suggestion that the mitochondria maybe critical in the proliferation of HP8 cells I used three inhibitors known to hinder mitochondrial function. A1214077, chloramphenicol and metformin-treated

HP8 cells all showed increased apoptosis and slowed proliferation (figure 47-50).

I cultured HP8 cells with increasing concentrations of chloramphenicol which is known to interfere with the large mitochondrial ribosomal subunit in eukaryotic cells (Cohen and Saneto, 2012). At increasing chloramphenicol concentrations, we observed slowed cellular proliferation of HP8 cells and increased apoptotic and necrotic cell death at 72 h in a dose-dependent manner (figure 47). To allude to whether this was a unique feature to MDV transformed T-cells I also looked into the influence of mitochondrial inhibitors on AVOL-1 cells. It was seen that this requirement of mitochondrial function was not isolated to HP8 cells, and the retrovirus-transformed T-cell line AVOL-1 cells saw a similar pattern (figure 47).

Metformin is known to specifically inhibit the mitochondria respiratory chain and to prevent mitochondrial-mediated apoptosis (Vial *et al.*, 2019). Therefore, HP8 cells were cultured with increasing concentrations of metformin and I assessed the growth of cells and the apoptotic/necrotic percentage of cells after 72 h. It was shown that increasing the concentration of metformin slowed cellular proliferation and increased apoptotic and necrotic cell death in a dose-dependent manner (figure 48). However, this was not isolated to HP8 cells, and the retrovirus-transformed T-cell line AVOL-1 cells saw a similar pattern (figure 48).

MCL1 inhibitor A-1210477 (Wang *et al.*, 2021) is known to localise to the mitochondria and facilitate mitochondrial function and ATP production (Perciavalle *et al.*, 2012). Culturing HP8 cells with A-1210477 showed reduced cellular proliferation and increased apoptosis and necrosis at A-1210477 concentrations of 10 μ M and above. This suggests that mitochondrial function is critical for the continued proliferation of HP8 cells (figure 49). However, this was not isolated to HP8 cells, and the retrovirus-transformed T-cell line AVOL-1 cells saw a similar pattern (figure 49).

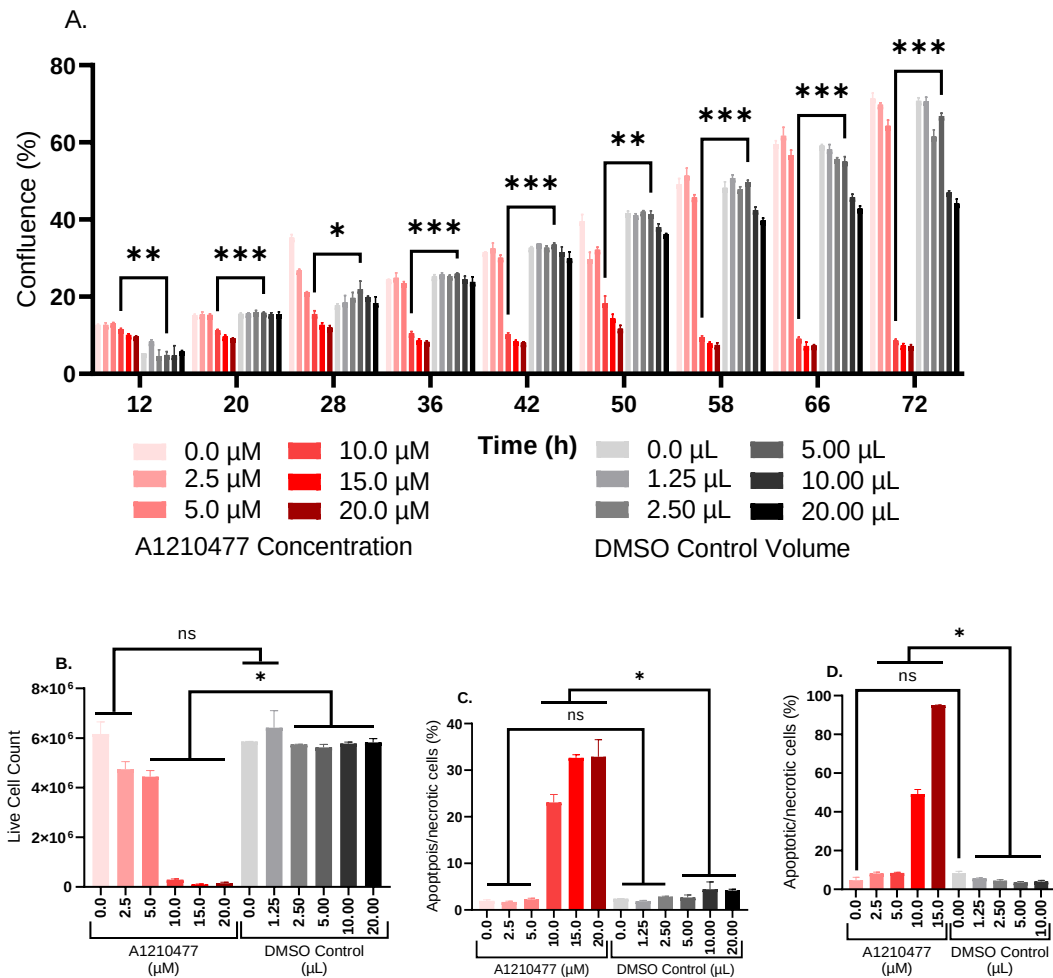


Figure 49. MCL1 inhibitor A1210477 affect on proliferation and apoptosis in HP8 cells and AVOL-1 cells. Proliferation assay HP8 (A) and AVOL-1 (B) and apoptotic assay HP8 (C) and AVOL1 (D) are shown. Mitochondrial function inhibitor A1210477 was added to the culture medium of HP8 cells for 72 h at concentrations of 0.0-20.0 μ M the growth was either recorded overtime using IncuCyte S3 scanner (A) or at 72 h timepoint using live cell counter (B). Also, at 72 h time point the percentage of apoptotic/necrotic cells were recorded using flow cytometry. (A) Significance calculated by paired t-test (first significant value shown only) compared to the control indicated on the graph. *p*-value indication: * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001, (B, C, D) significance calculated by unpaired t-test compared to the control indicated on the graph. Mean $\pm$ SD of two biological replicates.

It has been reported that mitochondrial stress induces lytic reactivation of latent viruses in the human *gammaherpesviruses* EBV and KSHV (Li *et al.*, 2011, Hu *et al.*, 2017, Zhang *et al.*, 2022a). Therefore, I hypothesised that upon mitochondrial inhibition lytic reactivation would be triggered in latently infected MDV-transformed cells (HP8). Using chloramphenicol, metformin

and A1210477 mitochondrial inhibitors, RNA or DNA extraction and RT-qPCR and PCR.

RT-qPCR was used to quantify the viral and host genes after treatment with chloramphenicol (500 µg/mL), metformin (100 nM) and A1210477 (7.5 µM). Sodium Butyrate (2.5 nM) was used as a positive control. Chloramphenicol showed viral genes Meq, gB and pp38 to be highly upregulated (p -value < 0.0001), whereas host genes saw only TP63 to be up regulated (p -value < 0.001) (figure 50A). Metformin treatment saw a similar trend, both gB and pp38 were highly up regulated (p -value < 0.001) with ICP4 and Meq also showing significant upregulation (p -value < 0.01). Whilst little upregulation of host genes, TP-63 and c-REL were significant to below a p -value of 0.05, whilst c-MYC was not significant. A1210477 showed a similar trend again with gB and pp38 showing high upregulation of their expression (p -value < 0.001), with a lower upregulation of Meq and ICP4. Again, the host genes TP-63 and c-MYC saw upregulation to a p -value below 0.05 and c-REL saw no significant up regulation. GAPDH was used as an internal control and saw no significant change between control cells and treated cells (figure 50).

Overall, the general trend of the data showed increased expression of viral genes (Meq, pp38, gB and ICP4) compared to the host genes that were either upregulated to a lesser extent or not at all (TP63, c-REL, c-MYC and GAPDH) (figure 50).

The viral DNA was also assessed using qPCR, which used the pp38 primers to identify viral genomes. Viral pp38 DNA saw significant (p -value < 0.05) up regulation compared to WT untreated cells. MSB-1 reactivation assays are an alternative approach to study whether virus has changed from a latent state to a lytic viral replication state. MSB-1 cells are used since HP8 cells are not receptive to reactivation. This involves the treatment of MSB-1 cells with mitochondrial inhibitor compounds at the following concentrations: chloramphenicol (500 µg/mL), metformin (100 nM) and A1210477 (7.5 µM) and Sodium Butyrate (2.5 nM) as a positive control. After 48 h of treatment, the cells were co-cultured on CEF monolayers for 5 days and then stained with antibody against MDV-1 and a secondary red fluorescent antibody for quantification. There was no significant increase in red fluorescent stain to any of the mitochondrial inhibitor compounds compared to the WT untreated control (figure 53).

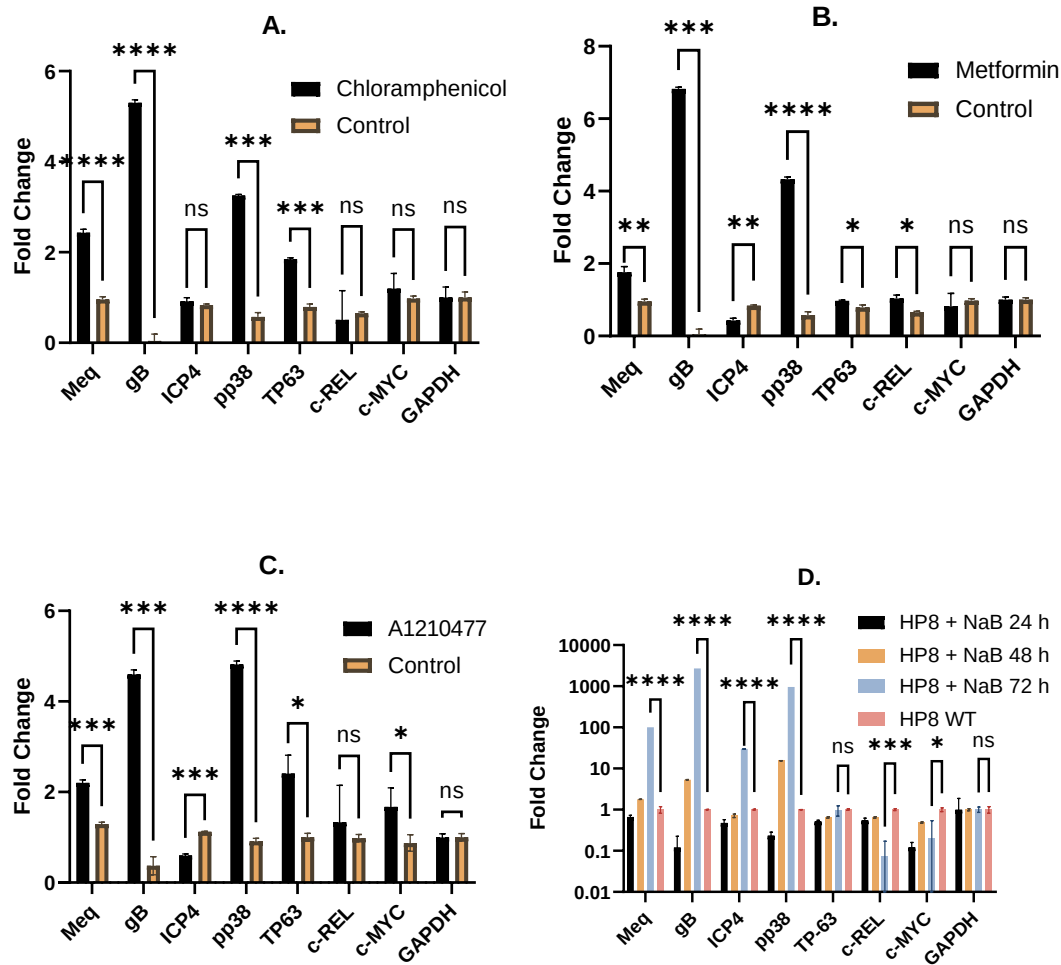


Figure 50. Chloramphenicol (A), Metformin (B) and A1210477 (C) inhibition affects on viral gene activation. Sodium Butyrate (NaB) is used as a positive control for lytic reactivation in HP8 cells (D). Treatments were at the following concentrations: chloramphenicol (500 $\mu\text{g/mL}$), metformin (100 nM) and A1210477 (7.5 μM). Sodium Butyrate (2.5 nM) was used as a positive control fold change of RNA above loading control cell samples. Meq, gB, ICP4 and pp38 are viral genes and TP-63, c-REL and c-MYC host genes. Mean $\pm$ SD of three repeats. Unpaired t-test *p-value* indication: * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

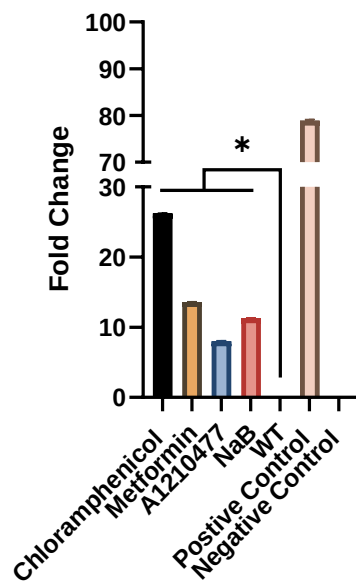


Figure 51. MDV DNA genome copy number at 48 h after mitochondrial inhibition or sodium butyrate treatment (NaB). qPCR normalised to GAPDH, DNA fold change above the control sample level (unpaired t-test p -value < 0.05). Positive control: sample known to contain MDV-1 (obtained from MDV reference laboratory), negative control: sample known to contain no MDV genomes (MDV reference laboratory). Mean $\pm$ SD of three repeats.

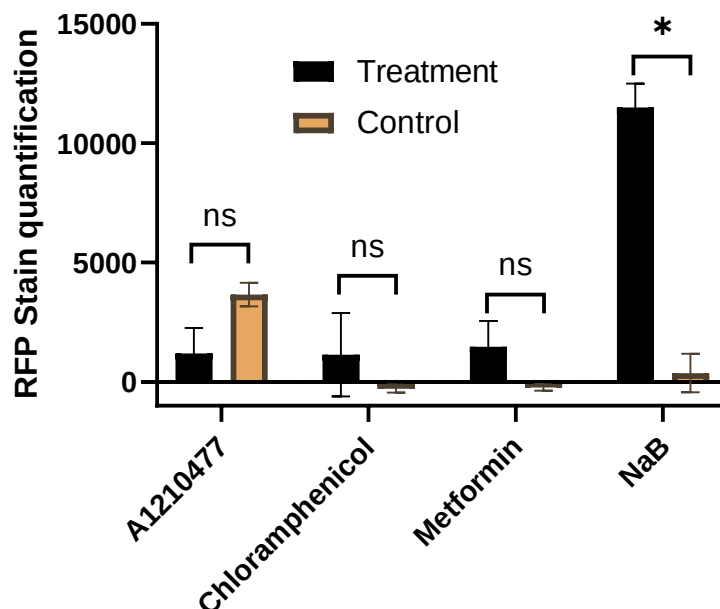


Figure 52. MSB-1 reactivation assay. MSB-1 cells were treated with the compounds A1210477, chloramphenicol, metformin and the control NaB. The treated cells were subsequently co-cultured on CEF monolayers for 5 days

when they were then stained with BD1 primary antibody (anti-MDV-1) and anti-mouse RFP fluorescent secondary antibody. Mean plotted of two technical repeats $\pm$ SD. Asterisk (*) indicates significance by unpaired t-test p -value < 0.05 .

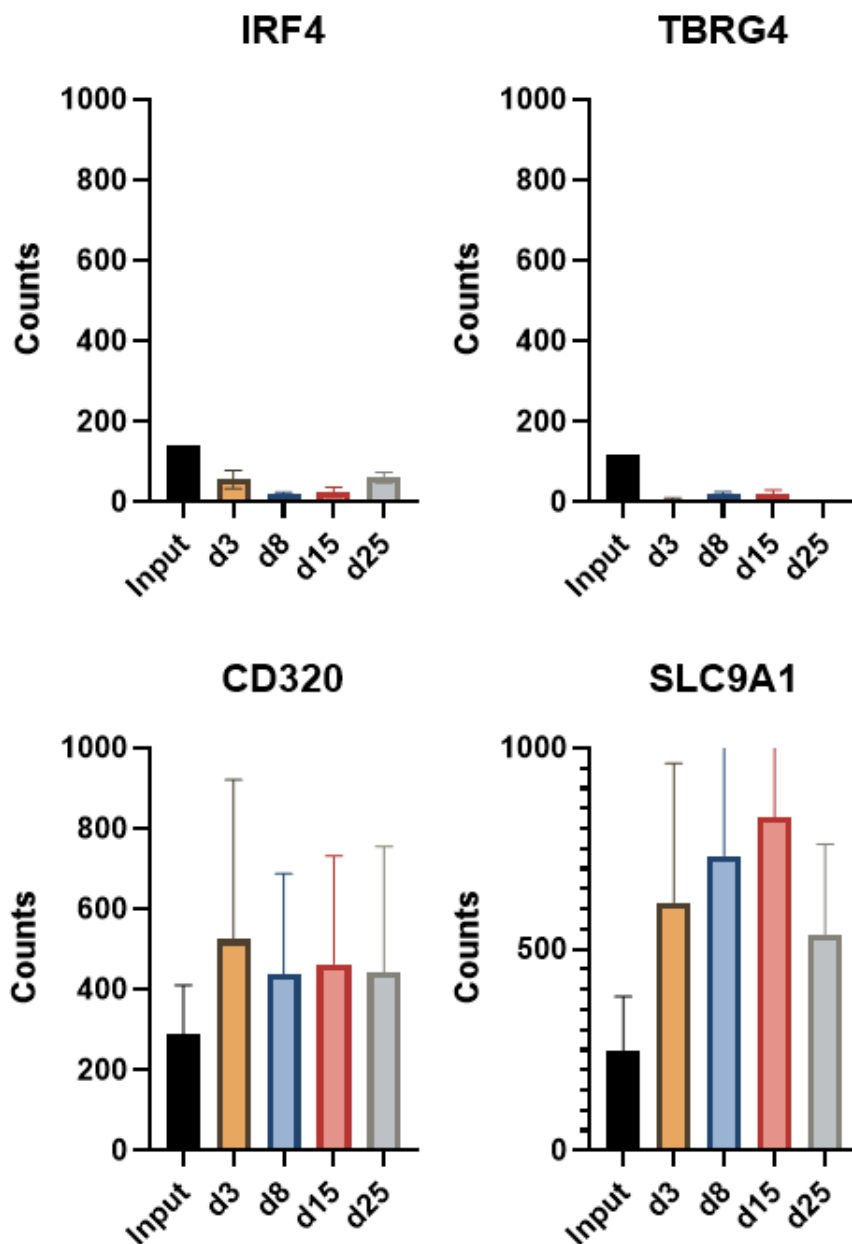


Figure 53. NGS sequencing sgRNA count data for each time point. Samples were taken during the GeCKO screen from day 3 to day 25, these samples were then analysed by NGS for the CRISPR sgRNAs that remained at each time point. NGS measures the number of times that a particular sgRNA is recorded called the count. This is plotted for IRF4 and TBRG4 genes, the input refers to the pKLV2 plasmids library that was amplified and used in this study. Mean plotted $\pm$ SD.

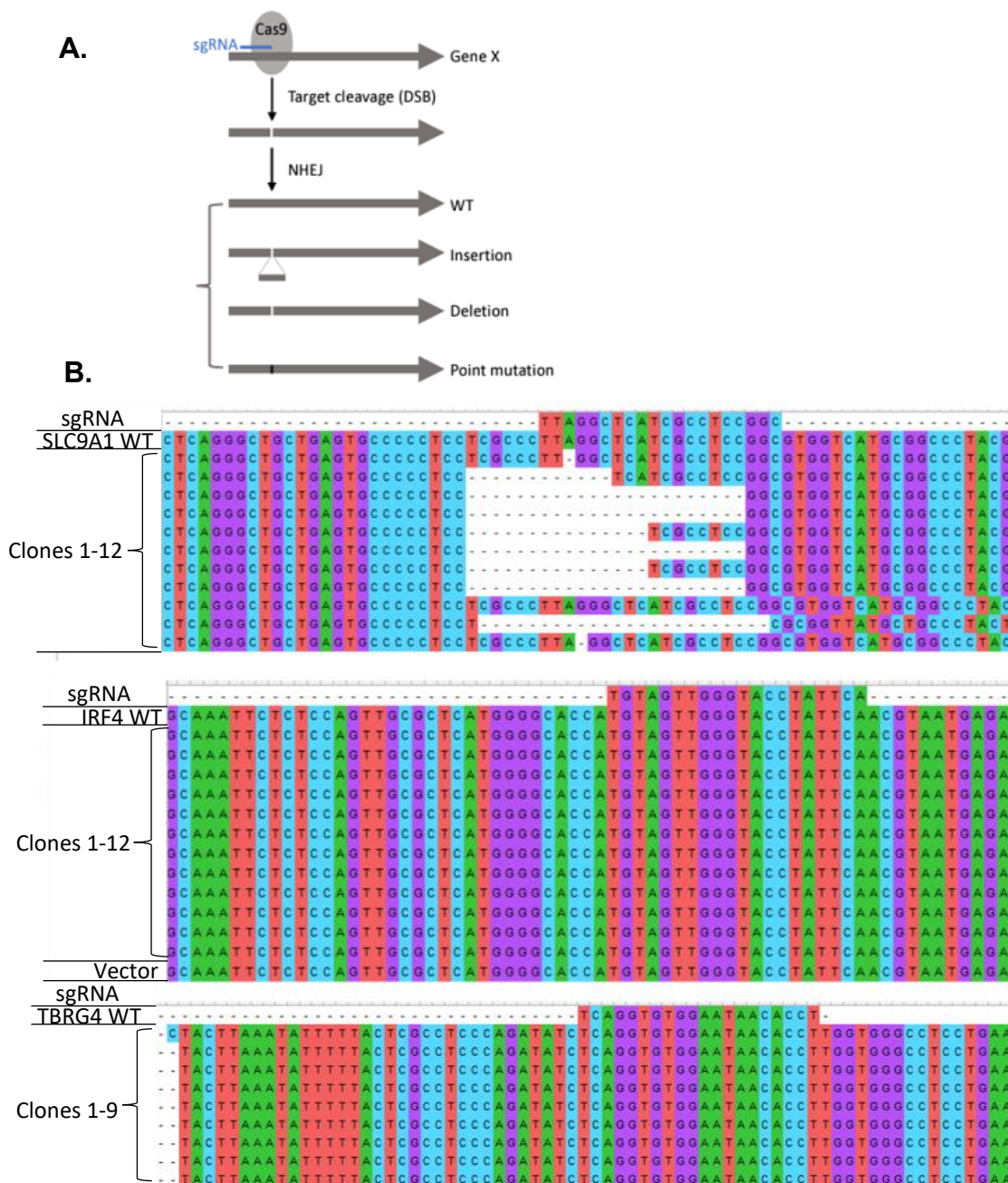


Figure 54. CRISPR-E experiment to test for gene essentiality. sgRNAs were cloned into the sgRNA expression vector px459, transfected into HP8-Cas9 cells and selected with puromycin to kill untransfected cells. The resulting cells were single cell sorted and cultured, gDNA extracted and PCR performed to amplify the region surrounding the sgRNA target. Non-essential genes should be permissive to gene editing whereas essential genes should not (A). The IRF4 and TBRG4 genes showed WT-only sequences whereas the control SLC9A1 sgRNA clones saw a mixture of edited and WT suggesting that IRF4 and TBRG4 sgRNA KO cause a cellular proliferation defect in HP8 cells (B).

TBRG4 and IRF4 were strong hits in my screen. Their function has also been investigated in EBV and KSHV; therefore, to validate these two hits I used sgRNAs against these genes and analysed their proliferation (Ma *et al.*, 2017a, Zhang *et al.*, 2022a). Firstly, looking at the NGS sequencing sgRNA count data can provide insights into the confidence of the hit (figure 53). A strong hit should show depletion in all time points sampled compared to the input and should decrease over time. For both IRF4 and TBRG4, a reduction in the sgRNA counts was seen at all time points compared to the input control, although a reduction over time was less clear (figure 53). For the two control genes shown CD320 and SLC9A1 no reduction was seen overtime, which would be expected in genes non-essential to cell proliferation (figure 53). To confirm the hits IRF4 and TBRG4 one method that was used was CRISPR-Essentiality sequencing methodology (CRISPR-E, figure 54A) (You *et al.*, 2020). This method assumes essential genes will not tolerate gene editing leaving only WT sequences whereas non-essential genes are permissive to gene editing and would allow substitutions and INDELs within their sequence. I saw that the control non-essential gene (SLC9A1) was permissive to cell editing whereas TBRG4 and IRF4 genes were not (figure 54B), suggesting the requirement of these genes for HP8 survival and proliferation (figure 54B). Through the transfection of sgRNAs conjugated to GFP into HP8 cells and subsequent FACS sorting for positive sgRNA-GFP expression, monitoring of cellular proliferation could be performed. It was observed that IRF4 depleted cells showed significant downregulation in growth compared to the non-essential control gene TAOK1/SLC9A1 ($0.05 < p\text{-value}$) (figure 55). TBRG4 depleted cells saw a proliferation decrease, this was not a significant result for TBRG4-sg2 vs SCL9A1-sgB but all other comparisons TBRG4-sg2 vs TAOK1-sgB and TBRG4-sg4 vs TAOK1-sgB and SCL9A1-sgB were significant ($0.05 < p\text{-value}$).

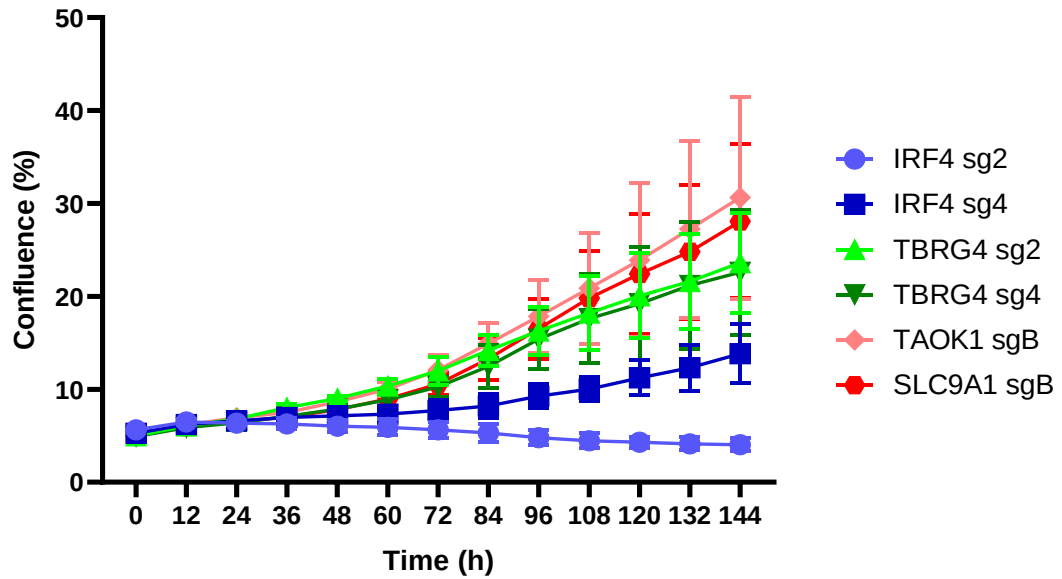


Figure 55. HP8 screen hit genes proliferation analysed after knock down by sgRNAs. IRF4, TBRG4 and control sgRNAs (TAOK1 and SLC9A1) were cloned into the px458-GFP expression vector, transfected into HP8-Cas9 cells, and sorted for GFP positives. The cells growth was then analysed over 6 days. IRF4-sg2 and IRF4-sg4 show significant difference compared to the non-targeting controls TAOK1-sgB and SLC9A1-sgB at 144 h (unpaired t-test p -value < 0.05). TBRG4-sg2/4 vs TAOK1-sgB and SCL9A1-sgB were not significantly different at 144 h (unpaired t-test p -value > 0.05). Mean plotted $\pm$ SD, 6 technical repeats.

4.5 Discussion

Genome-wide KO studies are often implemented in the determination of gene essentiality, enabling easy downstream analysis and validity tests due to the binary nature of the mutation. Previously CRISPR-Cas9 genome-wide-loss-of-function screens have been utilised to identify host dependency factors in herpesvirus-transformed B-cells and herpesvirus-transformed stem cells (Ma *et al.*, 2017b, Gruffaz *et al.*, 2019). I have now used CRISPR-Cas9 to perform the first GeCKO loss-of-function screen for MDV-transformed T-cells (HP8 cells) to identify host dependency factors required for cellular proliferation and the maintenance of the transformed phenotype. In this study, I identified a shortlist of genes specific to MDV-transformed cells that are predicted to be essential to the HP8-transformed phenotype.

Due to the nature of HP8 cells, it was not possible to have an equivalent transformed T-cell uninfected with MDV as a control cell line; although a primary T-cell screen could be performed as a control this was beyond the scope of this project. Additionally, if time permitted the AVOL-1 cell line transformed by REV could have been chosen as a potential control T-cell line. However, this would not be an ideal solution as there could be inherent differences between the isolated T-cells. Therefore, to ensure a robust list of genes of interest was identified, I used the non-MDV infected cell lines; the chicken fibroblast (DF1 cells) and chicken cord lining epithelial cells (CLEC) CRISPR-Cas9 dropout screen and transcriptomic data to remove common essential genes and non-expressed genes respectively from the shortlist of genes created. Even with the appropriate controls, the hit genes identified are required to be validated outside the context of a screen to be certain of the phenotype-genotype association. A caveat to CRISPR-Cas9 screens is that only a few have used culture conditions reflective of the solid-state tumour they represent, therefore screen results need to be extensively validated in various cell types or tissue culture and *in vivo* studies to be certain of a genotype-phenotype correlation. Furthermore, it has also been reported that screen hits have not been validated using the screening cell line or similar cell lines (Jain *et al.*, 2020, Thomas and Ashcroft, 2021), highlighting the importance of culture conditions, experimental design and validation experiments on study outcomes.

Mitochondria are organelles of eukaryotic cells involved in the critical processes of metabolism and programmed cell death and are associated with the innate immunity of the host against viruses. A significant number of studies have proven viral infection can modulate mitochondria metabolism, and regulate mitochondrial-induced programmed cell death and cellular immune response (Li *et al.*, 2021b). In cancer, it is clear that the metabolic pathways and metabolites within mitochondria are critical to supporting tumour growth and disease progression, for example, ATP synthesis and assimilating the precursors for biomass accumulation (Thomas and Ashcroft,

2021). Mitochondria are also involved in the regulation of cell survival through several methods of mitochondrial-driven apoptosis and resistance to mitochondrial-induced apoptosis, a hallmark of many cancers (Hanahan and Weinberg, 2011). However, how mitochondria contribute to cancer is not straightforward. *In silico* analysis of transcriptomic data saw 25% of cancer types downregulated Oxidative phosphorylation (OXPHOS) while 35% of cancer types upregulated OXPHOS (Gaude and Frezza, 2016), demonstrating the complexities and variabilities of the mitochondria's association with cancer.

The use of CRISPR deletion screens has allowed the identification of nuclear-encoded mitochondrial genes that are essential for cultured cancer cell survival. A caveat of mitochondrial research in this way is CRISPR libraries as of now have not incorporated mtDNA targeting guides, due to issues finding recognition sites and sgRNA delivery (Thomas and Ashcroft, 2021). Nonetheless, recent studies have described mitochondrial gene editing systems (Mok *et al.*, 2020, Hussain *et al.*, 2021). Work using the data provided by Dependency Map (DepMap) consortium has identified 1158 nuclear-encoded genes which localise to the mitochondria and 264 (23.2%) of these genes are commonly essential in >90% of 625 tumour cell lines tested (Calvo *et al.*, 2016, Tsherniak *et al.*, 2017). Although the DepMap study shows some significant conservation between cell lines, evidently nuclear-encoded mitochondrial genes are not all required in cancer cell lines and some maybe unique to a few cell lines. From these CRISPR screens, pathway-level agreement between studies has been strong for nuclear-encoded mitochondrial genes, however, there has been much less agreement at a single gene-gene level. Therefore, the variability of nuclear-encoded mitochondrial genes required for oncogenic growth is large. Since we removed genes that are commonly essential, the mitochondrial genes identified in this screen will show specificity to HP8 cells (Calvo *et al.*, 2016, Tsherniak *et al.*, 2017).

From gene ontology analysis of screen hits for the proliferative phenotype of HP8 cells, it was observed that there was a strong correlation to mitochondrial-related pathways (figure 47-49). This is perhaps not surprising for several reasons: Transformed cancer cells often have an elevated metabolic rate. A CRISPR-Cas9 screen of the related herpes virus KSHV identified 146 target genes which are depleted in KSHV-infected cells, many of which are known to localise to the mitochondria (Holmes *et al.*, 2020). Nuclear-encoded mitochondrial genes show some conservation across cancerous cell lines (Calvo *et al.*, 2016, Tsherniak *et al.*, 2017). This led me to hypothesise that mitochondrial translation is essential for the proliferation of HP8 cells. I used three known mitochondrial inhibitors, A-1210477, metformin and chloramphenicol to test this hypothesis. Metformin targets the mitochondrial respiratory-chain complex 1, the first enzyme in the respiratory chain (Vial *et al.*, 2019). Chloramphenicol targets mitochondrial ribosomes,

which are critical in the synthesising components of the respiratory chain (Sharma *et al.*, 2009). A-1210477 is known to localise to the mitochondria and facilitate mitochondrial function and ATP production (Perciavalle *et al.*, 2012). I showed that using mitochondrial inhibitors chloramphenicol, metformin and A-1210477 can induce cell death and reduce the proliferation of HP8 cells (figure 47-49). Interestingly similar findings have been observed in KSHV-infected B cells when cultured with chloramphenicol but not in the EBV-positive Burkitts Lymphoma cell line (Holmes *et al.*, 2020). However, other studies have made associations of mitochondrial function to cancer in EBV (Krishna *et al.*, 2020, Cao *et al.*, 2021). Metformin has been investigated as a cancer treatment and several studies have shown metformin to reduce cancer cell proliferation (Salani *et al.*, 2014, Wheaton *et al.*, 2014, Sharma and Kumar, 2018). Moreover, MDV infection has been seen to induce mitochondrial changes in chickens (Chu *et al.*, 2019). These findings taken together with the gene ontology analysis from the HP8 CRISPR-Cas9 screen suggest that HP8 cells require mitochondrial function to sustain their proliferative phenotype. This finding is consistent with work on other cancers for example glioblastoma (Sighel *et al.*, 2021). To investigate if this phenotype was specific to MDV latently transformed cells I performed the same experiments in the REV-transformed cell line AVOL-1, which showed similar levels of proliferation inhibition and increased apoptosis (figure 47-49). This suggested that this is not a unique phenotype to HP8 cells but also appears to be key in AVOL-1, another oncogenic virally transformed T-cell line. However, mitochondrial function and OXPHOS is not essential in all cancerous cell lines (Calvo *et al.*, 2016, Gaude and Frezza, 2016, Tsherniak *et al.*, 2017).

Specifically, A-1210477 targets the protein MCL1, suggesting MCL1 plays a role in the proliferative phenotype of HP8 cells and may mediate this process in part through the regulation of mitochondrial-induced apoptosis. Many solid and blood cancers are dependent on MCL1. Blood cancers such as Non-Hodgkin lymphoma, Myeloma, Acute lymphoblastic leukaemia and Acute myeloid leukaemia have a greater dependency on MCL1 than solid tumours (Bolomsky *et al.*, 2021). DepMap analysis shows that CRISPR-Cas9 knockout of MCL1 could significantly suppress the growth of tumour cell lines in 682/1078 (63.3%) analysed. Additionally, transcriptomic analysis in chickens has related MCL1 to phenotypes that include cell proliferation and differentiation, muscle growth, and cell division (Chen *et al.*, 2019). Therefore, taken together our findings suggest that the MDV-transformed lymphoblastoid cell line HP8 requires MCL1 gene for proliferation (figure 49). However further validation of this finding is required.

Mitochondrial stress has been associated with viral reactivation from latency in KSHV and EBV-infected cells (Zhang *et al.*, 2022a). Therefore, I speculated that mitochondrial inhibition may induce increased reactivation of latent MDV in HP8 cells. I showed that during mitochondrial inhibition using

three compounds, lytic genes are upregulated compared to the respective controls and to comparable levels to the positive control NaB at 24-48 h, a known lytic reactivation compound (figure 50). This was observed in both RNA and DNA samples by RT-qPCR and qPCR respectively (figures 50 and 51). However, using a co-culture experiment there was no significant increase in plaque formation post-treatment with mitochondrial inhibitors (figure 53). Therefore, we suggest that lytic reactivation can be triggered by mitochondrial-induced stress and can contribute to viral reactivation but has no impact on viral reactivation in cell culture in isolation using these 3 mitochondrial inhibitors trialled here (figure 52). This is the first report of MDV lytic reactivation by mitochondrial-induced stress and is the second stressor found that induces reactivation in MDV, after hypoxia (Mallet *et al.*, 2022a). This finding aligns with studies of the human oncogenic *gammaherpesviruses* EBV and KSHV which show comparable data (Chen *et al.*, 2011, Hu *et al.*, 2017, Zhang *et al.*, 2022a). However, more work is required, especially to gain further visions into mitochondrial defects. Additional comparative studies looking at mitochondrial network structure, transcript levels, genome copy number and protein levels in MDV-transformed cell lines and normal cells could provide more insights.

One mitochondrial-associated gene highlighted in our screen was transforming growth factor-beta regulator 4 (TBRG4). Although not highlighted in the screens of Ma *et al.* (2017) and Gruffaz *et al.* (2019) in their screens of EBV and KSHV transformed cells, TBRG4 has been shown to act as a cellular repressor of lytic replication in EBV and Kasposi sarcoma-transformed cells (Zhang *et al.*, 2022a). Since latency is the major determinant for transformation in MDV-infected cells, it is plausible the mutation of TBRG4 may allow the reactivation of viral replication and thus, reduced cellular proliferation. In human studies, TBRG4 has been found to regulate tumorigenesis of human lung, tracheal and osteoblast cancers (Wang *et al.*, 2018, Huang *et al.*, 2020, Wang *et al.*, 2020). In the current study it was observed that TBRG4 effected the proliferation however this was not significant ($p\text{-value} < 0.05$) (figure 55). This evidence with the support of TBRG4 studies in human cancers provides some evidence for the involvement of TBRG4 in cellular proliferation across species (Wang *et al.*, 2018, Huang *et al.*, 2020). Nevertheless, it is plausible the functionality may differ since human and chicken TBRG4 possess a 50.00 % nucleotide sequence identity (NCBI genome browser).

IRF4 is a member of the Interferon Regulatory Factor family of transcription factors, primarily expressed in T-cells and is involved in differentiation and effector function during clonal expansion of T-cells (Lu *et al.*, 2023). IRF4 transduces signals from several mitogenic stimuli, such stimuli activate NF- κ B or STAT6 resulting in IRF4 promoter activation (Shaffer *et al.*, 2009). IRF4 plays a critical role in the host immunity and the normal development of

lymphoid, myeloid and dendritic cells. Additionally, IRF4 acts as an oncogene in many lymphoid malignancies and is highly expressed in numerous types of B-cell and T-cell lymphoid malignancies (Gualco *et al.*, 2010). IRF4 induces several oncogenes, such as MYC, as well as genes that characterise each cell type by utilising its ability as a master regulator of immunity. IRF4 and its upstream factor NF- κ B form a transcriptional regulatory circuit, including feedback and feed-forward loops, to maintain the oncogenic transcriptional program in malignant lymphoid cells. Several studies have shown the functional requirement of IRF4 for oncogenic cell survival and proliferation (Wong *et al.*, 2022). The downstream effects of IRF4 are a consequence of its interaction with critical oncogenic transcription regulatory networks. One of the most notable genes upregulated by IRF4 is the MYC oncogene, seen in both neoplasms of B and T-cells (Wong *et al.*, 2022). As seen in MDV IRF4 is an essential survival factor in EBV-transformed cells (Ma *et al.*, 2017). One of the roles IRF4 plays in lymphoid cells is to induce MYC expression, a well-known nuclear phosphoprotein that is linked to cell cycle progression, apoptosis and transformation (Table. 21) (Ma *et al.*, 2017). Therefore, I suggest a similar role of IRF4 is seen in HP8 cells. Seeing the similarity between HP8 cells and the *gammaherpesvirus* EBV transformed cells gave confidence in the screening methodology due to the two viruses possessing evolutionary similarities. Additionally, a recent study observed the knockdown of IRF4 induced lytic reactivation (Bristol *et al.*, 2022). These studies elude a functional role of IRF4 in oncogene induction and lytic viral reactivation in herpes virus latently infected cells. Given the strong association of IRF4 with oncogenesis in several cancers, many studies have proposed IRF4 as a possible therapeutic target leading to the approval of lenalidomide in 2006/2007 (Zeldis *et al.*, 2011) and pomalidomide in 2013 by the FDA and EU (Hoy, 2017). Here I identify IRF4 and TBRG4 as a hit genes for the proliferation of HP8 cells. Subsequent sgRNA KD experiments outside the context of a screen provide evidence of this genotype-phenotype association (figure 55).

The shortlist of hit genes needs to be further validated and characterised. Once complete any validated hits can be further investigated into whether their mutation in chicken cells protects from the oncogenic proliferation seen in MDV-infected birds (table 23). Additional *in vivo* studies could be performed to investigate if these gene mutations protect against MDV-induced lymphogenesis.

4.6 Conclusion

A CRISPR-Cas9 GeCKO screen was successfully performed in an MDV latently infected transformed cell line (HP8), identifying a shortlist of genes that are specific to the HP8 cell's proliferative and latent phenotype. Selected

genes from this list were validated. The gene shortlist was seen to have a strong signal for mitochondrial-associated genes, which was investigated further with mitochondrial inhibitors. These inhibitors confirm the requirement for mitochondrial function for the effective growth of HP8 cells and mitochondrial inhibition increased HP8 cell apoptosis. However, this is somewhat conserved among cancer cell lines including AVOL-1 (retrovirus transformed T-cell) and KSHV latently infected cells but has not been fully investigated in EBV latently infected cells. Mitochondrial inhibition has also been shown to be a trigger of lytic reactivation. Furthermore, the growth of sgRNA-edited transfected cells was assessed which confirmed the hit gene IRF4 as critical in the proliferation of HP8 cells.

Chapter 5: RCAS-A and RCAS-J screen for host non-essential genes involved in viral entry and replication

5.1 Summary

The functionality of a gene can be investigated through the manipulation of gene expression, either by overexpression or reducing its expression. The reduction of a gene's expression can be used to reveal a phenotypic trait of interest. The phenotype of interest in this study was non-essential host cell genes required to support RCAS virus infection and replication. Many studies have revealed specific receptors for RCAS viruses which limit their tropism to specific organisms. However, little is known beyond these specific receptors. With the advent of genome-wide knockout (GeCKO) screens, it is now possible to systematically analyse host genes that prevent viral infection and replication. In this chapter, I developed a successful method to identify receptors and critical factors required in viral replication. This can be applied to other ASLV/RCAS viruses as well as other retroviruses such as the Reticuloendotheliosis virus where the receptors are not known. Furthermore, the only host genes required for viral entry and replication of RCAS-J and RCAS-A are the defined receptors.

5.2 Introduction

Since ALVs induce oncogenesis in infected chickens it is of interest to prevent infection and increase understanding of the biological processes involved to establish a propagating ALV infection. GeCKO screens can be applied to virus infection studies since a sgRNA library can be applied to host cells and subsequent viral infection. Simply, CRISPR-Cas9 relies on the Cas9 enzyme guided by single guide RNA (sgRNA; CRISPR RNAs) to mediate loss-of-function mutations through inducing frameshift mutations in coding region DNA. Following viral infection, the determinants for viral infection can be revealed. However, the caveat to these screens is having a marker for viral infection. For some viruses, this is relatively straightforward, when the invading virus induces cell death since the remaining living cells contain mutations which prevent viral-induced death. Such screens have been successfully used in influenza virus research (Han *et al.*, 2018, Song *et al.*, 2021) and HSV-1 for example (Suzuki *et al.*, 2022). Screens using highly cytopathic viruses have typically excelled at the identification of a virus's cognate receptor, for example, ZIKV CRISPR screen identified the previously defined receptor AXL as a highly enriched hit (Zhang *et al.*, 2016). Similar

has been seen in the CRISPR screens of Hepatitis C virus, Picornaviruses, Dengue virus, Norovirus and HIV-1 where known receptors or cell surface localised proteins utilised by the virus for binding or entry were all strongly enriched (Haga *et al.*, 2016, Marceau *et al.*, 2016, Savidis *et al.*, 2016, Kim *et al.*, 2017b, Park *et al.*, 2017).

However, when the virus does not induce cell death a more sophisticated marker for viral infection is required. In one recent study to look at host dependency factors of influenza-A virus, Li *et al.* (2020) used genome-wide CRISPR/Cas9 screen to identify host dependency factors of influenza-A involved in viral replication, using hemagglutinin A (HA) as a marker for viral replication and sorting based on levels of surface viral HA. Another study engineered an inducible cellular fluorescent marker system to respond to hepatitis C virus (HCV), which was successfully used to identify genes essential to HCV entry and cell-cell spread (Ren *et al.*, 2015). A similar approach was achieved when researching HIV host dependency factors (Park *et al.*, 2017) and in identifying host factors that when overexpressed are sufficient to inhibit influenza A virus infection (Heaton *et al.*, 2017). These studies provide evidence that the CRISPR-Cas9 screens using selective markers and FACS can be employed to investigate the requirements for pathogens to infect and replicate in cells of interest.

ALVs are characterised by having specific receptors depending upon subtype. ALV-A has been established to bind the TVA receptor (also known as CD320 in humans), a receptor for cobalamin (Cbl) uptake in host cells (Koslova *et al.*, 2021, Krchlikova *et al.*, 2021). ALV-J has been reported to bind the chicken Na^+/H^+ exchange 1 (chNHE1, also known as SLC9A1 in humans) to facilitate entry (Chai and Bates, 2006). But more recent findings have reported the chicken Annexin A2 (chANXA2) (Mei *et al.*, 2015) as a novel receptor and the chicken glucose-regulation protein 78 (chGRP78/HSPA5) to aid in ALV-J entry into DF1 cells (Wang *et al.*, 2016). Chicken DF1 cells have been previously edited using CRISPR-Cas9 technology in our lab and in the hands of others (Bai *et al.*, 2016). With the technology now available to perform unbiased systematic GeCKO screens in association with a viral infection this approach will be able to confirm the findings of others as well as to highlight any further potential non-essential host genes involved in the entry and replication of ALVs.

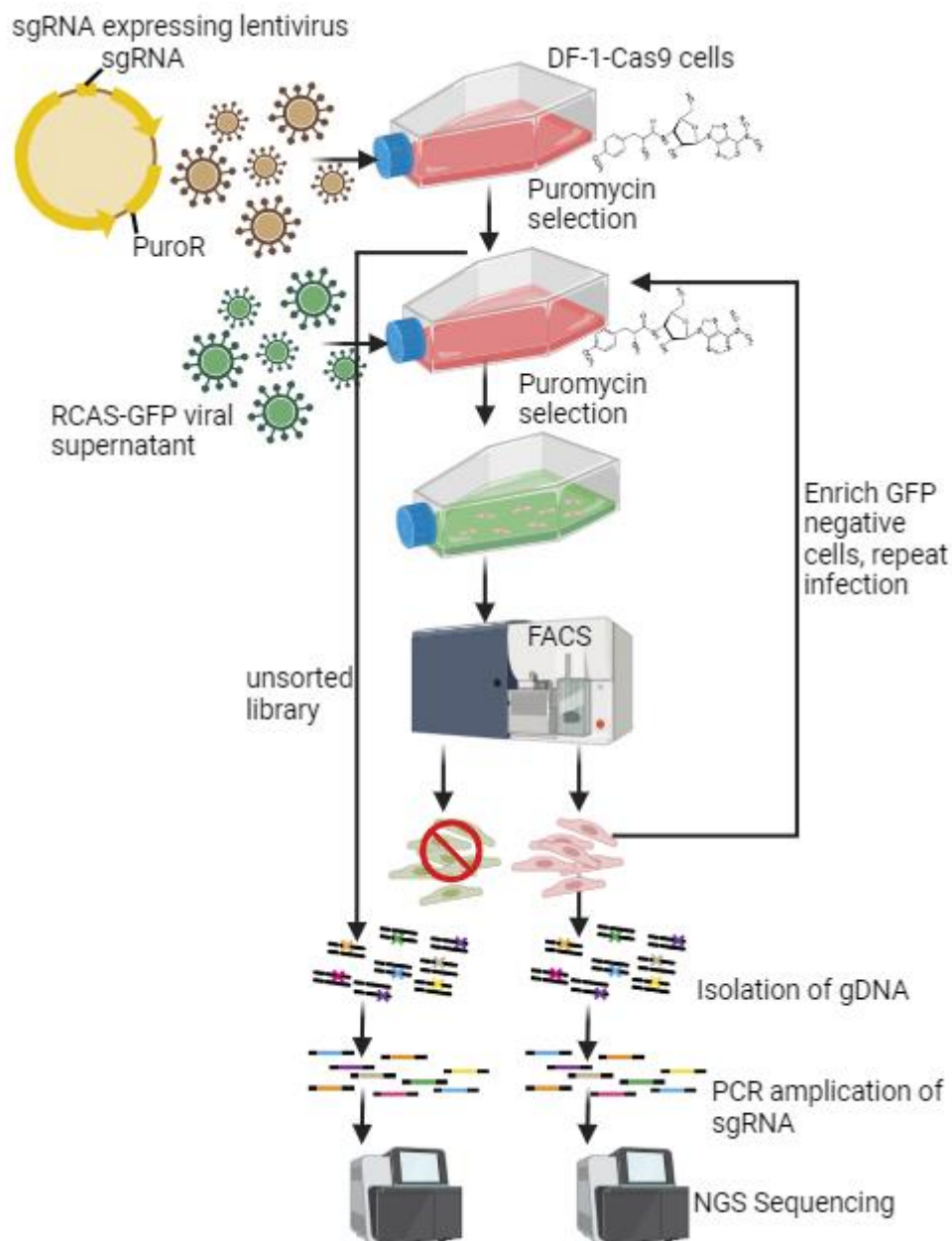


Figure 56. DF1-RCAS-GFP GeCKO screen workflow and screening strategy. Briefly, an sgRNA lentivirus library was transduced into DF1-Cas9^{Blast} cells and selected using puromycin to remove untransduced cells. After selection, an initial sample was taken before RCAS-GFP virus infection. Cells were then sorted for non-GFP fluorescence and cultured, this was repeated 3 times before a final sample of cells was collected. The gDNA of both the initial and final sorted population was performed, PCR to amplify the CRISPR identifier regions and NGS sequencing was used to distinguish the genes which were essential to the infection and replication of RCAS-J virus. Created with BioRender.

5.3 Aim of Chapter

In the present study, it was aimed to identify host cell factors required for the efficient entry and replication of the RCAS virus. I performed a FACS-based screen, using a fluorescently tagged virus. A loss-of-function pool of sgRNA cells was sorted for cells expressing no fluorescence to enrich for mutant cells refractory to RCAS infection or replication. Guides enriched in the final pool could be used to determine the non-essential host genes required for viral infection and replication. Additionally, I aimed to perform gene ontology analysis to reveal pathways required for ALVs entry and replication. Figure 56 summarises the principle of the experiment.

5.4 Results:

5.4.1 sgRNA-TVA blocks RCAS-A infection in DF1 cells

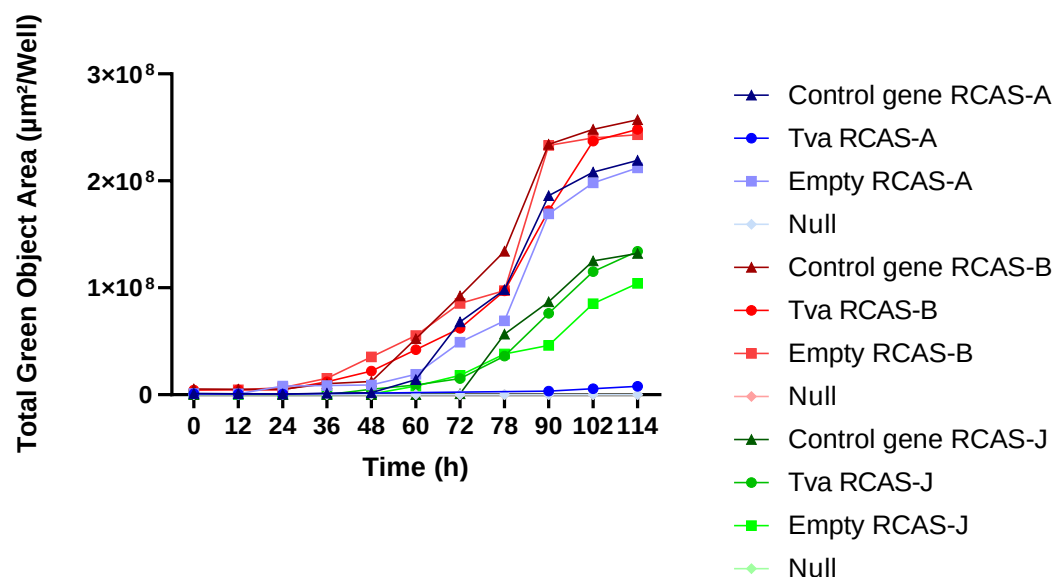


Figure 57. sgRNA-TVA transfected DF1-Cas9 cells prevent RCAS-A-GFP infection. sgRNA against TVA was cloned into a px459 expression vector, transfected into DF1 cells and selected with puromycin. The resulting cells were then infected independently with either RCAS-A -B and J virus and the infection was tracked. Control gene for RCAS-A: SLC9A1; control gene for RCAS-B: SLC9A1; control gene for RCAS-J: TVA. n = 1.

Before proceeding with a screen, I wanted to ensure the methodology proposed in figure 56 would produce efficacious hits. Using the TVA-sgRNA sequence published by Koslova *et al.* (2021) the sgRNA was electroporated into cells and subsequently infected with GFP expressing RCAS-A and B virus. As expected I saw that cells expressing sgRNA against TVA caused a noticeable reduction in viral GFP expression. A similar experiment was also performed with antibioticly selected DF1 cells expressing the TVA-sgRNA and subsequent infection with RCAS-A, RCAS-B and RCAS-J. Again the results showed that sgRNA-TVA reduced the expression of RCAS-A-GFP but not RCAS-B-GFP or RCAS-J-GFP (figure 57), confirming that TVA is a sufficient positive control and that my proposed method would enrich these cells sufficiently.

5.4.2 RCAS-A & RCAS-J CRISPR-Cas9 screen

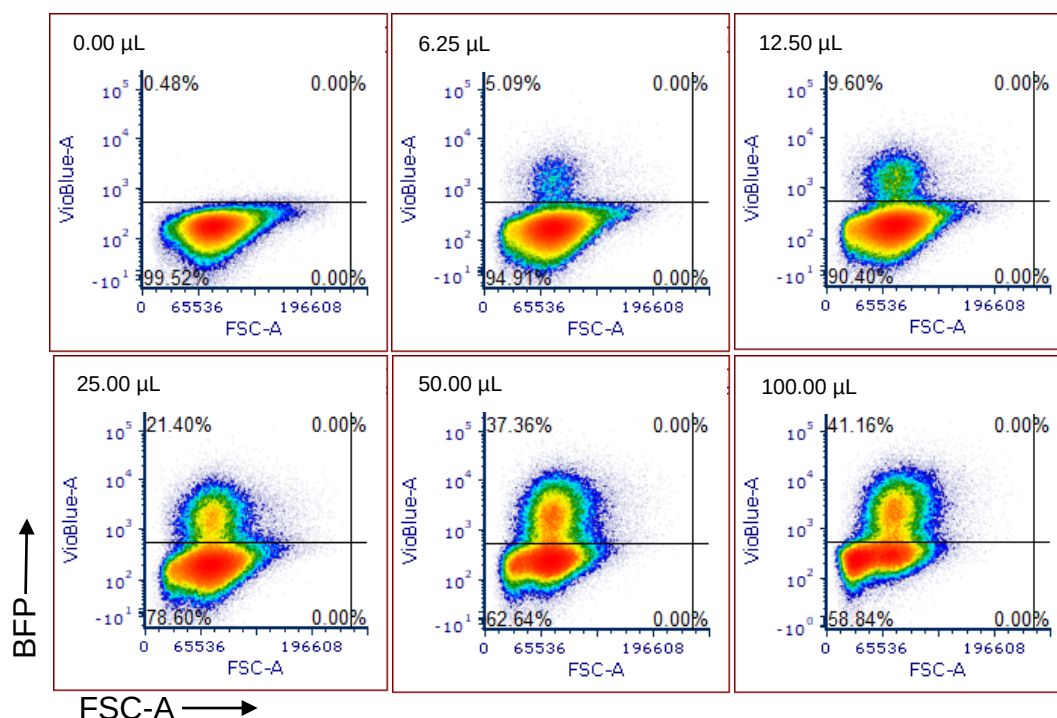


Figure 58. Lentivirus titration in DF1-Cas9^{Blast} cells, using BFP fluorescent marker. The concentrated lentivirus supernatant was titrated on HP8-Cas9^{Blast} cells using 6.25 µL, 12.50 µL, 25.00 µL, 50.00 µL, 100.00 µL /1 mL media. After 5 days incubation FACS was used to analyse the BFP expression from lentivirus infected cells. BFP = Blue fluorescent protein, FSC-A = Forward Scatter-Area.

The first stage of an effective screen is to produce a cell line that expresses Cas9 protein; this was achieved using the same method as described for HP8 cells. The cells were analysed using the same GFP/BFP reporter construct (pKLV2-U6sgRNA5(gBFP)-PGKGFP2ABFP-W). The results of figure 21 and 22 show a significant majority of cells transduced by the reporter lentivirus are expressing Cas9 activity. Therefore, these cells will provide good editing efficiency for the CRISPR-Cas9 screen. I first prepared a library of sgRNA lentiviruses and titrated the supernatant so the MOI could be calculated (figure 58). This library of lentivirus expressing sgRNAs was used to mutagenise DF1-Cas9^{Blast} cells by CRISPR-Cas9 at an MOI of 0.3. Which represented a library coverage of >350x per sgRNA.

Subsequently, the pool of DF1-Cas9^{Blast} cells was puromycin selected for 10 days and a control sample was taken before RCAS infection. This ensured the pool of cells remaining would all be expressing sgRNA and would remove any essential genes from the screen, a possible caveat of this methodology. Subsequently, I infected the selected pool of DF1-Cas9^{Blast} cells with RCAS-A-GFP or RCAS-J-GFP virus supernatant. After infection cells were FACS

sorted for GFP-negative cells. It was seen that after one sort many cells would become infected with RCAS-GFP virus after one sort thus, this was repeated three times to ensure a stringent selection of guides that prevented RCAS virus infection and replication. At each sorting stage, the percentage of cells positive and negative for GFP was recorded (figure 59). As expected after each sort the cells became more refractory to RCAS-GFP viral infection. After the final sort, a sample was taken as the endpoint of the experiment and gDNA was extracted. Before sequencing, I performed an infection assay to check the final pool of cells that were refractory to RCAS-virus infection. In both cases (figure 60), it was seen that the sorted cells were not conducive to RCAS-GFP virus infection compared to the control DF1-Cas9^{Blast} cells, confirming selection for cells refractory to RCAS-GFP infection had occurred. gDNA was extracted from the final 3rd sort cells and the pre-sort control sample. This gDNA was then used for PCR_1 and PCR_2 to prepare the amplicons for NGS (figure 61 shows the amplification plots for each of these PCRs and both RCAS-A and RCAS-J screens).

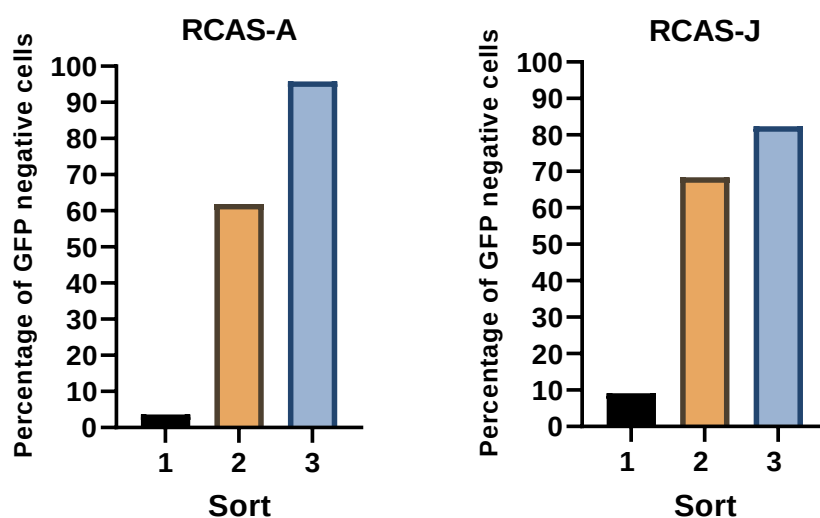


Figure 59. The percentage of GFP-negative cells increased at each fluorescent cell sorting stage. At each sort, the percentage of GFP-positive cells was recorded and plotted as a percentage of the total population.

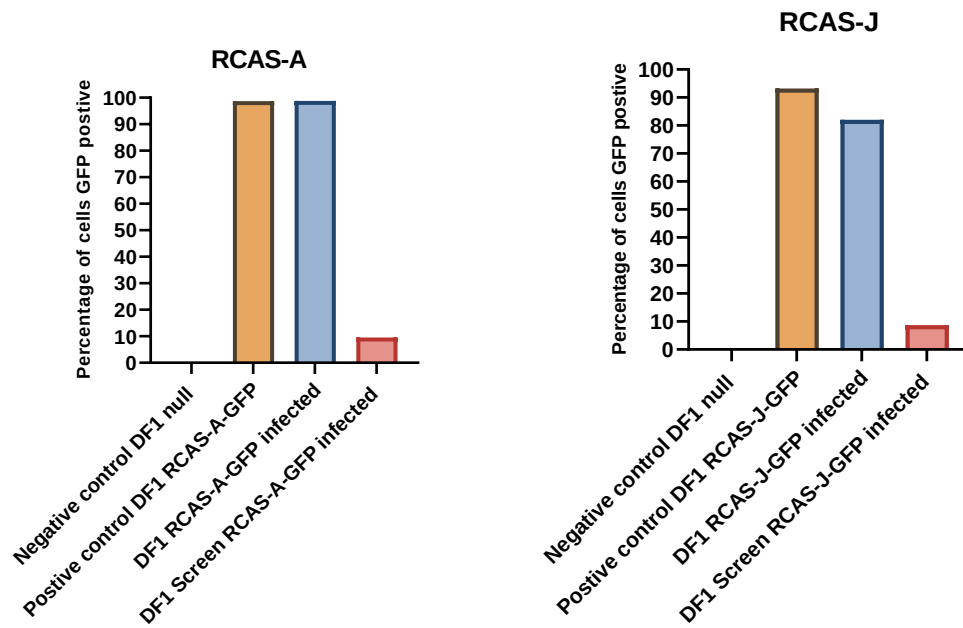


Figure 60. The post-FACS sorted cells were challenged with RCAS-A and RCAS-J for the respective screens. The post-sorted cells were challenged with RCAS-A-GFP virus for the RCAS-A screen or RCAS-J-GFP virus for the RCAS-J screen and compared to WT uninfected DF1 cells or RCAS infected DF1 cells. After 5 days incubation with RCAS viral supernatant the percentage of cells was quantified using flow cytometry.

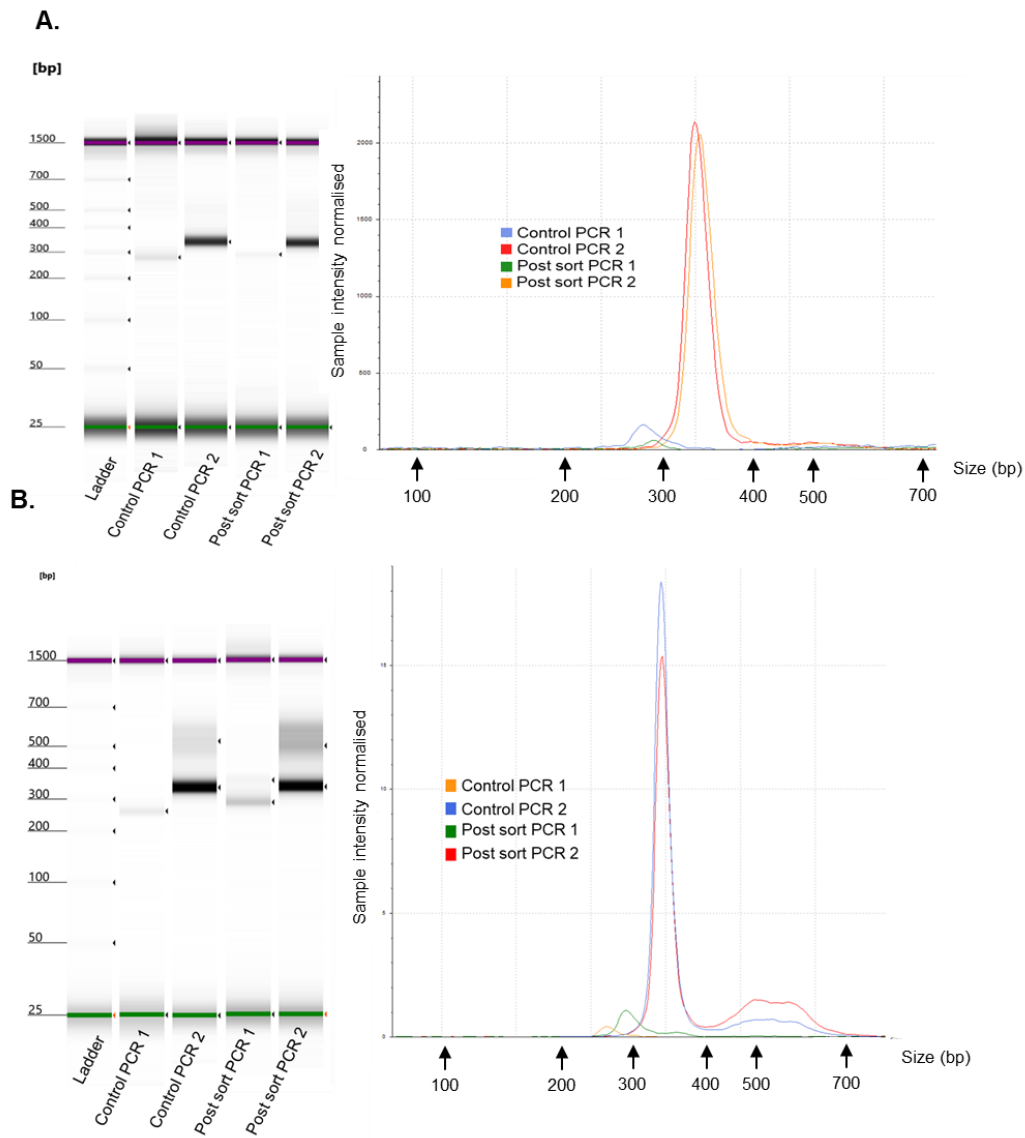


Figure 61. PCRs amplicon analysis for the control and selected cell populations. (A) RCAS-A, (B) RCAS-B. The gel image (left) and the electropherogram (right) depict the same samples. PCR_1 target was 280-bp and the PCR_2 target was ~350-bp.

5.4.3 RCAS CRISPR-Cas9 screen sequence analysis

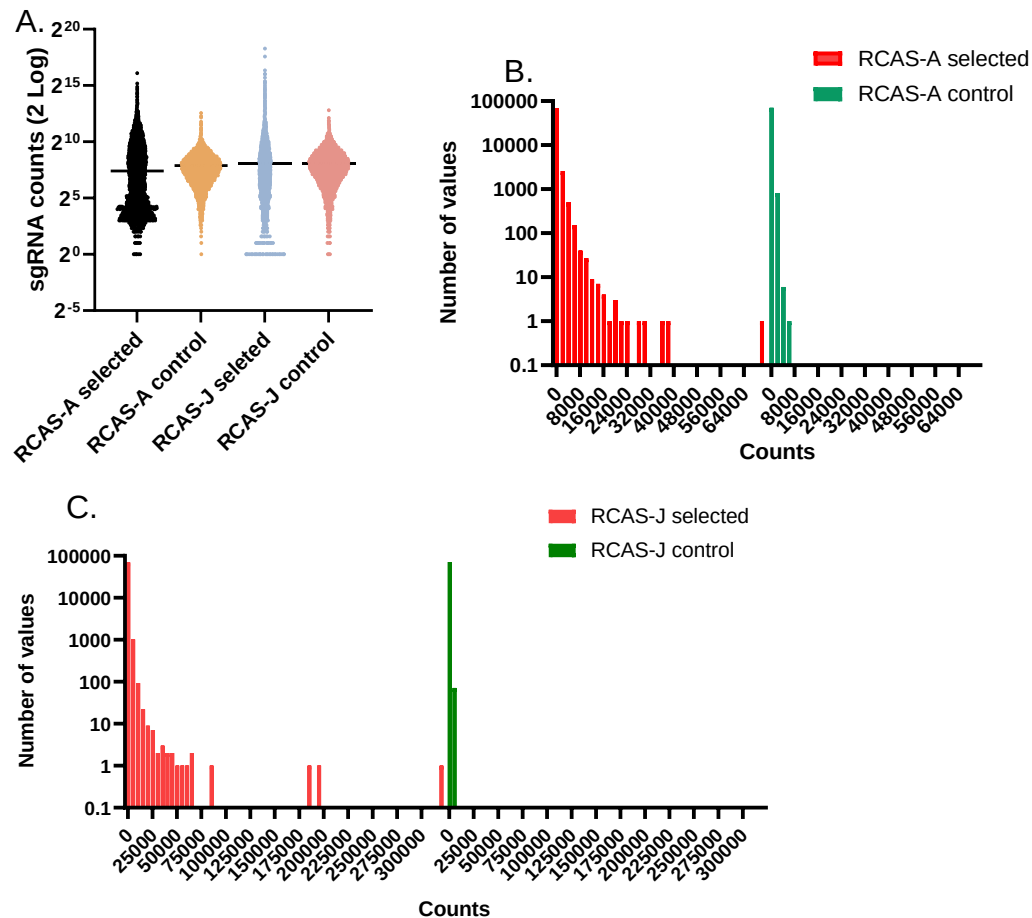


Figure 62. sgRNA read count distribution of RCAS-A and RCAS-J sorted population and control populations. (A) The read count distribution represents the frequency of NGS sequencing counting an individual sgRNA. B and C show the frequency of NGS counts for each gene (x-axis) against the number of genes counted at that value (y-axis).

The control sample and endpoint sample were then subjected to NGS sequencing to identify the genes that had been enriched in the endpoint sample. Figure 62A shows the NGS count data with the control and selected RCAS-GFP cell populations plotted. For the control samples, a normal distribution is expected, while a selected population would be expected to have a longer tail with more 0 count sgRNAs. As expected, it was observed the selected population differed in sgRNA guide distribution with more sgRNAs highly enriched than the control populations (figure 62A). The same data was plotted with the x-axis showing the NGS counts for each gene and the y-axis showing the number of genes within that count bracket. Both histograms (62 B and C) show control RCAS sample sgRNAs are similarly represented, however, after selection the sgRNA counts are more varied with some sgRNAs having very high counts, indicating these sgRNAs have been

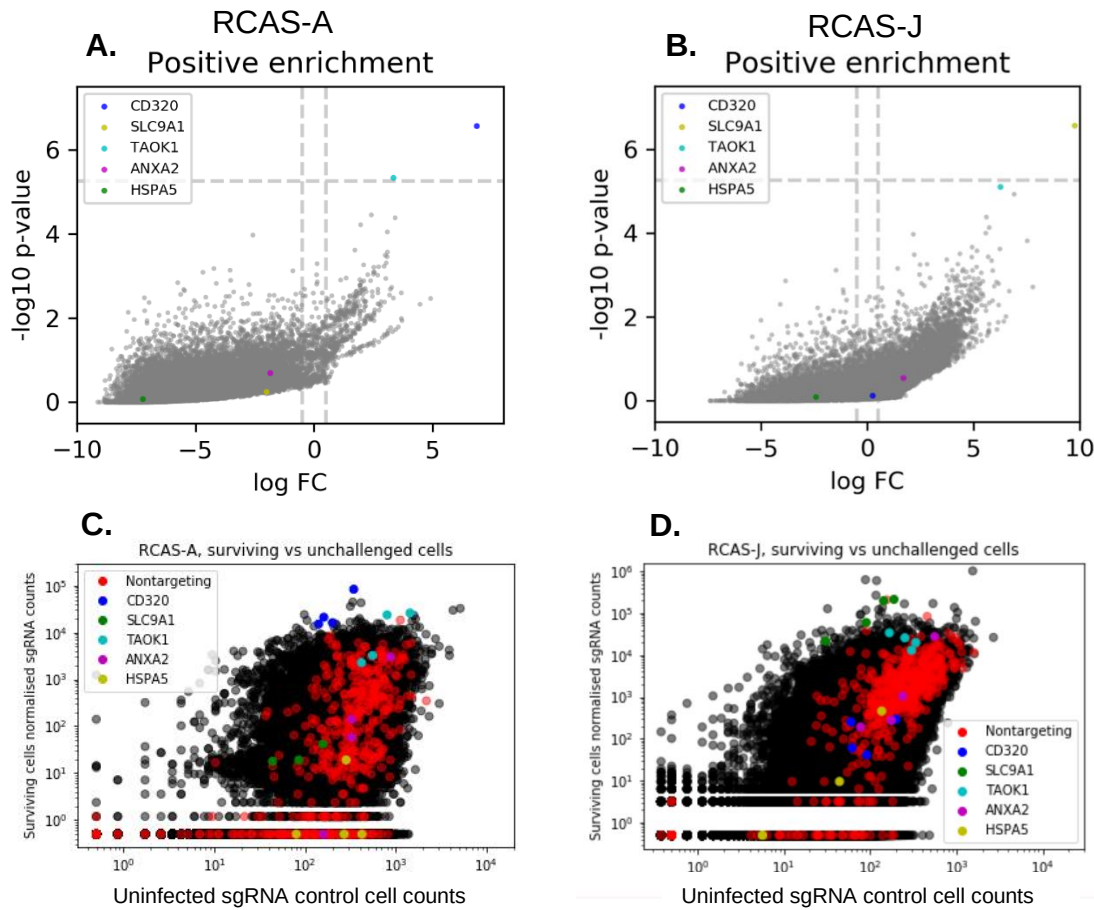


Figure 63. RCAS-A and RCAS-J primary receptor mutations are highly enriched in cells that prevent RCAS infection. A and B: *p*-value vs the log fold change of sgRNA counts. C and D: surviving cells post FACS sorting sgRNA counts vs uninfected sgRNA control cell counts. A and C: RCAS-A. B and D: RCAS-J. Non-targeting controls highlighted in red for reference. Log FC = Log Fold change of sgRNA counts. The dashed vertical lines represent 0.5 Log Fold change (which represents a 50% deviation) from the control sgRNAs. Horizontal dashed line represents the threshold for significance.

enriched. sgRNA read count distribution of RCAS-A and RCAS-J sorted population and control populations were plotted as a histogram to show sgRNA count distribution (figure 62 B and C). Both histograms show control RCAS samples sgRNAs are similarly represented, which would be expected at this point, with some sgRNAs showing a slight increase in sgRNA number, perhaps due to these genes conferring growth advantages. However, after selection, the sgRNA counts are more varied with some sgRNAs having very high counts. Also shown in figure 62A are long whiskers in the selected samples which show few sgRNAs are highly enriched rather than clustering around the mean which is seen in the control samples. Indicating that few sgRNAs have been enriched highly, which would be expected in a screen of this type.

In both RCAS-A and RCAS-J, the major receptors of both were significantly enriched (positive p -value < 0.01), *tva* (CD320) gene for RCAS-A and *nhe1* (SLC9A1) gene for RCAS-J (figure 63). The charts show in figure 63 that both RCAS-A receptor CD320 (A and C) and RCAS-J receptor SLC9A1 (B and D) are highly positively enriched, as well as TAOK1 (A and B), the same can be seen in C and D. The charts in figure 63 show that TAOK1 was a highly enriched gene in the input plasmid and the control sample, suggesting TAOK1 maybe a false positive hit (A and B). Both receptors were shown not to be enriched in the unchallenged control cells or input plasmid whereas TAOK1 in both viruses showed enrichment (C and D). A gene of note that was shared between both screens was TAOK1 and was significantly (positive p -value < 0.01) enriched although with a log lower p -value score (positive p -value: SLC9A1 2.75E-07, TAOK1 7.99E-06, CD320 2.75E-07, TAOK1 4.68E-06). However, TAOK1 proved to be a false positive result. This conclusion was made due to the high counts seen in the control sample and plasmid input sample (figure 64) suggesting TAOK1 sgRNAs were numerous in the transduced library and that TAOK1 sgRNA KO seems to confer a growth advantage (figure 63 and 64). Thus, leading to reduced TAOK1 depletion during the sorting stage of the screen. This was confirmed with sgRNAs against TAOK1 which were shown to have no effect on RCAS-A or RCAS-J infection (figure 65).

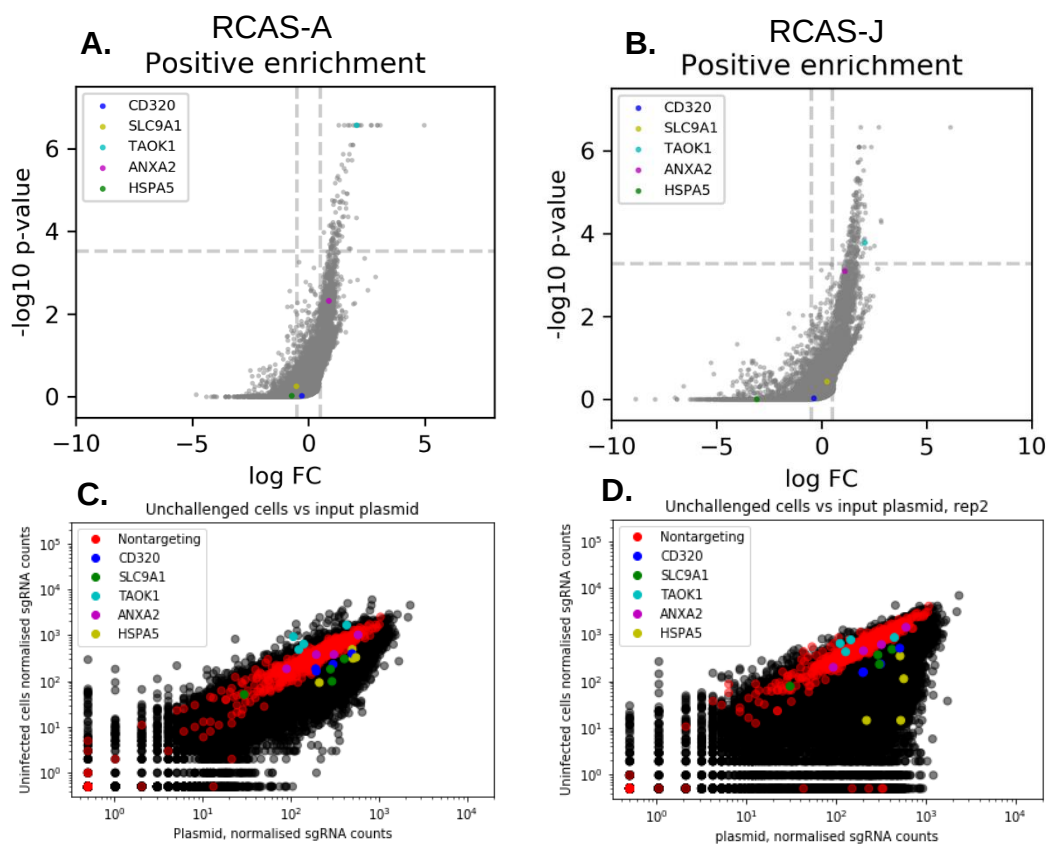


Figure 64. Unchallenged control cell sgRNA counts compared to input plasmid sgRNA counts. A and B: *p*-value vs the log fold change of sgRNA counts. C and D: surviving cells post FACS sorting sgRNA counts vs uninfected sgRNA control cell counts. A and C: RCAS-A. B and D: RCAS-J. Non-targeting controls highlighted in red for reference. Log FC = Log Fold change of sgRNA counts. The dashed vertical lines represent 0.5 Log Fold change (which represents a 50% deviation) from the control sgRNAs.

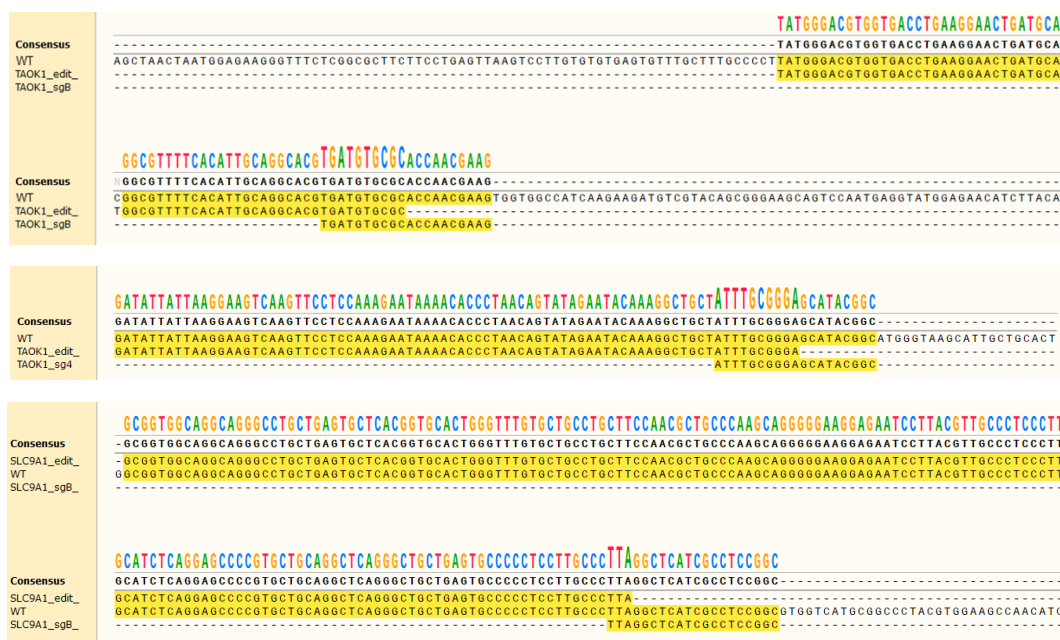
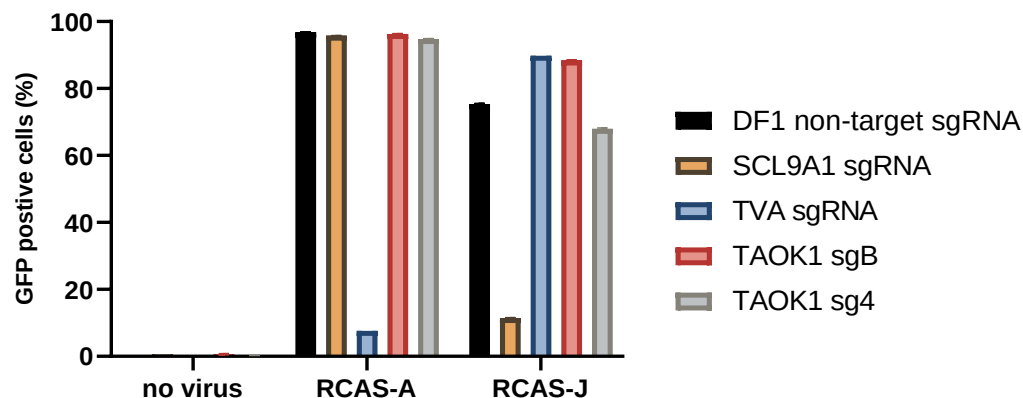


Figure 65. TAOK1 CRISPR editing and RCAS-A and RCAS-J infection. sgRNAs were cloned into the px459 expression vector and transfected into DF1 cells. After puromycin selection to remove untransduced cells, the cells were infected with RCAS-A/J virus. The bar chart shows an infection assay, with sequencing below to confirm successful sgRNA on-target gene editing. sgB and sg4 were the two sgRNAs used.

5.4.4 RCAS-A and RCAS-J CRISPR-Cas9 biological pathways analysis

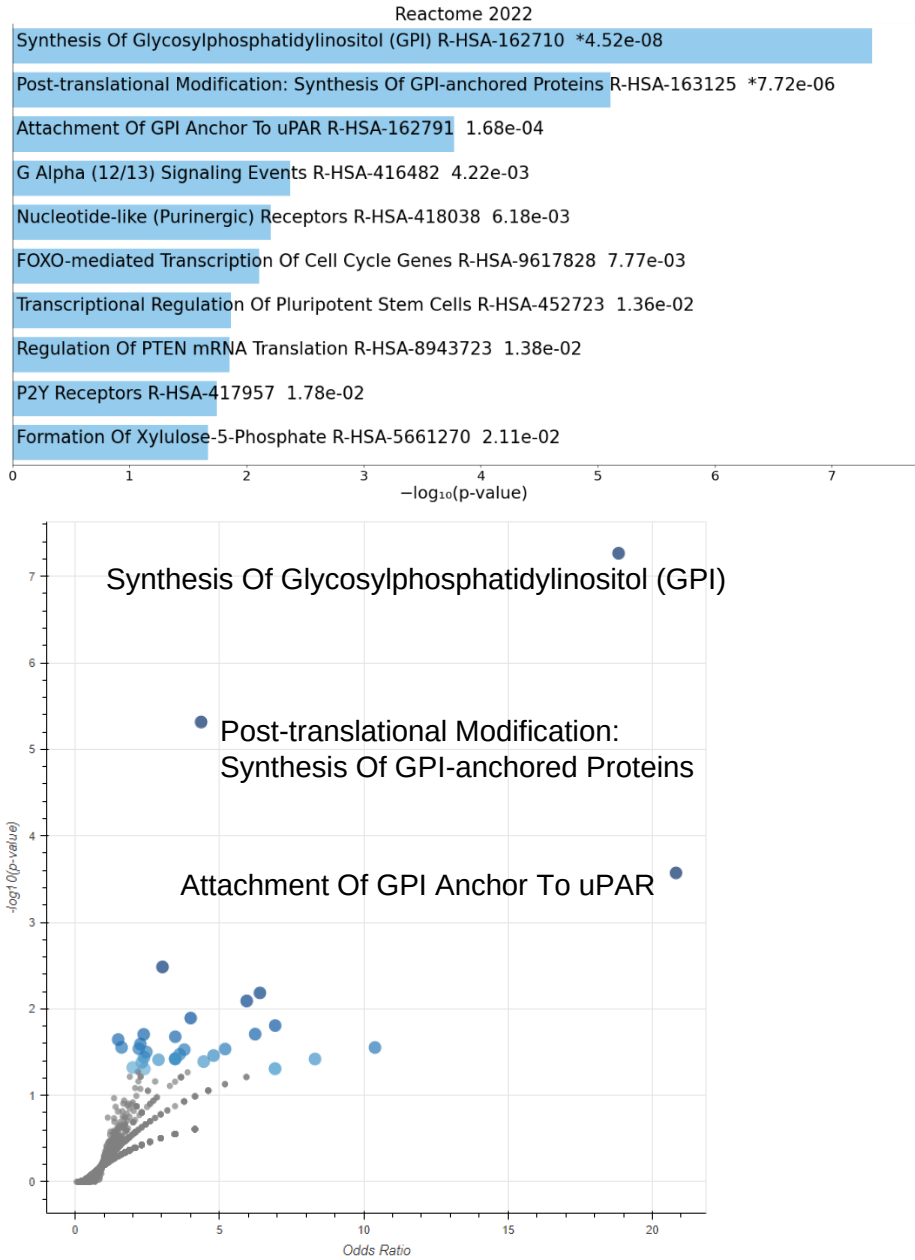


Figure 66. RCAS-A screen Reactome pathway analysis bar chart and volcano plot. Bar chart of top 10 enriched terms and volcano plot of terms from the Reactome_2022 gene set library. The input genes represent those enriched with a $p\text{-value} < 0.05$. The top 10 enriched terms for the input gene set are displayed based on the $-\log_{10}(p\text{-value})$, with the actual $p\text{-value}$ shown next to each term. For the volcano plot, each point represents a single term, plotted by the corresponding odds ratio (x-position) and $-\log_{10}(p\text{-value})$ (y-position) from the enrichment results of the input query gene set. The larger and darker-coloured the point, the more significantly enriched the input gene set is for the term.

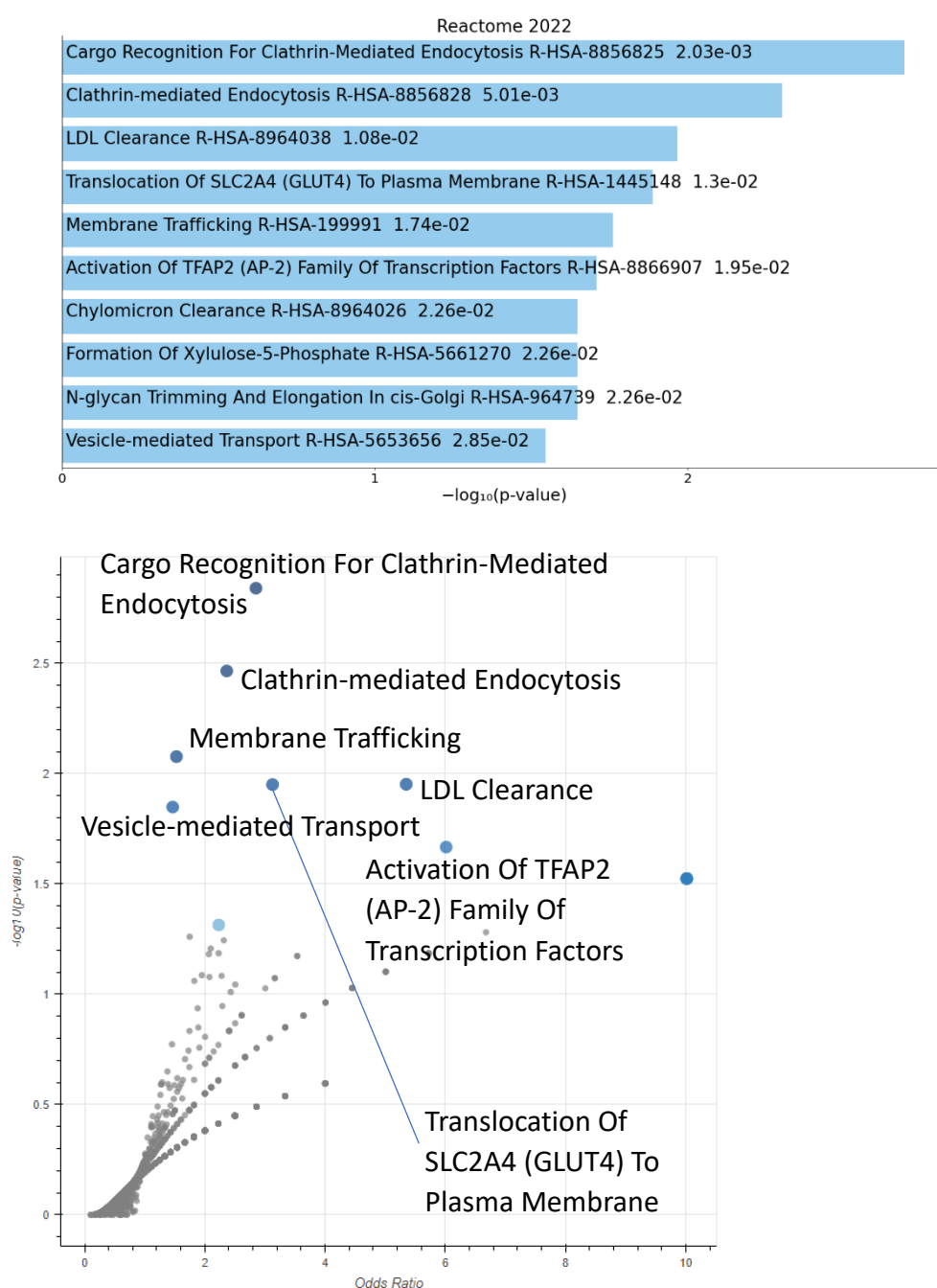


Figure 67. RCAS-J screen Reactome pathway analysis bar chart and volcano plot. Bar chart of top 10 enriched terms and volcano plot of terms from the Reactome_2022 gene set library. The input genes represent those enriched with a $p\text{-value} < 0.05$. The top 10 enriched terms for the input gene set are displayed based on the $-\log_{10}(p\text{-value})$, with the actual $p\text{-value}$ shown next to each term. For the volcano plot, each point represents a single term, plotted by the corresponding odds ratio (x-position) and $-\log_{10}(p\text{-value})$ (y-position) from the enrichment results of the input query gene set. The larger and darker-coloured the point, the more significantly enriched the input gene set is for the term.

Genes enriched by a $p\text{-value} < 0.05$ were put through gene pathway analysis (enrichr), and for RCAS-A glycosylphosphatidylinositol (GPI) involved pathways were significantly enriched (figure 66). For RCAS-J pathways involving clathrin were most highly enriched (figure 67). It was noted the pathways involved in RCAS-A infection provided many more significant pathways than that of RCAS-J. RCAS-A showed 30 significant pathways ($p\text{-value} < 0.05$) whereas RCAS-J has 6 significant pathways ($p\text{-value} < 0.05$). It was also seen that there were no shared significant pathways between RCAS-A and RCAS-J.

5.4.5 RCAS-A & RCAS-J combined hits

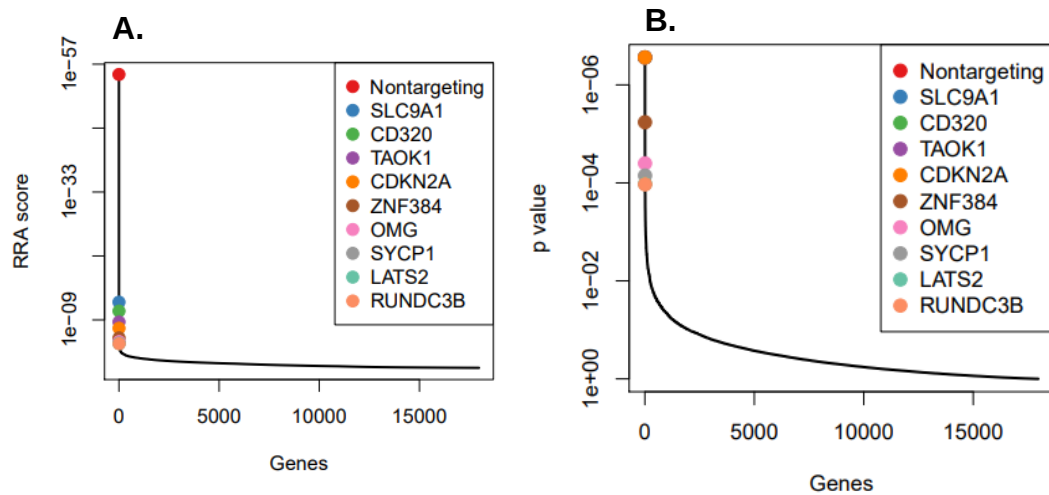


Figure 68. RRA score and $p\text{-value}$ of RCAS-A and RCAS-J screens. DF1-Cas9^{Blast} cells were transduced with pKLV2 lentivirus library expressing sgRNAs against host genes. A control DF1-Cas9Blast cell population was taken after which several rounds of RCAS-GFP and FACS sorting for non-GFP cells was performed. The cells refractory to RCAS-GFP infection were collected. Analysis of the differences between the starting and end cell population produced a ranked list of genes that are involved in the entry and replication of RCAS-A/J viruses. (A) RCAS-A/J (RRA; Robust Rank Algorithm), (B) RCAS-A/J.

5.4.6 RCAS hit confirmation.

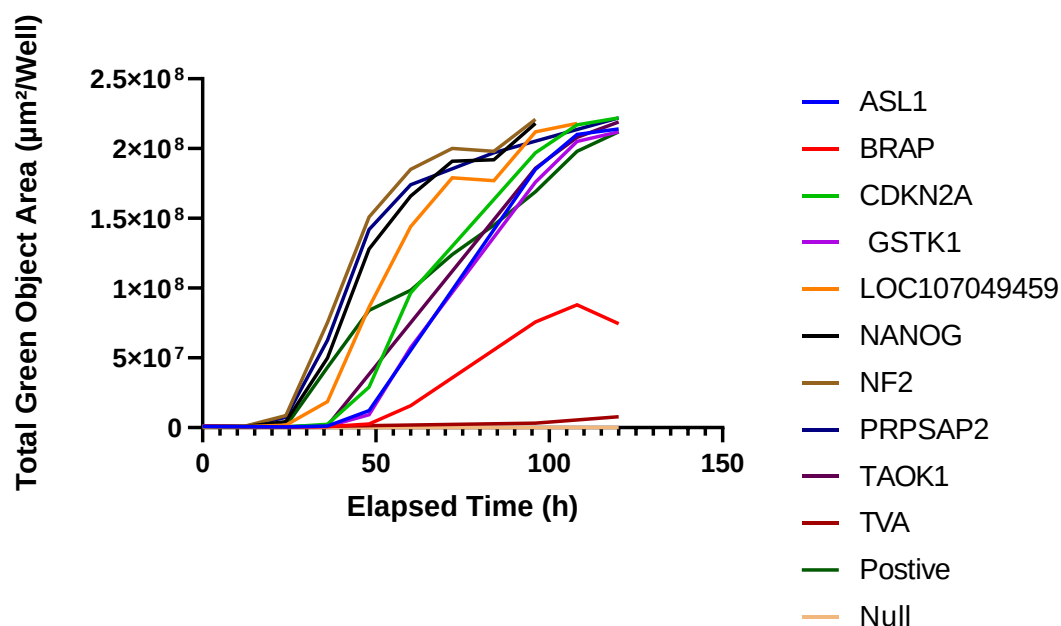


Figure 69. sgRNAs against specific genes highlighted as essential in the RCAS-A screen. The most enriched genes in the RCAS-A-GFP screen were validated using the most enriched sgRNAs. sgRNAs were cloned into the px459 expression vector and transfected into DF1 cells. After puromycin selection to remove untransduced cells, the cells were infected with RCAS-A virus. Null: uninfected cells. Positive: RCAS-A infection in the presence of no sgRNAs.

To confirm whether hits other than the defined receptors SCL9A1 and TVA were real we cloned the top-performing guides into the sgRNA vector px459. These sgRNAs plasmids were transfected into DF1 cells and selected with puromycin. The resulting cells were challenged with RCAS-A virus or RCAS-J virus. None of the top 'hits' including the second most enriched gene TAOK1 in both studies proved to be required for viral infection and replication in these screens (figure 68 and 69). The sequencing results show the sgRNAs used made successful on-target gene editing for TAOK1 guides and SLC9A1 sgRNA, while the TVA guide had been previously validated by Kosolova *et al.* (2021). Thus, giving confidence in the genotype-phenotype association. It was noted that BRAP (figure 69) showed a marked reduction in GFP fluorescent intensity, however under fluorescent microscopy there was no observable difference to the other genes tested (figure 69).

5.5 Discussion

A CRISPR-Cas9 mediated genome-wide screen of non-essential host cell genes was successfully performed for two RCAS virus sub-groups RCAS-A and RCAS-J. The combined use of CRISPR-Cas9 technology and FACS allowed the set up of a stringent assay to identify non-essential host genes required for the infection and replication of two RCAS viruses. For both viruses a sufficient body of literature describing already known receptors or genes involved in the infection of cells in cell culture exists. For example, it has been established that TVA (CD320) is a membrane-bound receptor for the RCAS-A virus and chNHE1 (SLC9A1) is a membrane-bound receptor for the RCAS-J virus (figure 70) (Chai and Bates, 2006, Lee *et al.*, 2017b, Lee *et al.*, 2019, Koslova *et al.*, 2021). For the first screen, carried out on RCAS-A-GFP, the positive control was validated (figure 57) to ensure that in our hands the proposed method could corroborate any findings. These established genes involved in RCAS infection provided positive control genes for each screen. Both screens used a genome-wide pKLV2-Chicken sgRNA library that was first selected with puromycin, and in doing so, eliminated any genes which are essential to DF1 cell maintenance and growth. Furthermore, due to the length of this study (approximately 2 months) any genes involved in the proliferation would also be removed during this selection. The selected DF1 cells were sorted 3 times and infected approximately 4/5 times over 10-14 days. A long infection period and using multiple sorts ensured that only cells that possessed the desired phenotype were selected. However, a caveat of the long infection/sort is that this cycle could allow cells to increase in representation that are tumour repressor genes or any other gene where mutation leads to increased proliferation of the host cell. To mitigate against this phenomenon, analysis of the enriched genes was compared to a control chicken cell line where library selection had occurred. At each subsequent sort, GFP expression decreased showing enrichment for cells expressing sgRNAs against genes that block entry or replication of RCAS-GFP (figure 56). Furthermore, the endpoint infection assay showed the remaining cells were significantly refractory to viral infection (figure 60).

For the RCAS-A virus screen as expected *tva* gene KO was enriched most significantly (positive *p-value* 2.68E-07), indicating the technical quality of the screening process and confirming the results of others using a non-biased genome-wide screening approach (Koslova *et al.*, 2021). However, beyond TVA, no other genes produced a significant enrichment to the same extent as TVA.

From gene pathway analysis of genes with positive *p-value* < 0.05, it was seen that GPI anchor biological processes were highly selected (figure 66). GPI plays a critical role in the trafficking of translated protein from the ER to the Golgi to the plasma membrane (Kinoshita, 2016). TVA is seen in two

isoforms: a type 1 transmembrane protein and an isoform which is linked to the membrane surface by a GPI anchor (Krchlikova *et al.*, 2021).

Given that TVA is a membrane-bound receptor, this provides further evidence that membrane-bound receptors are the critical non-essential genes involved in RCAS-A viral infection. These results with the studies of Koslova *et al.* (2021) provide strong evidence that the RCAS-A infection is

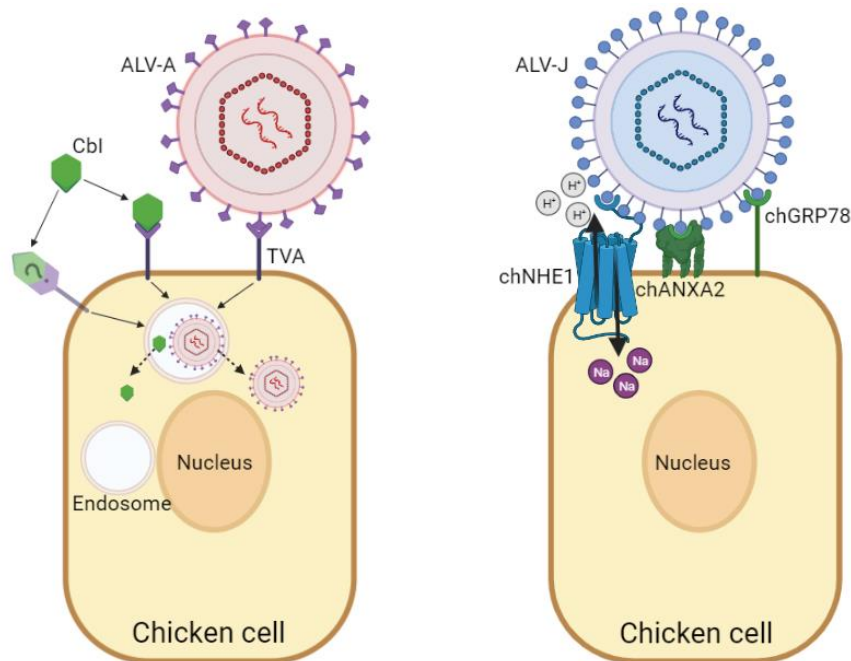


Figure 70. Diagrams illustrating the receptors in chicken cells for ALV-A and ALV-J. Left, TVA receptor and a possible alternative Cbl receptor is shown. Right, the chNHE1 ALV-J receptor and two other suggested receptors are shown. Created with BioRender.

solely mediated by the TVA receptor. Therefore, it can be concluded that the genomic deletion of TVA in chickens would confer complete resistance to RCAS-A/ALV-A infection. Due to the discrete correlation of TVA with RCAS-A infection, this would suggest a minimal evolutionary risk for entry via other receptors. It remains to be seen if TVA Δ has long-term implications but since the uptake of the essential vitamin cobalamin is redundant, little to no adverse phenotypic effects would be expected (Krchlikova *et al.*, 2021). This is corroborated by the work of Chen *et al.* (2022) where amino acid substitution blocked ALV-A and ALV-K infection but did not hinder cobalamin uptake. Of course, essential receptors may play a role, however, the data shown here and by Koslova *et al.* (2021) show that *tva* mutation is sufficient to completely block ALV-A infection. These results with the studies of Koslova *et al.* (2021), Lee *et al.*, (2017b) and Lee *et al.*, (2019) provide strong evidence that the RCAS-A infection is solely mediated by the TVA receptor and thus it can be concluded that the genomic deletion of TVA in chickens would confer complete resistance to RCAS-A/ALV-A infection. To further

investigate this hypothesis a GeCKO screen could be performed in a DF1- Δ TVA cell line which would provide a more stringent threshold for genes making cells permissive to RCAS infection. Significantly enriched hits could then be validated using KO experiments.

In the RCAS-J screen as expected, the chNHE1 (SLC9A1) which has been previously identified as the receptor for ALV-J was the most enriched gene (positive *p-value* 2.75E-07) (Chai and Bates, 2006). However, like the RCAS-A CRISPR-Cas9 screen, few other genes were significantly enriched to the same extent as the receptor. The other novel receptor reported to aid in RCAS-J infection chANXA2 saw no significant positive enrichment in our screen (positive *p-value* 0.28658, position 5147), contrary to the suggestions of Mei *et al.* (2015) which showed increased ALV-J infection in chANXA2 expressing non-permissive cells. Antibodies or siRNA against chANXA2 in DF1 cells significantly inhibited ALV-J infection (Mei *et al.*, 2015). However, when analysing the sgRNA count data only 1/4 sgRNAs were significantly enriched, with 3 others not reaching the threshold for significance, perhaps due to inefficient guides. Thus, more sgRNAs would be required in the library to investigate if this is a true co-factor in RCAS-J infection in the context of a screen. Another cellular factor reported to aid in ALV-J infection is chGRP78 (HSPA5) which saw no significant positive enrichment in our screen (positive *p-value* 0.8132, position 14032). This would be expected as HSPA5 which is an essential gene (depmap.org). Nonetheless, the study by Wang *et al.* (2016) which used siRNA and antibodies against chGRP78 significantly inhibited ALV-J infection and replication, in addition to overexpression of chGRP78 in non-permissive cells, enabling infection of ALV-J (Wang *et al.*, 2016).

A gene ontology analysis was performed using the most enriched genes with a positive *p-value* < 0.05. Pathways that showed the most significant enrichment for RCAS-J were those involving clathrin-mediated endocytosis and vesicle transport (figure 67). The RCAS-J receptor chNHE1 is a membrane receptor and functions as a Na⁺/H⁺ exchanger (Putney *et al.*, 2002). Clathrin is a protein heavily involved in endocytosis and has been linked to chNHE1 endocytosis (Szadvari *et al.*, 2019). Thus, it is speculated that RCAS-J is internalised by clathrin mediated endocytosis. It was also observed that no significant pathways involved in the entry and replication of RCAS-A and RCAS-J were conserved between the two viruses. The viruses only differ in the nucleotide sequence within the envelope gene, thus it is interesting that the viruses appear to be dependent on their specific receptors for viral entry and replication. However, it is important to note the caveat that host essential genes would not be revealed in this screen.

During hit validation (figure 65 and 69) no other genes, other than the primary receptors, were found to inhibit RCAS infection. All hits identified are likely false positives since they were also found to be positively enriched in the

plasmid input sample and control sample (figure 64). In addition, KO of genes using sgRNA in cell culture showed no significant decrease in viral infection (figure 65 and 69).

One gene that saw high enrichment in both screens was TAOK1 (positive *p-value* 1.33E-06 RCAS-A screen and 2.67E-06 in RCAS-J screen) albeit, the positive *p-value* being a factor of 10 below that of the receptors. TAOK1 localises to the microtubule cytoskeleton and perinuclear region of the cytoplasm. TAOK1 enables alpha-tubulin binding activity, beta-tubulin binding activity and kinase activity. TAOK1's roles include mitotic G2 DNA damage checkpoint signalling, regulation of microtubule depolymerisation and positive regulation of JNK cascade (NCBI gene). Due to TAOK1's role in the cell cycle, perturbation of this gene may lead to an increase in cellular proliferation. Explaining TAOK1s enrichment in the screen and thus a false positive hit (figure 64, 65 and 69).

It maybe expected that screens for host determinants of viral infection do not produce an array of hit genes beyond those already defined as critical to the virus entry or replication given the nature of viruses and their hosts. Firstly, when screening for host genes any essential genes to the host will be negatively selected against and thus, only host non-essential but viral essential genes can be identified. Therefore it could be suggested that all RCAS virus genes essential for viral replication and spread in cell culture are host essential genes except for their entry receptors since no genes aside from the entry receptors were seen to be significant. Secondly, through the evolutionary time of host mutation, viruses would evolve to utilise only critical gene products of the host since a non-essential gene could easily mutate and confer resistance to viral infection. Perhaps, these issues of any essential gene or gene which confers a growth disadvantage would be exacerbated in a study such as ours where selection lasted up to ~ 6 weeks. In the future, it maybe beneficial to design screens where the start-to-end point of the experiment is minimised to gain the best chance of finding genes that maybe depleted over selection.

5.6 Conclusion

A successful CRISPR-Cas9 GeCKO screen was performed to identify host non-essential genes for virus replication and infection, and correctly identified the two known receptors for RCAS-A and RCAS-J infection, TVA for RCAS-A and chNHE1 for RCAS-J. However, co-factors identified by previously published work were not strongly enriched in our screen, due to poor sgRNA design and gene essentiality (chANXA2 and chGRP78). Further, gene ontology analysis suggests that GPI anchor-related pathways are essential in RCAS-A infection and replication, and clathrin-mediated vesicle trafficking pathways are essential in RCAS-J infection and replication. Both viruses

appear to rely heavily on their defined receptors and few other genes were enriched to a significant extent. One of these TAOK1 was conserved across both viruses but was likely a false positive, and from subsequent experiments, TAOK1 appears not to be critical in the infection or replication of RCAS viruses. This methodology described here can now be applied to other ALV/RCAS viruses as well as other retroviruses such as Reticuloendotheliosis virus where the receptor is yet to be described.

Chapter 6: MEQ binds to gga-miR-21 promoter but does not regulate miR-21 expression in HP8 cells

6.1 Summary

Marek's disease virus (MDV) is an *alphaherpesvirus* and is commonly associated with the oncogenicity of T-cells. MDV encodes a basic leucine zipper protein (bZIP), MDV EcoQ (Meq). Meq forms homo or heterodimers with c-Jun, which regulates viral and host gene expression, resulting in the oncogenic phenotype seen in infected cells. However, less is known regarding the mechanisms of Meq oncogenicity. MDV has been reported to regulate gga-miR-21 expression via AP-1 response elements. We confirm the findings that the gga-miR-21 promoter can be upregulated through Meq dimer binding. However, *in vitro* cellular assays when Meq levels are reduced or increased show no correlation to gga-miR-21 expression. It was also demonstrated that mutation of gga-miR-21 promoter AP-1 had no significant impact on gga-miR-21 expression. The data, therefore, suggest that although the gga-miR-21 promoter can be bound and upregulated by Meq dimers, at the cellular level, the gga-miR-21 expression is not significantly regulated through Meq binding.

6.2 Introduction

As one of the most effective oncogenic viruses in susceptible birds, MD first highlighted a role for several viral oncogenes (Davison *et al.*, 2004) such as MEQ, pp38, viral IL-8, LAT, viral telomerase RNA and virally encoded miRNAs (Nair, 2013). Of greatest importance is the MEQ oncogene, where studies using MDV *meq* mutant have undoubtedly shown that MEQ is responsible for the transformation of lymphocytes and is present in all MDV-induced tumours and latently infected cells, however; it appears dispensable for viral replication (Jones *et al.*, 1992) (Lupiani *et al.*, 2004b).

As part of the basic leucine zipper family of proteins, Meq has a proline-rich region in the transactivator domain at the C-terminal end and a comparable N-terminal domain to other bZIP Jun/Fos family proteins (Jones *et al.*, 1992, Qian *et al.*, 1995). It has been established that Meq can form both homodimers and heterodimers. Homodimers bind to Meq response elements I and II (MEREI & MEREII) and have been seen to have repressive functions (Levy *et al.*, 2003). However, c-Jun seems to be the primary dimerisation partner for MEQ, forming MEQ/C-JUN dimers which promote activational gene regulatory functions (Levy *et al.*, 2003). The MEQ/C-JUN heterodimers are more stable than individual homodimers and transactivate promoters containing AP-1 motifs, which has been seen to occur at chicken IL-2 promoters of MDV transformed cells (Levy *et al.*, 2003, Levy *et al.*, 2005).

Meq may also regulate Bcl-2 and CD30 and other cell cycle and apoptotic pathways (Levy *et al.*, 2003, Ajithdoss *et al.*, 2009). Additionally, AP-1 activation has been seen in T-cell lymphomas (Mori *et al.*, 2000, Iwai *et al.*, 2001) and it is known that c-Jun is a retroviral oncogene and AP-1 transcription factor (Wisdom, 1999, Vogt, 2001). This suggests a Meq/AP-1 interaction may contribute to MDV-induced viral oncogenicity.

Traditionally, tumour-inducing viruses have been associated with viral oncoproteins e.g. MEQ, but increasingly, attention has shifted to the role of miRNAs in tumorigenesis (Yao *et al.*, 2009). Viral miRNAs are predominately characteristic of herpesviruses. MDV-1, an *alphaherpesvirus*, has also been identified to encode 25 miRNAs, derived from 13 pre-miRNAs located within the *meq* oncogene and LAT region of the terminal and inverted repeat long and short regions of the genome (Burnside *et al.*, 2006, Yao *et al.*, 2008). Differential miRNA expression patterns have consistently been observed in MDV latent and tumour cells, indicating a possible association of these miRNAs in proliferation control (Burnside *et al.*, 2006, Yao *et al.*, 2009, Luo *et al.*, 2011). A well-characterised *Herpesviridae* example is EBV. EBV infection induces the cellular miR-155 which is well established to play critical roles in immune regulation, and lymphocyte homeostasis including the immortalisation of B cells (Muylkens *et al.*, 2010). The functional ortholog of miR-155, MDV-1-miR-M4 has been shown to be highly expressed during both lytic and latent MDV replication and is crucial in MDV-induced tumorigenesis (Zhao *et al.*, 2011). This high expression of MDV-1-miR-M4 is in part to compensate for the repression of miR-155 in MDV lymphogenesis (Gottwein *et al.*, 2007, Skalsky *et al.*, 2007, Zhao *et al.*, 2009a). Additionally, miR-21 has been linked to viral latency during EBV infection (Cameron *et al.*, 2008).

miR-122, miR-222, miR-146b, miR-454, miR-7b, miR-34a, miR-18a, miR-133a, miR-29a and gga-1et-7d are just some of the host miRNAs that are upregulated in MDV infected compared to non-MDV-infected T cells (Lambeth *et al.*, 2009, Yao *et al.*, 2009). In the MSB-1 cell line latently infected with MDV, it was reported that nearly a third of miRNAs matched the *Gallus gallus* (red Jungle Fowl) genome and was noted that one of the most abundant was gga-miR-21 (Yao *et al.*, 2008). Additionally, miR-21 has been highly associated with expression in various vertebrate tumours, also being consistently expressed in human cancers as well as overexpression during MD lymphomagenesis (Fujita *et al.*, 2008, Kumarswamy *et al.*, 2011, Padhi and Parcells, 2016).

In humans, the hsa-miR-21 promoter is predicted to have a 79.4 % identity to the *Gallus gallus* gga-miR-21 promoter (Stik *et al.*, 2013). hsa-miR-21 activity is mediated via promoter transcription factors, for example, AP-1, STAT3 and Foxo3a (Loffler *et al.*, 2007, Fujita *et al.*, 2008, Wang and Li, 2010). In the MDV-transformed MSB-1 cell line, it has been reported that MEQ can bind

response elements, some of which such as the AP-1 sites exist in the gga-miR-21 promoter region (Stik *et al.*, 2013). MEQ binding to gga-miR-21 promoter AP-1 response elements resulted in the transactivation of the gga-miR-21 gene, which was subsequently observed to mediate apoptosis through PDCD4 control (Stik *et al.*, 2013). Stik *et al.* (2013) also report that gga-miR-21 is overexpressed in natural infection of virulent MDV infection in chickens and not when infected with the CVI988 vaccine strain.

In this study, we found that the gga-miR-21 promoter interacts via AP-1 response elements with MEQ. However, *in vitro* cellular assays of MDV-infected T-cells (HP8 cells) showed no significant difference in gga-miR-21 expression in response to MEQ expression or mutation of AP1 response sites in the gga-miR-21 promoter.

6.3 Aim of Chapter

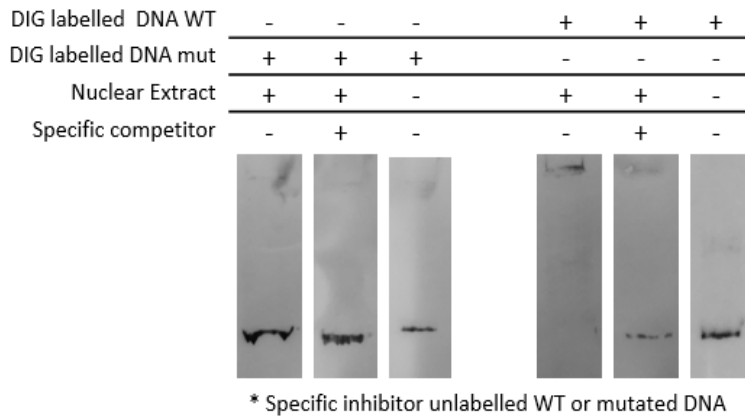
Previously, upregulation of gga-miR-21 had been associated with MDV infection in birds. Furthermore, evidence suggested that gga-miR-21 was upregulated by the Meq oncoprotein binding to the gga-miR-21 promoter. Here we aimed to use CRISPR-cas9 technology to investigate the relationship between Meq oncoprotein and gga-miR-21 at the cellular level.

6.4 Results:

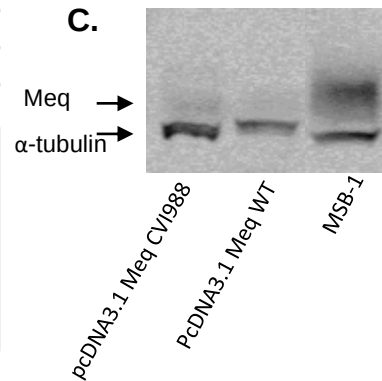
6.4.1 Meq binds the gga-miR-21 promoter and transactivates the gga-miR-21 promoter

Stik *et al.* 2013 had previously identified the position of the gga-miR-21 promoter and suggested it is controlled by various response elements (REs), one being AP-1. It is known that AP-1 specifically binds dimers from the Jun/Fos family of transcription factors including the MDV oncoprotein Meq. The findings of Stik *et al.* (2013) were first corroborated to show that gga-miR-21 is bound by transcription factors including Meq. This was done by cloning a synthesised mutant sequence and a WT sequence of the promoter of gga-miR-21 into a pGL3-vector. The mutant sequence contained AP-1 sites that were mutated in line with the gga-miR-21 promoter mutation as reported by Stik *et al.* (2013). These vectors were then used in an Electrophoresis mobility shift assay (EMSA). This was performed with a DIG-labelled gga-miR-21 promoter which was either the WT or mutant sequence that was then incubated with HP8 nuclear extract. There was a detectable chemiluminescence shift in the band position when the mutant gga-miR-21 promoter was incubated with nuclear extract (lower band) since the transcription factors cannot bind the DIG-labelled DNA, thus passing further down the gel. This suggests the mutations of the fragment prevented the binding of transcription factors which may include Meq (figure 71A). The transactivation was then assessed of the gga-miR-21 promoter through an *in vitro* dual luciferase assay. Upstream of the promoter-less PGL3 luciferase plasmid, a mutant or WT gga-miR-21 promoter was cloned. WT or mutant PGL3-L plasmid were co-transfected into DF1 cells with pcDNA.3.-Meq, pcDNA3.1-c-Jun or both (figure 71B). An additional PGL3-R control plasmid was also transfected and Meq expression was verified by Western Blot analysis (figure 71C). Luciferase levels measured relative to PGL3-R control were seen to be significantly higher when c-Jun and Meq were added to the WT promoter PGL3 plasmid compared to the mutant promoter plasmid. This suggested the AP-1 RE mutations reduced the binding of transcription factors c-Jun and Meq.

A.



C.



B.

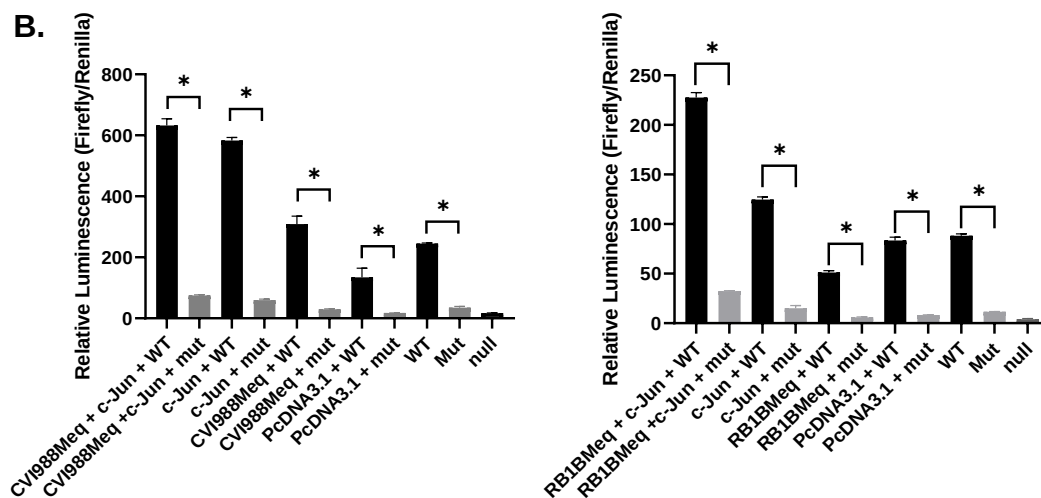
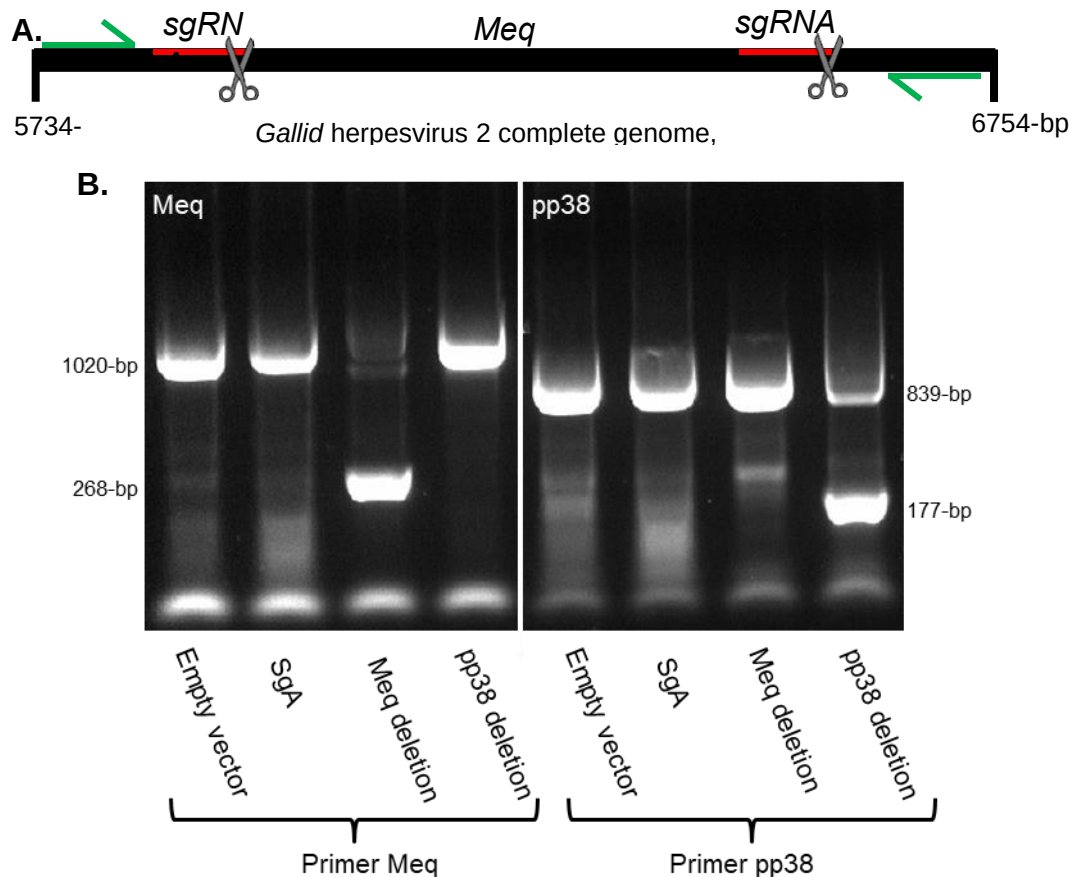


Figure 71. Meq binds the gga-miR-21 promoter and transactivates the gga-miR-21 promoter. (A) Electrophoresis mobility shift assay (EMSA) of complexes formed between MSB-1 nuclear extract and either WT or AP-1 sites with mutated gga-miR-21 promoter fragment. PCR amplicons of either WT or AP-1 site mutated gga-miR-21 promoter were tagged with DIG-labelling. The labelled fragments were then incubated with MSB-1 nuclear extract. These samples were then run on a protein gel electrophoresis system. (B) The effect of mutating AP-1 sites in the promoter of gga-miR-21 on luciferase activity for both MDV vaccine strain CVI988-Meq (left) and virulent MDV strain RB-1B-Meq (right). The gga-miR-21 WT and AP-1 edited promoter sequences were cloned into the promoter region up stream of the luciferase gene in two pGL3-Luciferase plasmids. These plasmids were then transfected into DF1 cells either alone or with pcDNA-3.1-CVI988 Meq, -RB1B-Meq, -c-Jun, or both Meq and c-Jun. Mean plotted +/- SEMs of three replicates, asterisks (*) indicates unpaired t-test p -value < 0.05 . (C) Western Blot to show Meq expression was present in the dual luciferase assay (B).

6.4.2 Meq deletion *in vivo* cellular assay does not affect gga-miR-21 levels

Since it was observed that the gga-miR-21 promoter mutation can reduce transcription factor binding, it was next looked at whether gga-miR-21 levels could be manipulated in cellular assays. For this, I first took the approach of deleting *Meq* from the HP8 cell line. Using HP8 cells and guides targeting the C and N terminus (figure 72A), *Meq* was shown to be deleted from the genomic DNA of bulk sorted cells (figure 72A and 72B). Taqman RT-qPCR of cellular RNA from the deleted cell populations revealed no significant change when compared to the unessential control deleted gene pp38 (figure 72C) (Reddy *et al.*, 2002b). This indicated that in cellular assays *Meq* levels did not play a significant role in the transactivation of gga-miR-21.



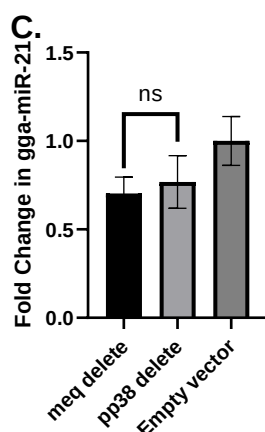


Figure 72. Deletion of Meq by CRISPR-Cas9 editing in MSB-1 cells. (A) Schematic diagram of a *Gallid herpesvirus 2* genome MDV0005/Meq gene, with approximate guide RNA target sites and PCR amplification primer locations. (B) PCR amplification of the edited region, using primers Meq-N-F and Meq-C-R on digested Cas9 cells transfected with lenti U6-sgRNA/EF1a-mCherry-Meq-C-F-sgRNA and lenti-U6-sgRNA/EF1a-mCherry-Meq-N-R-sgRNA after sorting for mCherry 48 h later. A shorter band size of 268-bp for Meq deletion and 177-bp for pp38 deletion (a non-essential control gene) indicates successful deletion of each gene. sgA is a non-targeting control sgRNA. (C) Relative expression of gga-miR-21 normalised to endogenous control U6 snRNA in cells with deleted Meq and pp38 and expressed as fold change ($2^{-\Delta\Delta CT}$) relative to the empty vector control. Asterisks (*) indicates unpaired t-test p -value < 0.05 , mean plotted $\pm$ SEMs of three replicates.

6.4.3 Over-expression of Meq does not affect gga-miR-21 levels

Having demonstrated that decreasing levels of Meq did not affect the gga-miR-21 level, it was decided to explore the effect of overexpression of Meq. For this, RCAS vector was used to deliver Meq to HP8-Cas9 WT cells and miR-21 promoter mutated HP8-Cas9 cells (see 6.4.4). WT and mutated HP8-Cas9 cells were co-cultured with DF1 cells expressing RCAS-Meq-GFP or RCAS-GFP as a control, which enabled HP8-Cas9 cell RCAS infection. GFP HP8-Cas9 cells were sorted for GFP-positive markers and used for RNA extraction and RT-qPCR to analyse miR21. Significantly more Meq could be detected in RCAS-Meq-GFP infected HP8 cells compared to control RCAS-GFP infected cells via Western Blot analysis (figure 73A). However, the Taqman RT-qPCR analysis of gga-miR-21 levels showed no evidence that the increased Meq expression had any significant role in up-regulating the gga-miR-21 promoter (p -value > 0.05) (figure 73B). Thus, this data indicates that *in vitro* cellular assay analysis for gga-miR-21 levels shows no evidence of a Meq gga-miR-21 interaction in MDV latently infected cells.

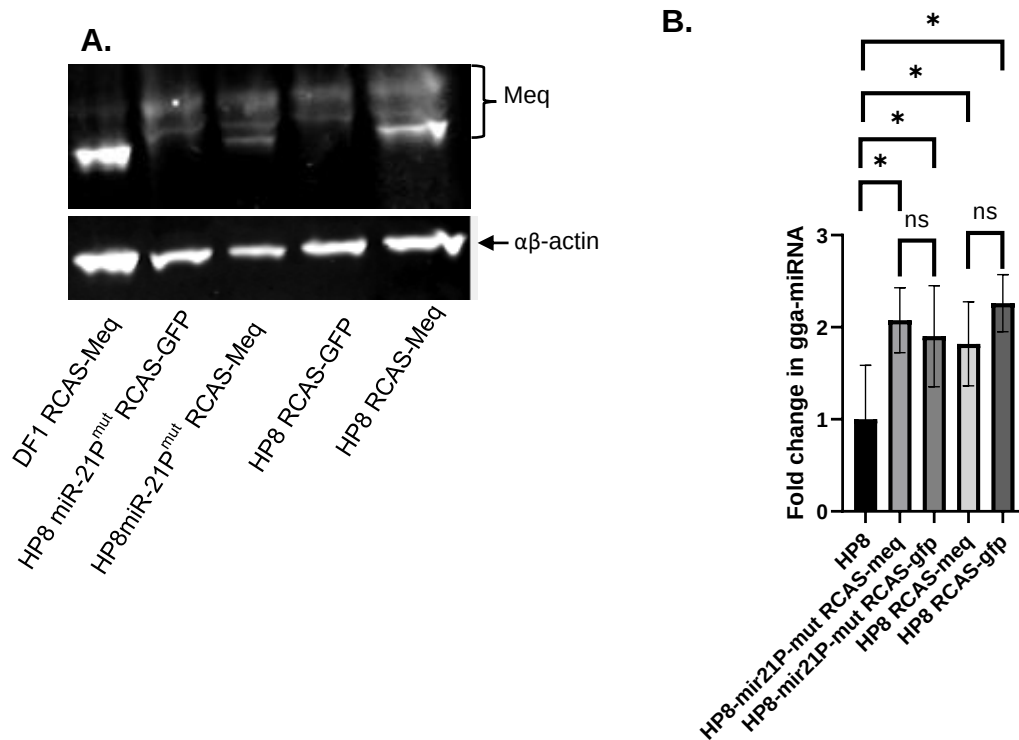


Figure 73. Meq upregulation by RCAS-meq virus infection. (A) Western blot showing upregulation of Meq, the blot was probed with FD7 (anti-Meq) monoclonal antibody and goat anti-mouse Indye680 IgG (Licor), $\alpha\beta$ -actin was used as a loading control. (B) Relative expression of gga-miR-21 normalised to endogenous control U6 snRNA in cells infected with RCAS-Meq-GFP or RCAS-GFP. Asterisks (*) indicates unpaired t-test *p*-value < 0.05, mean plotted +/- SEMs of three replicates.

6.4.4 gga-miR-21 promoter edited HP8 cells show no decrease in gga-miR-21 levels

Next, it was investigated whether editing the gga-miR-21 promoter AP-1 Response Elements (RE) impacted the gga-miR-21 cellular levels in line with the findings by Stik *et al.* (2013). This was carried out on HP8-Cas9 cells, using sgDNA and gga-miR-21 promoter edited fragment. RT-qPCR analysis of the gga-miR-21 levels in the edited cell line showed no significant difference between WT and edited cells (figure 74).

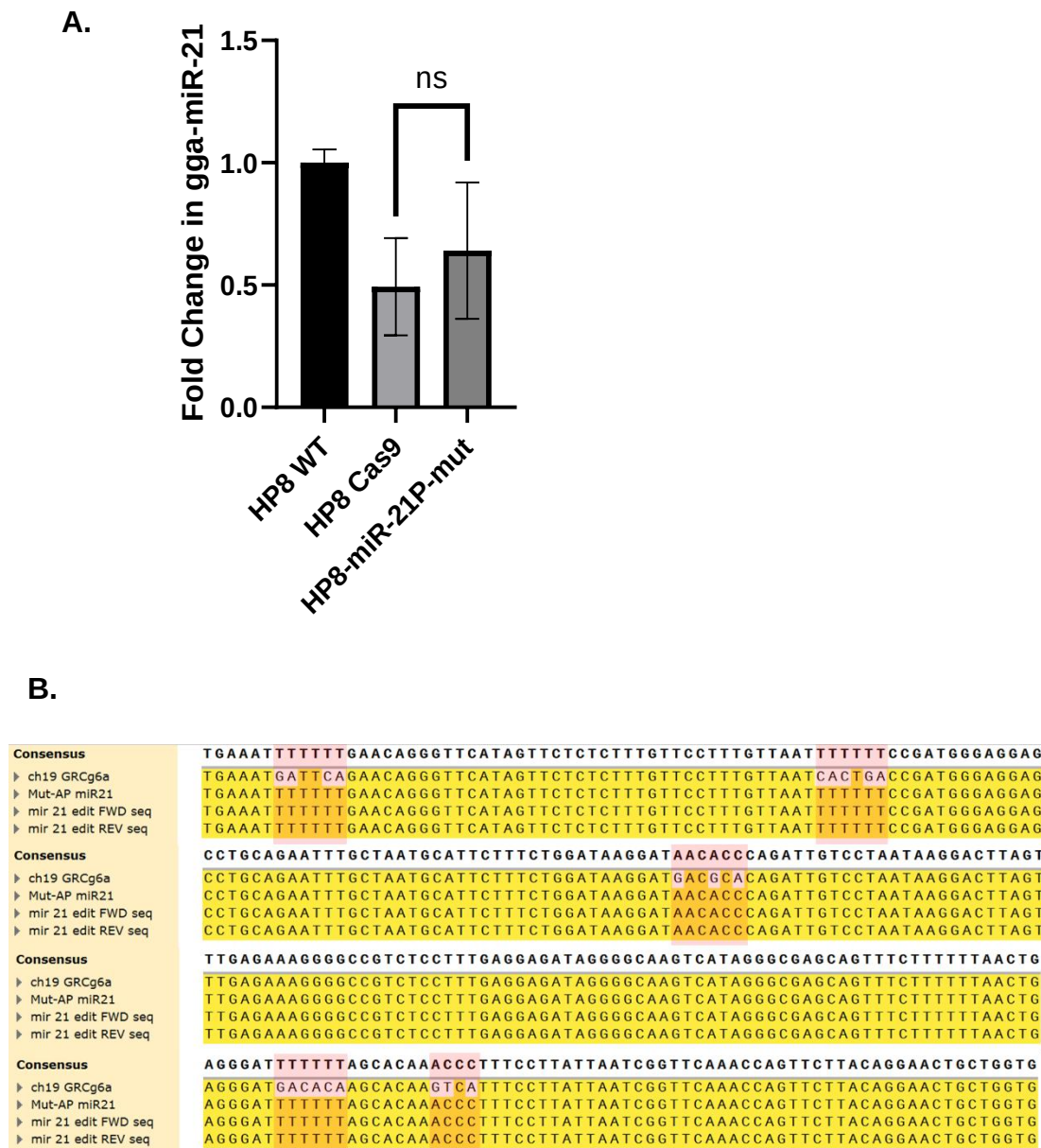


Figure 74. gga-miR-21 promoter edited cells. gga-miR-21 promoter-mut had been edited at all 5 AP-1 response elements. (A) Relative expression of gga-miR-21 normalised to endogenous control U6 snRNA in cells with an edited gga-miR-21 promoter and unedited. HP8-miR-21P-mut showed no significant difference in gga-miR-21 levels compared to the control HP8-Cas9 parent cells. unpaired t-test preformed, mean plotted +/- SEM 3 technical repeats. (B) Sequencing result shown of HP8-Cas9 miR21 promoter edited cells, with AP-1 sites mutated highlighted in red.

6.5 Discussion

Stik *et al.* (2013) showed how mutation of the gga-miR-21 promoter reduced the transcriptional activity. The gga-miR-21 promoter binds transactivator proteins such as MEQ via AP1 response elements *in vitro* (figure 71). Although both assays focused on the AP-1 response element (RE) interaction and Meq is present in HP8 cells and *in vitro* delivery of Meq is shown, it is plausible that the MEQ transcriptional regulator was reduced in its binding capacity despite only AP-1 sites being mutated. Therefore, the significant decrease in the luciferase signal seen in figure 71 maybe a cumulative effect of several response elements being hindered in their binding. For example, AP-1 protein has been seen to bind the miR-21 promoter and decrease the expression of mir-21 (Mudduluru *et al.*, 2011), as well as other transcription factors binding the miR-21 promoter including Jun/Fos, STAT3 and ETs protein families (Loffler *et al.*, 2007, Iliopoulos *et al.*, 2010). This could explain why gga-miR-21-promoter controls (figure 71B) show reduced transactivation of luciferase without the addition of the stimulant transactivators Meq and c-Jun (figure 71B). Nonetheless, these findings support those found elsewhere (Stik *et al.*, 2013). Also, CVI988-Meq is seen to induce higher levels of transactivation than RB-1B Meq. The reason for this is not fully understood but could be due to this Meq construct being codon optimised, resulting in more potent transactivation. Interestingly, this finding contradicts that of Stik *et al.* (2013) who found lower gga-miR-21 levels in CVI988-meq transfected DT-40 B cells compared to virulent RB-1B-Meq transfected cells and a similar pattern was seen in isolated PBLs from CVI988 or RB-1B infected chickens (Stik *et al.* 2013).

It is clear that Meq dimerisation and transactivation of RE in gga-miR-21 promoter occurs *in vitro*. Therefore, I continued to look at the gga-miR-21/MEQ interaction *in vitro* cellular assays. In figure 72 deletion of Meq revealed no decrease in the gga-miR-21 levels compared to the control (non-essential gene pp38) (Reddy *et al.*, 2002b). Additionally, overexpression of Meq saw no significant increase in gga-miR-21 expression (figure 73). This is further corroborated by figure 74, where mutation of the gga-miR-21 promoter in MDV-transformed cell line HP8 showed no significant change to the gga-miR-21 levels. These data, therefore, do not support the hypothesis that gga-miR-21 is significantly regulated by Meq through the gga-miR-21 promoter in MDV-transformed cells. It was acknowledged that Stik *et al.* (2013) observed significant miR-21 miRNA changes in line with this hypothesis *in vivo* MDV infection studies of chickens. However, we speculate there maybe additional factors involved in host chickens contributing to this phenotype which is not present at a cellular level.

It is seen that miR-21 significantly increases in active T-cells lineages; however, it is still to be revealed whether this is cause or effect (Cobb *et al.*, 2006, Wu *et al.*, 2007). Therefore, if gga-miR-21 is a marker of T-cell

activation, an *in vivo* natural infection would activate immunity and thus miR-21 levels would increase. However, in *in vitro* cellular assays, where cells are latently infected and not challenged with a lytic infection, miR-21 may not increase, unless the MEQ and/or C-JUN significantly upregulated miR-21, which was not observed here, suggesting that gga-miR-21 is not linked to tumorigenicity in latently transformed MDV T-cell line HP8.

Other lymphotropic herpesviruses in humans have been seen to transactivate hsa-miR-21 through EBNA2 and K13 proteins, which may play a role in the transformative potential of these viruses (Mrazek *et al.*, 2007, Tsai *et al.*, 2009). This and the evidence from Stik *et al.* (2013) does not rule out a possible role for gga-miR-21 and Meq in the oncogenic process but perhaps through regulatory mechanisms beyond the gga-miR-21 promoter.

6.6 Conclusion

In conclusion, the *meq* oncogene appears to bind through AP-1 response elements. However, it was shown that Meq binding the gga-miR-21 promoter response elements does not have a significant effect on gga-miR-21 levels in HP8 cells. The explanation for this is unknown, but we suggest other oncogenic transcriptional regulators within an MDV infection contribute to the elevated levels of gga-miR-21 seen *in vivo*.

Chapter 7: Thesis Final Discussion

7.1 Final Discussion and Summary

Chickens now number 34.4 billion in 2023, up from 13.9 billion chickens in 2000 (FAO), and their eggs and meat are an essential source of high-quality protein, particularly for malnourished and impoverished people (Poultry Development Review FAO). Therefore, research into chicken disease is essential to allow the industry's continued growth and to increase its sustainability and productivity. Viruses represent one of the key threats to poultry production and unlike humans where ~80% of cancer is caused by non-infectious diseases, in poultry oncogenic diseases are caused by viruses. Two such viruses are MDV and ALV and were the topic of this thesis. Understanding the genetics of host-virus interactions and the function of viral and host genes which lead to symptomatic disease is fundamental to understanding the pathological mechanisms of viruses. Which could lead to improved vaccines and therapeutics in the future. Therefore, our broad aim was to reveal some of the genetic determinants for avian viral oncogenic diseases.

In my PhD, I have successfully applied CRISPR-Cas9 genome-wide screens to reveal genes of interest relating to several phenotypes. These included: (i) a screen for essential and non-essential genes in the MDV vaccinal strain CVI988, (ii) a screen in the MDV infected latently transformed cell line to reveal gene dependencies for oncogenicity, and (iii) a screen in DF1 cells to determine the requirements for infection and replication of RCAS-A and RCAS-J viruses.

Marek's Disease Virus has been extensively studied in the past with many pathogenic genes such as Meq, vTR and vIL8 as well as genes critical for MDV replication such as MDV0.5 and pp38. However, to date, no study has systematically reviewed gene essentiality for any MDV strains replication and spread in cell culture. Therefore, the first screen was designed to reveal and rank genes required for MDV CVI988-strain in replication and spread *in vitro*. The CVI988 screen was successful in producing comparative data on gene essentiality for virus replication in cell culture across all ORFs of the CVI988 genome. The screen was shown to be robust due to several factors. First, numerous MDV genes have already been characterised, among these are the MDV glycoproteins, these MDV genes provide verified positive (gB, gE, gH, gI, gM, gL) and negative (gC and dD) controls and our results were in concordance with previous studies. As expected, all previously characterised genes in the top twenty essential and non-essential lists agreed with

published literature on gene essentiality in MDV. The gene vIL-8 highlighted the clear requirement for validation, especially validation *in vivo*. vIL-8 has been seen in an *in vivo* infection model to be required for a productive viral infection (Cui *et al.*, 2005). However, was a gene listed in the 'non-essential' list, since it is not essential in cell culture but vIL-8 is known to be critical in pathogenicity and replication of MDV through recruitment of B and T-cells to the site of infection. Additionally, I knocked out two genes, MDV19 and MDV20, shown to be essential in the MDV latently infected cell line MSB-1. Upon triggering lytic viral reactivation, I saw a significant reduction in viral plaques after co-culture with CEF monolayers. This was consistent with the results of the screen and proved a successful methodology that can be used in future validation of essential genes in MDV. Non-essential genes could be verified through gene editing of the virus used in this experiment. Although KO was achieved in one case it was not stable; however, this is not strong evidence that this would be an essential gene due to the inherent difficulty of engineering gene deleted viruses in CVI988. Further genes from the CVI988 screen such as MDV11.5 should be validated using other cell lines and *in vivo*. MDV11.5 may be of significant interest since little literature has been published on MDV11.5 to date.

Screens have been readily employed to study host genes but there have been many fewer publications relating to screens involving viral genes. This is likely to be an artefact of the methodologies used. The major problem is paracrine effects where an unedited virus originating from one cell could infect a neighbouring cell resulting in the selection of that cell even though it may harbour sgRNAs against an essential viral gene and, thus would not be identified as essential during the selection process. For viral gene screens in a pooled GeCKO like methodology would require high viral infectivity in the cell type of interest which can be a challenge in cell associated viruses that require cell-cell contact for viral spread in cell culture. A pooled screen against viral genes would also require an effective viral identification method such as the use of a fluorescently tagged virus, a cytopathic virus or a highly efficacious antibody marker of viral infection to distinguish between infected and non-infected cell populations. For arrayed screens, the ability of high throughput is hindered, however can be mitigated if lab automation is possible. Thus, may reduce the number of genes a single individual can reasonably screen. For both arrayed and pooled screens against viral genes the availability of off-the-shelf sgRNAs libraries are currently not widely available. Therefore requires the synthesis of sgRNA plasmid libraries or the synthesis of crRNAs for each specific gene. Both methods are time consuming to prepare and highly expensive to obtain. Although arrayed screens can limit the total number of genes perturbed, arrayed screens can

provide more insight than an equivalent pooled screen. Arrayed screens mitigate the possibility of paracrine effects and competition from other cells with perturbed genes. Additionally, as each gene is perturbed in a single well phenotype selection can be tailored to each gene's function. As DNA synthesising costs fall and lab automation becomes more common, arrayed screens will become easier to carry out. Screens of viral genes have significant untapped potential to uncover many viral gene functions, which may include the identification of new entry factors, viral replication genes and pathogenic genes. In the future, this arrayed CVI988 screen methodology can be applied to other viruses. Within our lab, this has included HVT to reveal essential and non-essential genes. This data can then be used to reveal essential viral replication genes, inform sites for recombinant virus production or help unpick the molecular virology of viruses including genes that contribute to their pathogenic potential.

The viral gene screen presented here could be modified in several ways to change the phenotypic identifier. We used viral growth/spread in cell culture as the phenotype to be measured, however, the major pathogenic factor of MDV is the transformation of CD4⁺ T-Cells therefore would be interesting to adapt the current CVI988 screen to investigate the transformation potential of viral genes. It may be plausible to carry out an arrayed screen on CD4⁺ T-cells and measure the increase/decrease in cell proliferation post transfection of sgRNAs against viral genes and vMDV infection.

GeCKO screens have been widely used since CRISPR entered into the molecular biology field in 2014. Since the major pathogenic phenotype of MDV is lymphoma and the major viral oncogenes have been established it was of interest to investigate if host genes contribute to the oncogenicity of a latent MDV infection in T-cells (HP8 cells). The HP8 screen was successful in shortlisting hit genes responsible for the oncogenic phenotype of MDV latently transformed cell line HP8. Here it was shown that a GeCKO screen could be applied to transformed virally infected cells, identifying 339 genes at the day 25 time point or a more strict list of 155 genes at day 3 post selection that are non-essential in chicken cells but essential in transformed cells such as HP8. Up to 14 of these genes may be unique to HP8 cells or chicken cells from comparison to a publicly available database (DepMap portal) of CRISPR screens in 1078 cell lines. From the shortlist, it was clear that mitochondrial-associated genes were critical in the maintenance of the transformed phenotype of HP8 cells. This was corroborated when 3 mitochondrial inhibitors were applied to HP8 cells, showing a marked reduction in cell growth and an increase in apoptotic cell death for all 3 inhibitors. Furthermore, RT-qPCR showed that viral lytic reactivation

occurred when the mitochondria were inhibited, however, this was not sufficient to form viral plaques in cell culture. This result was a new finding in the field of MDV but not unexpected as it has been known that cellular stress reactivates a number of herpes viruses, such as hypoxia in MDV, and the mitochondrial protein TBRG4 KO causes reactivation of KSHV and EBV (Mallet *et al.*, 2022b, Zhang *et al.*, 2022a). On further analysis of singular genes, KO of IRF4 using sgRNAs was proved to significantly inhibit the growth of HP8 cells. This has also been observed in EBV-transformed cells (Ma *et al.*, 2017a). TBRG4 was also seen to reduce the proliferative phenotype of HP8 cells but to a lesser extent (non-significant). To further confirm this genotype-phenotype correlation the IRF4 and TBRG4 protein expression knockdown should be confirmed by WB in the future. This result should also be corroborated in the widely used MDV transformed cell line MSB-1. This would enable more confidence in hits and that the genotype-phenotype correlation observed is robust. Additional work should concentrate on validating further hits from the HP8 screen, these include the genes seen to be specific to HP8 cells and those common genes between HP8 cells and EBV (LatI and LatII) infected cell lines (table 22 and table 23). From the current data set of the HP8 screen and RCAS screen, this could be used to reveal common and differing essential genes between chickens and other mammals or birds. If the time is available after a pooled screen it can be recommended to complete an arrayed screen of shortlisted genes from the pooled screen, this is used to further reduce the number of hits to validate. Following the work presented here, follow-up screens would be recommended in MSB-1 cells (another MDV transformed cell line) and the AVOL-1 a REV transformed T-cell line. The MSB-1 cell line would be useful to validate the current hit genes from the HP8 cell line and the AVOL-1 cell line would be useful to allow the identification of genes specific to HP8/MSB-1 cells and AVOL-1 cell lines specifically.

After the GeCKO screen had been performed in HP8 cells, it was looked into how else GeCKO screens could be implemented to study avian oncogenic viruses such as the retrovirus ALV/RCAS. It was chosen to screen host factors involved in the entry and replication of ALV/RCAS for two reasons: (i) RCAS-GFP virus was available allowing a marker for viral infection and (ii) the RCAS virus infects and spreads in DF1 cells easily. The RCAS-A/J screen successfully identified the known receptors for each virus, providing strong evidence that the CRISPR-Cas9 screen works on RCAS viruses to identify genes required for viral infection and replication when combined with FACS. Aside from the well-established receptors for each virus, TAOK1 was the only other gene enhanced in both screens. However, validation experiments showed TAOK1 to be a false positive gene. These screens

show that no other non-essential chicken genes are involved in the entry and replication of RCAS-A/J viruses and have established a successful screening protocol for avian viral disease receptors or replication dependencies when the virus is tagged with a fluorescent marker. This screening procedure can be transferred to other viruses such as other RCAS subtypes or viruses where the receptors or entry factors are unknown, this could include the chicken viruses REV and MDV.

One drawback worth highlighting is that both these GeCKO screens will fail to reveal host essential genes in the hit read, this is key to remember when analysing the data. Although speculative, many of the genes required for the RCAS-A/J replication are host essential genes apart from each virus's dedicated receptor, since only the receptor genes showed as significant hits in our screen. However, one benefit of only non-essential host genes being identified in a screen is that they may be of more interest therapeutically since their deletion or inhibition would be expected to have lesser effects on normal cell functions and thus minimize prophylactic or treatment pathology in birds. A further drawback of all these screens is the use of the *in vitro* cell culture system and how comparable these are to *in vivo* infection models, thus validation is key to producing indisputable genotype-phenotype associations. In the future, it may be possible to perform targeted *in vivo* CRISPR screens to confirm *in vitro* screen findings.

These approaches proved that GeCKO screens on both the chicken and avian viral pathogens can successfully identify genes specific to a phenotype of interest. Each of these screening approaches could easily be modified to investigate other avian or mammalian viruses and their host cell lines. The potential of similar screens to these presented here could be vital in increasing the knowledge of viral replication, life cycles, and pathogenicity, improving vaccines as well as completing the annotation of livestock genomes, by linking unknown genes to a particular phenotype. One current threat is the continued evolution of avian neoplastic viruses and in the case of MDV, this may be exacerbated by the widespread use of vaccination leading to more virulent strains. CVI988 has been the 'gold standard' vaccine since the 1990's however, with incomplete knowledge on the correlates of protection this has hampered research for the next generation of MDV vaccines. One notable development in MDV vaccinology has been the Prevexxion® RN vaccine created from the insertion of the LTR region of REV into CVI988, however, it remains to be seen whether this modification will offer significantly improved protection. Therefore, continued efforts like the ones presented in this thesis will be critical to unravelling the pathogenicity and replication mechanisms of MDV and host factors involved in the

proliferation of lymphocytes to aid in identifying possible vaccine improvements. ALV has no current vaccine, however, RCAS-A is known to enter only by interaction with the non-essential CD320/TVA, it has also been shown that an amino acid substitution can block ALV-A/K entry but retains cobalamin uptake (Chen *et al.*, 2022). Therefore, no adverse effects would be expected, thus, gene editing may provide an alternative approach to confer resistance to ALV-A and -K in chickens. However, the situation with ALV-J may be more complex, since evidence has presented two additional co-receptors (chANXA2 and chGRP78) that have been shown to contribute to ALV-J infection, the latter of which is an essential gene (Mei *et al.*, 2015, Wang *et al.*, 2016). Therefore, will require further investigation to see if like TVA these co-receptors can be edited without adverse consequences on their function. With the recently published UK Genetic Technology (Precision Breeding) Bill 2022-23 (House of Commons Library, UK) allowing for gene editing that produces beneficial traits in livestock animals, this research and similar studies may pave the way for gene-edited chickens that can resist viral diseases such as ALV.

The final body of work carried out as part of this PhD was to investigate the interaction of Meq with the gga-miR-21 promoter in HP8 cells. This could be an important mechanism by which Meq mediates its oncogenic effects in cells. The study by Stik *et al.* (2013) showed that gga-miR-21 was upregulated in MDV infection and Meq binds the promoter of gga-miR-21. Our findings show that although Meq binds the gga-miR-21 promoter in HP8 cells, decreasing or increasing Meq levels does not significantly alter gga-miR-21 levels. Therefore, it was concluded that in HP8 cells Meq does not mediate a significant increase in gga-miR-21 at the cellular level. Thus, at least at the cellular level, Meq mediates its oncogenic effects in alternative ways. In the future, the work carried out here should be investigated in MSB-1 cells to confirm the findings of the work presented here or to confirm the findings presented here are cell line specific. Furthermore, CHiP-seq/CHiP-PCR assay should be performed to further evidence the Meq binding partners and if mutation of the gga-miR-21 prevents Meq binding.

Overall the data presented in this thesis shows how CRISPR and CRISPR screening techniques can be used successfully to enhance our understanding of avian oncogenic viruses. This has included methods for screening viral genes and host genes alike. Some of these hits identified once studied further may provide insights into the correlates of protection, pathogenicity or replication of avian oncogenic viruses. The hope is this will give future researchers previously understudied genes to study and allow them to delve into their role in avian viral disease.

[illegible]

MDV2.6_seqenced_	1	-----CGATGCCCGGGGTATCACCCTCCCGCGATCCCGTGCACGGGGTCAAGTGCATCTT-----	59
CV1988_MDV2.6	1	CTTGGGCTGAGCGCAGTGCAGTGCCTCCCGGGGTATCACCCTCCCGCGATCCCGTGCACGGGGTCAAGTGCATCTTGTGGTCTCGGCTTCTTTTCCCTTTTTC	110
MDV2.6_seqenced_	59	-----GTGGGGATAGAGAAATCCGATACCTCAGAAATGTGCGA	98
CV1988_MDV2.6	111	ACGAAGAGTCAGTGAACCTTGGGGTACTTAATCGTGCTTTAATGTCCGATGGAGAACCGTGCTAGAAATATGTGGGGATAGAGAAATCCGATACCTCAGAAATGTGCGA	220
MDV2.6_seqenced_	99	GTCCTGCGGGTGAATC-----	115
CV1988_MDV2.6	221	GTCCTGCGGGTAGAATCGCGCAGCAGTGAATAAACCGCGGGGCCCTAAACCTCTCGCGCCGACAGACAGTTGTACACCTGCTGCATTACTACATCCAGTTCGTGA	330
MDV2.6_seqenced_	115	-----	115
CV1988_MDV2.6	331	ACCGTATCCCTGCTCCATCCAAATAGCTACATTATCGATATATGACGAGGAGTCAAAATCGGAAAAAAGTGCCTCTTTTAATTACAGGA	422

200

MDV28_sequenced_	1	-----GGCTTTTATCCACATACAT	19
CVI988_MDV28	221	GATTTGTTATCAGTCGGACAGAGTTAACCATTGTCATGAATAGAACGCAACCGAGGCTTTATCACCTTCACCCAAGAGATCCCTTTGACGGGCTTTTATCCACATACAT	330
MDV28_sequenced_	20	GGTCCTCGACAGAGACATGTATGTCCAAATGAACCACATTCCCATAAACATTATATGCGTCTATGTGTAGATCACAATGTTACAGGCAATTCACGACATCCACTCTCGTG	129
CVI988_MDV28	331	GGTCCTCGACAGAGACATGTATGTCCAAATGAACCACATTCCCATAAACATTATATGCGTCTATGTGTAGATCACAATGTTACAGGCAATTCACGACATCCACTCTCGTG	440
MDV28_sequenced_	130	ATTAATAAATTTTGCTAGAGTTGCACCTTAGGCTTCGTTGCTGCCAGCCAGCGGGACATATTGCCAGTAAACTCTCTCCATTCT	213
CVI988_MDV28	441	ATTAATAAATTTTGCTAGAGTTGCACCTTAGGCTTCGTTGCTGCCAGCCAGCGGGACATATTGCCAGTAAACTCTCTCTCCATTCTGAGATAATAGAACCTGCGTTTAGAGA	550
MDV28_sequenced_	213	-----	213
CVI988_MDV28	551	AAGACCACCAAACTCCGATGGATATTGGTATGCAAGTTGTGGGGATCATCAGGTGGCTGTGTACGTTCAACAAAAACATCGATGTCAAAGTCTATGCAAGGTGTCTGTCGGT	660
MDV28_sequenced_	213	-----	213
CVI988_MDV28	661	AAAACTCAGTGTACATCAGGGATAATGGCATATGCCTCAGTAGACGAGACATTGGAAAAAATATGACATCTGCAGAGATCCCTCATGTAGAATAAGGGGTATACG	770
MDV28_sequenced_	213	-----	213
CVI988_MDV28	771	TAGTGTGGCAGTCACAGAATATGCAAGTGTCCATTGTGCGAGGAACAAGATAGAAATCTTTTTCTACTCAAATACAAGTCTTTGGTCGCGTGAGGTACATAAATATT	880
MDV28_sequenced_	213	-----	213
CVI988_MDV28	881	CCACCCATTTTGCTTCCGCGGTTGCTTATTGCGGTGTTACGTATGTACATAAGCTACTATCTAAGGCGATTAAATGCAAGCATTACAACCAAACGGGATTCGGCTCGCAGT	990
MDV28_sequenced_	213	-----CAATCTCTTCNNNAGGGCTGGGGCGGGAAATCTGGGTATCTTCAGTCT	263
CVI988_MDV28	991	GTACGCCATATGATGATACGTTGCTTAACTACGAACGAATTCGATATCCATCCCATCACAACTCTCTCTGAGTGCTGGTGGCGTGTAGATCTTGGTATCTTCAAGTCT	1100
MDV28_sequenced_	264	CTGCGAAGTCATAATTCAATACCTGGGTTGAGGATGGGTACATATACACCTCTGAAACGTAAGGTC	330
CVI988_MDV28	1101	CTGCGTAGTCATAATTCAATACCTGGTGTGGGATTGGTCACATATACACCTCTGAAACGTAAGGTCGTAGCACCTGTTGCATCTTGGTGTGCGACAGAGCGTGAAGAG	1210

MDV56_Sequenced	1	-----TTGCCATTCCAAAGACCCGTTCTTTTAAATACCAAAACCGAA-ACCGGTATCAAAAT-GAACA-GGATTTT-TCAACAACCTCATC-----	83
CVI988_MDV56	441	AAACTCATAGTTGTGCCAATTCCAAAGACACCGTTTCATCTGAACACCAAAACGAGATACCGCTATCAAATTGGAACATGGATTCTGTCAACAACCTCATCATTACCTCCGT	550
MDV56_Sequenced	83	-----	83
CVI988_MDV56	551	CTTATACAACCACTGGTAGAACATATGGACATTGTCTGCAATGCTCACATGCCCTCGAGCCACCGTGTACAGCAACAAATGGAAACGGAATATCAACAATCGATGTCTA	660
MDV56_Sequenced	83	-----	83
CVI988_MDV56	661	AAATGTATCGTAGTAACCATGTGTTCGATATTCTCCATTGCAAGCTCATTGGCTATCACCTGTCTATGATAACCTTGATTCAATTTATTGACCAAAAAATTATCTATAT	770
MDV56_Sequenced	83	-----	83
CVI988_MDV56	771	AAACTGTACTATTTATGCTATACCGGATTCTCAATTGCCCTCATCTGTGCGTCTTACGATAAAATCGTCAGAAGTCTGACATCAATTGGCAAAACCGGCACAATTATAT	880
MDV56_Sequenced	83	-----	83
CVI988_MDV56	881	TTGCTTTAATCTCATCCATAGCAGATACGCTTATTACAAGAAATATGTTAATTGACAGTAATCCATCTTATGTAAAAATATTGAGAGCAATAGAGATGACATCTTTGATG	990
MDV56_Sequenced	83	-----	83
CVI988_MDV56	991	TGCTTTGTCATGCTTGGAGGCATTCATTGCATCCTACCACTATGTCTGCTGGCAACGCTCGAGATTAACTTGGAAAGCTGGGTTTTGATATTGACCGCCGGAAACAAT	1100
MDV56_Sequenced	83	-----	83
CVI988_MDV56	1101	TATCGGAATATAGCTCCATATGGAAACATTTCCCTCCTATTGCGATTCTATTTCATATATACATATATTAGCATAAAGCTGTGAAGGATGCAAGTAAAGCACTGATGA	1210
MDV56_Sequenced	83	-----	83
CVI988_MDV56	1211	ATACATGCTATTATCGCATTGTGCTGCAACGACTCTACGCCATCCCTCTGCGCTCGGCTCGGTCGTATGTCTCGACTCAAGATGTCAATGCAACGCTGAAGAAAGCC	1320
MDV56_Sequenced	83	-----	83
CVI988_MDV56	1321	ATATCAAGCGCAGATACGATTGATGGTCAGATTCCATGGTAGTTATGAGCCACGCGACAGGCGATTAAATCCAGTTGTTATTGCTTGCAAGGTACATGACAAAGGA	1430
MDV56_Sequenced	83	-----TTAGTGGGGCGAGTGTTTC-----	103
CVI988_MDV56	1431	GACTGTTAGTTTGACATCGACTGATATGTTACAGGGAGTCTGTGGCGTTTTAGTGGGGCGAGTGTTTCAATATTTATCCCGTCACGTCGCGACGAAAGTTTATCCCGTC	1540

MDV13/13.5_sequenced_	1	-NTNNCTTTCCTTCGTGCTAGGTATGTTTACTAAGACCAATACGGTTCTCTG-----	50
CVI988_MDV13/13.5	221	TGCATCTTTCCTTCGTGCTAGGTATGTTTACTAAGACCAATACGGTTCTCGCATGGTCGAAATACGACCTGGTACACGGCTTTATGAGGGTAGCGAACATAGCTCCATT	330
MDV13/13.5_sequenced_	50	-----	50
CVI988_MDV13/13.5	331	ATGCGGCTCGACTGCCTCCGAACCTATTATCTTCGAACGCGGGGATGCGGCACTCCATCAGACGATATTCCACGGGCACTTTTATTAAAGTCAACTGCAGCATACC	440
MDV13/13.5_sequenced_	50	-----	50
CVI988_MDV13/13.5	441	CGAATTCATTCTATGGTATGAACAAAAAGCCATGGCAGCGTGGATCAATCCTATTATGGGCACTGTTCTAATGATGAATGACGTTTTGAAAAGCGGATTGGAGAATTC	550
MDV13/13.5_sequenced_	50	---GGTGGGACTCTTACATTTCTAAAACGGATAGTGTAAAAGGAGCCCAATGGACCTCTTAGAAATCGCGGATCGGGTTGCATCAATTTAATTCGCGCTGCCGATATT	156
CVI988_MDV13/13.5	551	TGAAGGTGGGACTCTTACATTTCTAAAACGGATAGTGTAAAAGGAGCCCAATGGACCTCTTAGAAATCGCGGATCGGGTTGCATCAATTTAATTCGCGCTGCCGATATT	660
MDV13/13.5_sequenced_	157	TCAA-----	160
CVI988_MDV13/13.5	661	TCATGCTATGGCTCAACTAGACTTGACAGGTTTAAACGAGACTTCGAAAGATGATAGTCGGGGAATGCCATGTCAGGCTAAAGCGATGCGCGCAACCTCTGGCAGCCG	770

MDV3.4_sequenced_	1	-----NCAANCTAAATGAACAGGAGTTTGCATCAAAAGGTGATAACTGGGAACACAAGACTATGAGG	64
CVI988_MDV3.4	441	GTTTATTGAAAATTTCCATTGCGTGGGGCATAAACTATAGCATGAAACACTAAAATGAACAGGAGTTTGCATCAAAAGGTGATAACT-GGAAACACAAGACTATGAGG	549
MDV3.4_sequenced_	65	ACCCCTTAAATGTATATGAGAAAATTCACCAGACCCCTGCGATAGCAGA-	111
CVI988_MDV3.4	550	ACCCCTTAAATGTATATGAGAAAATTCACCAGACCCCTGCGATAGCAGAAAACCAAGGACCAATAAAATTTGCGATACGCATGCGCCCTCTACACAGACCCGCGATGCGCA	659
MDV3.4_sequenced_	111	-----	111
CVI988_MDV3.4	660	GACCACCTTACTGCAGTACGCATGCGCCCTCTACACACAAAACGCATGCGCAGTCCAAAACGCATGCGCAGACCGCTTACGCGAGTACGCATGCGCCCTCTACACACAAAA	769
MDV3.4_sequenced_	111	-----AATACGCAT	120
CVI988_MDV3.4	770	GCGATGCGCAGACCAATTACCCAAAACAAGCATGCGCCGTAAAGTATGTTATCTGCGCATGCGTGTTTTGACACACGCGCATGCGCAGATACACTCCACCGTACGCAT	879
MDV3.4_sequenced_	121	GCGCGTCATGCATAACTGGCGCATGCGTGAACGCCCTACCCGAACGAGCCCTGCGCGCTTGGCGCTATATACGCCCTA-----	197
CVI988_MDV3.4	880	GCGCGTCATGCATAACTGGCGCATGCGTGAACGCCCTACCCGAACGAGCCCTGCGCGCTTGGCGCTATATACGCCCTGCGCACTCATGCGCATGCGCAGACGTTCAA	989

MDV20_Sequence_	1	-----AAATTGCGCGGGGGTTGTAACTAAACAGTC-----	30
CVI988_MDV20_	2311	CCATTTTGCTAAGTAGCTAATAGAGCAACAAGCGAAAATTCGGCGGGGGTTGTAACTAAACAGTCAAAAGACGTGGGGACGAGAGACAATGCTCTTAGCCGAGGCTGT	2420
MDV20_Sequence_	30	-----	30
CVI988_MDV20_	2421	AATGGTCTAAACCAACGTATTGTCCACAACCTTAAATATTGTATTGGAATTGTTGCACATTAAATTTCTTATAGGGCTGTTACATTTTTTATATTACGAAGAAAT	2530
MDV20_Sequence_	30	-----	30
CVI988_MDV20_	2531	CGTCTCTCTTTTGCTGTATTTCAATTCTCATGTGCAAACTGTGAATTTGACGGGTACGTTTAAAGGGCTGACAGTTTAATGTGGGCCAGGGGTGCACCTCCGCGATGCTT	2640
MDV20_Sequence_	30	-----	30
CVI988_MDV20_	2641	CATGCGAGTCGAGATAAGACATGACTCTTCAACGCTGATCATTCGTCGCTTTTCAATAATCCGGGGCAGGGGATAGATCTTCTCCTGATACCGGTTGCGAAGGTGTA	2750
MDV20_Sequence_	30	-----GTCTTAATTTTGCTAGAGTCGTAGTGAAGTAAACGCTTTTCCATAGC	74
CVI988_MDV20_	2751	CTAACCCAGTTGACTGTGATATAGATGGTTACAGGGTATAATAAGCATGATATCCAGACGGTGCTTAATTTTGCTAGAGTCGTAGTGAAGTAAACGCTTTTCCATAGC	2860
MDV20_Sequence_	75	ATGTGTTGGTCCGAAGGGCAAGATGCGCATAATCCGGTTACAGATGCGGGAATTGGTCATACCTGCTAACATTGTATCAGTAATCATATCTGGGTTACGGCCCAAAGTTT	184
CVI988_MDV20_	2861	ATGTGTTGGTCCGAAGGGCAAGATGCGCATAATCCGGTTACAGATGCGGGAATTGGTCATACCTGCTAACATTGTATCAGTAATCATATCTGGGTTACGGCCCAAAGTTT	2970
MDV20_Sequence_	185	AGCCATGGATTTTCCGGAGAGGTTTACTGTAGAATATTGTGCCGAGTATTGACCGCATGTATTTGAAAAAGTAGATAGCATAATGCAGTGAGTCCCTCGGTACGATTTT	294
CVI988_MDV20_	2971	AGCCATGGATTTTCCGGAGAGGTTTACTGTAGAATATTGTGCCGAGTATTGACCGCATGTATTTGAAAAAGTAGATAGCATAATGCAGTGAGTCCCTCGGTACGATTTT	3080
MDV20_Sequence_	295	TATTAGTCCAAATTTCTATACACAGTCGTATAACAAATACATCCTCGCATTTATCTGCATCATATTAGTTTTTGACGATGCTGCCATCGTTGATATGGTTTT-----	393
CVI988_MDV20_	3081	TATTAGTCCAAATTTCTATACACAGTCGTATAACAAATACATCCTCGCATTTATCTGCATCATATTAGTTTTTGACGATGCTGCCATCGTTGATATGGTTTGACGATTCGG	3190

CVI988_MDV84.5	111	ACGGTTCAAAGTCGGGAAATTTGCGATGCTGAAAAGTGAACATAAAATGTAGGTGTCAGCTGCGGATATAATCCCGAACACGTAAGCATCTGGTTTTGTATTAGGCCATG	220
MDV84.5_Sequenced_	1	-----ATGTAGGTGTCAGCTGCGGATATAATCCCGAACACGTAAGCATCTGGTTTTGTATTAGGCCATG	64
CVI988_MDV84.5	221	CTCCAATACCAAGCCCGCTCCACAGCATTGTTATACGTATCTGATTGCTGCCGAGATGGATGTCGCGGCTAATGTTCTCGATCCCCCCCCCCCCCATTCGAAAGTGC	330
MDV84.5_Sequenced_	65	CTCCAATACCAAGCCCGCTCCACAGCA-----	91
CVI988_MDV84.5	331	GGTATCAGGTTTGATACCGCGCCGGTGTACTTCAAATAGTTTAAATGCCAGCATTGCAATTTCTAGCACACAAACTAGACCGGCCACCGAGTCAAATTTGTCTCTGCTGA	440
MDV84.5_Sequenced_	91	-----AATTGTGCTCTGCTGA	107
CVI988_MDV84.5	441	ACGATTGCATAGTTTGAAGATGCCATGCCAGTAAACAAATCTTGAATACCATGACATAAGCACAAAAGGCTCTTATCATCTACTAGCAGAGTTGTGAAGTAGATGAGC	550
MDV84.5_Sequenced_	108	ACGATTGCATAGTTTGAAGATGCCATGCCAGTAAACAAATCTTGAATACCATGACATAAGCACAAAAGGCTCTTATCATCTACTAGCAGAGTTGTGAAGTAGATGAGC	217
CVI988_MDV84.5	551	GATTGTGGGGCCAGCCGACCAAGCCCTGTAGGCAAACTTCTGTACTGCGGGTTAAGCAGATTAACACTCATCGCTA-GAGCGCAAAATATTTCGGATGACAAC	659
MDV84.5_Sequenced_	218	GATTGTGGGGCCAGCCGACCAAGCCCTGTAGGCAAACTTCTGTACTGCGGGTTAAGCAGATTAACACTCATCGCTATAAGCCGCAAAAT-----	310
CVI988_MDV84.5	660	TTTATGCGCGGCTGCGGTTTGTTTTAATTAATACCTAATGGGCAATATTACGCTCCACAAAATAAACACAAATCAGCCAGTGTTCACACATCA	756
MDV84.5_Sequenced_	310	-----	310

MDV3.8_Sequenced_	1	-----GGNNANNNNNT--GATTTCTACAAAAGATCTAAAT	34
CVI988_MDV3.8	221	TAAAGCCCTCCCGTTCACTCTTTACGCGCGGCACTATCGGTACACAGTGGCACACATCAAAACAAAGTAAAAAGGGGAAGGGAATTTGAATTTCTACAAAAGATCTAAAT	330
MDV3.8_Sequenced_	35	TTACGTAATGGATCCCGTCCCGCTGCTCCCTCACACGTGGCACTGCGCGTTAAACAATGCCACATCGTAGAGAAAAGCATA-----	115
CVI988_MDV3.8	331	TTACGTAATGGATCCCGTCCCGCTGCTCCCTCACACGTGGCACTGCGCGTTAAACAATGCCACATCGTAGAGAAAAGCATAAGGGGGAGGCCATCGGGAGATTATCCCGA	440
MDV3.8_Sequenced_	115	-----	115
CVI988_MDV3.8	441	GAAATCAAATATCTTGTAAATGTCGTACGAGCTCGTTCGTTGCTCTTTTCATGCGCAGCATTACCGGTGTAATGTAATAATGCCGCACAGTGAACAAGCAAAAGGG	550
MDV3.8_Sequenced_	115	-----BATGAGGTATTTTCCATATGTTCTCGAGGTCCGTG	151
CVI988_MDV3.8	551	GAAAGAGGTCTCCGGATAGCGGTTACAATCCAGTAACGACTTAACGCAGATTACTCTGCTAGAGCGTCTCGCATGAGGTATTTTCCATATGTTCTCGAGGTCCGTG	660
MDV3.8_Sequenced_	152	CCTGTATATTGCTA-----	165
CVI988_MDV3.8	661	CCTGTATATTGCTAATGTGACATCTGCCATGTGCCGTGTATCGGTGCATAGGAGTATGCTGTGCTATCCCTGTATATTATCTGTGCTATTTTGCTAATATCATA	770

MDV31_sequenced_ CV1988_MDV31	1	-----ATTGGTGCCGATATTATCATTTTCCCGTATGTAAACAGGTACTCTAGCAGAAATATCCACCACATCAAGAATTCAGATAACAATTTCTGTCTTACCATTG- 101 	3191	GTACTAGATTGGTGCCGATATTATCATTTTCCCGTATGTAAACAGGTACTCTAGCAGAAATATCCACCACATCAAGAATTCAGATAACAATTTCTGTCTTACCATTG 3300
MDV31_sequenced_ CV1988_MDV31	101	-----	101	3301 AGTTGTCACTAATACACCATCCACAAGACGCCCTTTGAATCGGAATGTGTAACTGTGCATTGAAATTGTTTGGTGAATACAGTTGACCATTTTGTGAGGAATGAAA 3410
MDV31_sequenced_ CV1988_MDV31	101	-----	101	3411 TTATAGTGTCCCGGTTGGCGTTTGTCTTATTATGATGAAGAAGCTCTGTGTGCACACTGTACGTTTGAGCTCGGAGACCAAATTTGCTCGCATACCTGTCCAGCAATTCTC 3520
MDV31_sequenced_ CV1988_MDV31	101	-----	101	3521 CCTCGGCTTGACTGCGGAAAGCGGTATCAGTAGAAGAGGTGGTGGAGCTTTCTCTAGTAGAATTCTAAGCATTTGATCTACCGTACCTCTTCAAATGAATCTAATAT 3630
MDV31_sequenced_ CV1988_MDV31	101	-----	101	3631 CGTTCTTACATTTCTAGCTAACTGTTGATGAGCTCTCATTCGGAGATGAGTGGATATTGCTGTCCTATCAAAGACACTTCAGATAATAATGCGAGGGCTTCTGCCGCGA 3740
MDV31_sequenced_ CV1988_MDV31	101	-----	101	3741 CAACGAAAGCTGCCTCAAAGATCTTCGACATAGACGTTTTACCATATAGGTATGAATGGGCTGATCCGCGGAGTGTGGCCCTTCGCGCGCAATCATTGGCTGTTGTATT 3850
MDV31_sequenced_ CV1988_MDV31	101	-----AGAAAGGCCCGTTTCTAGAAATCGAATTAAGATAATGATTGCA 146 	3851	TCAAATTGAACAGTTCCTTCTCTCATGTACATCAGATCTGGGGCGCGGTGTACAAACACATGCAACGAAAGGCCCGTTTCTAGAAATCGAATTAGAGATAATGATTGCA 3960
MDV31_sequenced_ CV1988_MDV31	147	GTAAATCCCAGAAAGAAATCGAATTGTGCAGCATAACGTCATTATCATCAAACCTGAATTGCTTGAAATAGTCGAACATACATCGATGAGATGACATCTCTATAGGGC 256 	3961	GTAAATCCCAGAAAGAAATCGAATTGTGCAGCATAACGTCATTATCATCAGAAGCTGAATTGCTTGAAATAGTCGAACATACATCGATGAGATGACATCTCTATAGTG 4070
MDV31_sequenced_ CV1988_MDV31	257	TCAAGAGTCTACCTGTAGGCGCAA----- 280 	4071	TCAAGAGTCTACCTGTAGGCGCTAAAGTCGCTCCTATGTTGAACGGGGTTGAAATGTACATGGTGCACCGGCGGACAAATCCCCTGCCGGGGGACAAATGGCATCCGGCC 4180

MDV37_Sequenced_ CV1988_MDV37_	1	TCATGGC-AACTTTATTTGGGACGCGAGAATATT----- 33 	1	--ATGGCAAACTTTATTTGGGACGCGAGAATATTGACAGGCGATGGAATGGAATGTTCCCTGCCGATGTGAAGAACTTTATAGTCCCCATTGGCCCATCGAGTTCGG 108
MDV37_Sequenced_ CV1988_MDV37_	33	-----	33	109 AAGGAACCGGTATTACATCTAACGCGCCAATATGGAACGACAACCTTGCATCATTACTGCACGAAACAATGCCGCCATGCAACATTAAATAATTGATGTCATAC 218
MDV37_Sequenced_ CV1988_MDV37_	33	-----	33	219 CGACTCTATTGCAATAGAAATTGACCGCAGACTTCGACCAATAGAGAAAAAATTGAACATATCGCAACAGCGTTAGCGGATCTGGAACATGCAAGGGCTGCAAGCAAC 328
MDV37_Sequenced_ CV1988_MDV37_	33	-----	33	329 TTGCCGACGCTGCAATGGATATTGCCGTTAGTGCTGTTGAAAAATCTGGACAGCACAGGAAGACGTTAATATCACTTCGGCCCGTGAGATTCAAATCGTAAAAATGAT 438
MDV37_Sequenced_ CV1988_MDV37_	33	-----	33	439 CCTTTGCTCAGATACGACTCTAATTTGTCTGTGGATCTCTTGAACTTAGTCTATGCAAAATAGGAATGTAGTGAACCTCCGGTGTGTTTTTCGGCACGTGGTATCGTACTTT 548
MDV37_Sequenced_ CV1988_MDV37_	33	-----	33	549 ACAAAATGCTCTGGTTGCCGACAAGCCCAAGCGTTGCCAGAAAAATAGACTATCATGGTGGAGAATGTGCGGAACGTTTATTGTGACGGCAATCAGATCTTTACAATCAT 658
MDV37_Sequenced_ CV1988_MDV37_	33	-----	33	659 GCGGACGTTTATATGTAGGCAACCGTTATTATTCTCTCTAGAATGTGCGATTCTTTGTTTATATGCATTCTATGCAAAAACCTGGGACTAATATTTCTCATCCCACTAAC 768
MDV37_Sequenced_ CV1988_MDV37_	33	-----	33	769 TTTATGAGTGCATTGAGTCGGTTCCCACTTATTTGGAATCTGTCAACGAGACTTGCTAGTTCTGATAGCAGACAAAATATAGTTTCGATTGGGCTGACATTCCTCAA 878
MDV37_Sequenced_ CV1988_MDV37_	33	-----ACGGGGTGCAATTACAGCATATAGCATTTTAAAGGGCTTTAGTAAATTCGGAAGTTTGGCCCGGGTGCGGGATCCC 110 	879	AGACACTTTCGACTCACCGTGTGAAAAATACGAACGGGGTGCAATTACAGCATATAGCATTTTAAAGGGCTTTAGTAAATTCGGAAGTTTGGCCCGGGTGCGGGATCCC 988
MDV37_Sequenced_ CV1988_MDV37_	111	TTCCCGGTGGAGATGTAATTCAGAAATTTGATGCTGAACAAAGGCATACGCAACGACGA----- 168 	989	TTCCCGGTGGAGATGTAATTCAGAAATTTGATGCTGAACAAAGGCATACGCAACGACGAAGTTAACAGGGCTGCTGCAAGCTTACTGGGACGAGCACAACTCTCTTTCTC 1098

CV1988_MDV2/79 Sequenced_MDV2/79_	221	CTCCCTCCGCCCGCTGTTTACTCGTCTGACTTTTCAGCGGGCTAGGGAGCGCCCGCAGGGGGCGCCGCGGGGAGGGGGTGGGGCGGACGCGGGAGAGAAAGACCGAAAG 330 	1	-----GGGCGCCCGCGCGGGGAGGGGGTGGGGCGGACGCGGGAGAGAAAGACCGAAAG 52
CV1988_MDV2/79 Sequenced_MDV2/79_	331	GGGCTCCACGGCAAACAAAAAAGAACGTCAAGCAAGGGTCTCTCTGCCCCATCCGCCCTGGGGTCTCTGCCCGCAGCGCGGTGCGGGCGGACCCGCCATTGCGG 440 	53	GGGCTCCACGGCAAAC-AAAAAAGAACGTCCGCGAGGGGGCCCTCCCCCCCCCGC----- 111
CV1988_MDV2/79 Sequenced_MDV2/79_	441	CCGCGAAGAGTTCCGCTCTGTACGCTCGCGGGCGCCCGGAGATGCGGCGCGCGCCCGCGCCCGCAGCAGAGCAACAGGAGCGCGCGCCCGGGGCAACCCCGCG 550 	111	----- 111
CV1988_MDV2/79 Sequenced_MDV2/79_	551	GCCCCCTGCGCGGTGGGGCGCGCGGAGGCGCTGCTCCACACGCGCGGCCCGCGCGCACAGCCGTTGGAAGCCGTTGAGCGCGCGCGGGGCTCTGTGATAGACCGA 660 	111	----- 111
CV1988_MDV2/79 Sequenced_MDV2/79_	661	ACGGGCCCCCGCGGAGGTGGGCGCTCGAGCGCGTCCCTGCGCAGGTGGTGGCCGCTGGGCGCCGCTCGGGGCTACCGGGGTCGCTCCGCGGCGGCTGCCGGGCGCCGAG 770 	111	----- 111
CV1988_MDV2/79 Sequenced_MDV2/79_	771	GCGCGAGTCTTGGCTGGAAGTGTGGCGGTGCGCAGCGCGGAGCCCTGTCCGGGAGGAGCCGCTCTCTGCTCGGCACGCTCCGCAATAAGCGTGGGACACAGTGTGG 880 	111	-----CCTGGACGTGTGGCGGTG----- 129

MDV27_Sequenced_ 1 -----ATCGTTTATGCGCGAGTCA----- 19
CVI988_MDV27_ 1 ATGTTTGGTGGTTTACTTGGCGAAGAGACGAAGAGGCACTTCGAACGGCTAATGAAACTAAGAACGATCGTTTATGCGCGAGTCATAGAAATGAACGGTCAATACGAGA 110
MDV27_Sequenced_ 19 ----- 19
CVI988_MDV27_ 111 CGGTGATATGGTTGACGCCCTTTTAAATTTTGCATTCGGTGCCGACGACATCAGACAGTGATGCTGCCATAGGCATATTACATAACTGCTGCGATGTTTGG 220
MDV27_Sequenced_ 19 ----- 19
CVI988_MDV27_ 221 GTATTTATTCTGCGATTACTACTCGTATGTTGTATAGCAGCATGCTTGTAGTGAATTTGATGAACACGTCGTGATTGCTGCTACCTAGATGTTATCCACGTATTACAAAT 330
MDV27_Sequenced_ 19 ----- 19
CVI988_MDV27_ 331 GCACAGGCTTTCCTATCTCCGATGATGATGCGTGTGGCGAATTCATAATATTTCAAGAAATATGACGAGATGGAATGTGACGCCCATCGCAATGCATATTACAGTACAAT 440
MDV27_Sequenced_ 19 ----- 19
CVI988_MDV27_ 441 GAATTCCTTTATATCAATCGAACATCTGATGCAATTTAAGCAGTTAACTGTTTTTCATTAGTCGTTTTTCGAAATATTAAATCGCTTCGTTCCGAGATGTAATAAATTAG 550
MDV27_Sequenced_ 19 ----- 19
CVI988_MDV27_ 551 ATGATCACACAGTTAAAAACGTGCAAGAATCGACGCGCCATCCTATGATAAACTTCACGGAACCTCTCGAGCTCTTCAAAAAATGATATTGATGCATGCCACTTATTTT 660
MDV27_Sequenced_ 19 ----- 19
CVI988_MDV27_ 661 GTAACCTCTGTTTTACTTTGGAGATCACGCGAGGCGTGCCGAACGTTTGCTTCGTGTTGCTTTGATACCCCCATTCTCAGATATAGTTACTAGACATTTCCGTCAGAG 770
MDV27_Sequenced_ 19 ----- 19
CVI988_MDV27_ 771 GGCTACTGTTTTTTGGTTCCGAGGAGGCATGGAAGAACTTGGTTTTTGGTTCCATTGATTGCTCTTGCAATGTCTTCATTGGAAGGTATACGCATTGGATATACATCAC 880
MDV27_Sequenced_ 19 ----- 19
CVI988_MDV27_ 881 ATATCCGTAAAGCAATCGAACCTGTATTTGAAGACATTGGGGACGCTCTTAGCGATGGTTTTGGTGCCATCGCGTGATCATGTCAAAGGAGAAACCATACGTTTTTCA 990
MDV27_Sequenced_ 19 -----TGGATTGAAAAGTACAGTCACGTTTGTCATCTAGCCATAACACGAAACGTAAGTATCGCATCCTTACAAACCTGTTTTGTATACATTATACGACGTTGGGGCT 121
CVI988_MDV27_ 991 TTTCCGAGTGGATTGAAAAGTACAGTCACGTTTGTCATCTAGCCATAACACGAAACGTAAGTATCGCATCCTTACAAACCTGTTTTGTATACATTATACGACGTTGGGGCT 1100

8.1.2 HP8 screen hit shortlist

Gene	CRISPR (DepMap Public 22Q4+Score, Chronos):	Mitochondrial association
• ACAD9	140/1078	y
• ACSBG2	0/1078	y
• ADH1C	no data	y
• ADSS	3/1078 566/1078	
• AP1M1	234/1078	
• ARHGAP45	38/1078	
• ASPM	234/1078	
• ATP2B1	115/1078	
• ATP5A1Z	no data	
• ATP5J2	1078/1078	y
• ATP5PD	491/1078	y
• ATP5PF	522/1078	y
• ATP7A	26/1078	
• BSG	57/1078	
• C9H21orf2	76/1078	y (human gene)
• CAB39	222/1078	
• CCDC47	26/1078	

• CEPT1	288/1078	
• CIAPIN1	1059/1078	
• CIT	634/1078	
• CLSTN3	1/1078	
• COQ6	13/1078	y
• COX19	206/1078	y
• CSNK2A1	51/1078	
• CUBN	0/1078	
• DHRS7B	13/1078	
• DHX30	241/1078	y
• EARS2	427/1078	y
• EFR3A	427/1078	
• EMC3	803/1078	
• EMC8	26/1078	
• ENSGALG00000004363		
• ENSGALG00000005682		
• ENSGALG00000009792		
• ENSGALG00000015352		
• ENSGALG00000047203		
• ENSGALG00000050405		
• ENSGALG00000053889		
• ERAL1	383/1078	y
• ESCO2	186/1078	
• FARS2	489/1078	y
• FAU	1078/1078	
• FECH	633/1078	
• FKBP4	10/1078	
• FLVCR1	41/1078	
• FZR1	204/1078	
• GCLC	51/1078	
• GFPT1	551/1078	
• GNAS	7/1078	
• GPATCH8	44/1078	
• GRSF1	200/1078	
• GTPBP6	no data	
• HEATR3	272/1078	
• HERC2	9/1078	
• HTD2	no data	y
• IPMK	33/1078	
• IRF4	66/1078	
• ITFG2	0/1078	
• ITPK1	146/1078	
• KIF14	734/1078	
• KMT2E	24/1078	

• KNSTRN	3/1078	
• L3MBTL2	2/1078	
• LAMTOR3	679/1078	
• LAMTOR5	17/1078	
• LCMT1	310/1078	
• LETM1	976/1078	y
• LIG3	181/1078	y
• LIPT1	133/1078	
• MAPK4	1/1078	
• MARCH5	no data	y
• MGA	32/1078	
• MIOS	442/1078	
• MKLN1	17/1078	
• MPC2	5/1078	y
• MRPL10	947/1078	y
• MRPL54	483/1078	y
• MRPL57	851/1078	y
• MRPS17	304/1078	y
• MRPS21	1045/1078	y
• MRPS33	162/1078	y
• MTERF3	214/1078	y
• MTG2	622/1078	y
• NDRG3	15/1078	
• NDUFA6	502/1078	y
• NDUFAF7	176/1078	y
• NDUFB1	204/318	y
• NDUFB10	671/1078	y
• NDUFS1	372/1078	y
• NDUFS3	336/1078	y
• NDUFS5	706/1078	y
• NDUFV2	187/1078	y
• NMNAT1	229/1078	
• NPEPPS	525/1078	
• NSFL1C	15/1078	
• OXSR1	16/1078	
• PDCD2L	9/1078	
• PDSS1	216/1078	
• PIM1	17/1078	
• POLG2	290/1078	y
• POMP	693/1078	
• PPAT	687/1078	
• PPCDC	122/1078	y
• PPP2R3C	799/1078	
• PPP4R2	707/1078	

• PRDM1	20/1078	
• PRELID3B	1070/1078	y
• PRKAA1	16/1078	
• PRKAG2	2/1078	
• PTAR1	229/1078	
• PTPA	862/1078	
• PUM1	114/1078	
• PYM1	690/1078	
• RAPGEF5	0/1078	
• RBMXL1	1074/1078	
• RNASEH1	21/1078	y
• RNF4	768/1078	
• RPL36	1078/1078	
• RPRD1B	410/1078	
• RTN4IP1	114/1078	
• SAR1A	46/1078	
• SDHB	490/1078	
• SEC22B	no data	
• SENP2	10/1078	
• SH3GL1	68/1078	
• SLC16A1	135/1078	
• SLC25A1	112/1078	y
• SLC25A19	106/1078	
• SLC25A22	79/1078	y
• SLC25A3	751/1078	y
• SLC38A1	22/1078	
• SLC39A9	41/1078	
• SLC7A3	0/1078	
• SRM	57/1078	
• SRPK1	20/1078	
• SSB	1043/1078	
• SSR2	153/1078	
• STEAP3	42/1078	
• STXBP5L	3/1078	
• SYNJ1	5/1078	
• TADA1	351/1078	
• TAPT1	100/1078	
• TBRG4	37/1078	y
• TCF3	37/1078	
• TIMMDC1	226/1078	y
• TMEM242	277/1078	
• TNFRSF1A	0/1078	
• TSPAN9	0/1078	
• TSTA3	13/1078	

• TXNRD2	5/1078	y
• UBE2T	102/1078	
• UBR5	593/1078	
• UROS	112/1078	
• YME1L1	240/1078	y
• ZNF511	154/1078	
Total:		45
Yellow highlights are genes specific to HP8 cells, and orange that this gene is essential in ≤ 5 cell lines from the DepMap portal database.		

8.1.3 CVI988 screen sgRNAs

Sequence Name	Sequence	Gene
cvi988 g1	AAGACATCTTCAGTATGAGT	0.5/80
cvi988 g2	GGTATGTGCCGGTTCCAGTG	0.5/80
cvi988 g3	AACCCCCCAAAAACCTCTCG	0.5/80
cvi988 g4	CTGTGTCTAACCCTAATCGG	MDV-1/80
cvi988 g5	GAAAGTCAGCGAGTAAACAG	MDV-1/80
cvi988 g5-1	TGCGGGAGTTTGGAACCTCCG	MDV-1/80
cvi988 g6	TCTCGCCCCCATCCGCCCTG	MDV-2/79
cvi988 g7	TACTCACAGAGCCCCGCGCG	MDV-2/79
cvi988 g7-1	CAGAGGCGAACTCTTCGCGG	MDV-2/79
cvi988 g8	GTCCTGGCCTGGACGTGTGG	MDV-2.3/78.6
cvi988 g9	ATAAGCGTGGGCACACGTGT	MDV-2.3/78.6
cvi988 g10	AGAAAGCCGAGACCACAAGA	MDV-2.6/78.5
cvi988 g11	AACCGTTGCTAGAATATGTG	MDV-2.6/78.5
cvi988 g12	TGCCTGGAAATGGTAAGTAG	MDV3/78
cvi988 g13	CTCCTGCAGTGTATACCCGG	MDV3.2/78.4
cvi988 g14	CAGTACTCGTAAAAGCTGAG	MDV3.2/78.4
cvi988 g15	CCCCTGCATAGCAGAAAACC	MDV3.4/78.3
cvi988 g16	GCGTACTGCAAAATTTATTG	MDV3.4/78.3
cvi988 g17	CATGACGCGCATGCGTACGG	MDV3.4/78.3
cvi988 g18	GTATATAGCGCAAGCGCGCA	MDV3.6/78.2
cvi988 g19	GCATGCGCGATGAGTGCGCA	MDV3.6/78.2
cvi988 g21	ATCGTAGAGAAAGCATAAGG	MDV3.8/78.1
cvi988 g22	GCTGCGCATGAAAGAGCGAA	MDV3.8/78.1
cvi988 g23	GCATAAGCGTCTCTGCGATG	MDV3.8/78.1

cvi988 g24	CGGTAACAGGACACAATGCA	MDV4/77 /5.1/77.5
cvi988 g25	ATATGGGGAGTAGAGATGGG	MDV4/77 /5.1/77.5
cvi988 g26	GATCTCGAATTTCCCTTACGT	MDV5/76
cvi988 g27	AGGAACGGGATCGTGCGGGG	MDV5.2/76.8
cvi988 g28	TGAGGAGGAGAAACAGAAGC	MDV5.3/76.4
cvi988 g28-1	CCCGAGAGAAAGATCGAGGG	MDV5.3/76.4
cvi988 g29	AGCGATCATTTCCCCCAGAA	MDV5.4/75.92
cvi988 g30	ATTCGTGGTCACCGAACGAA	MDV5.4/75.92
cvi988 g31	AGAATATACAAAGTACAATG	MDV5.4/75.92
cvi988 g32	CGTATGCGATCACATTGACA	MDV5.5/75.91
cvi988 g33	GTTGTATGTGTGTGAGCAGT	MDV5.5/75.91
cvi988 g34	ACGAACGCGGACGTGTAGCG	MDV5.6/75.9
cvi988 g35	AAACGATTGCGGAAGTACGG	MDV5.6/75.9
cvi988 g36	TCGATAATACGGAAGCAAAG	MDV5.6/75.9
cvi988 g37	CAGACGAAGAAGCCTGACGT	MDV5.7/75.8
cvi988 g38	ACCGCCGAATGATACAAATG	MDV5.7/75.8
cvi988 g39	ATGACGTCACACCGAACAGG	MDV5.7/75.8
cvi988 g40	CAGTATAAATAGTAGCTAGG	MDV5.8/75.7
cvi988 g41	ATCCGTTTCGAGATGGAAAG	MDV5.8/75.7
cvi988 g42	GTTCTACTAACAAAGTGTGC	MDV6/75.3 /6.1/75
cvi988 g43	CATGCGTGATCTATTTGTGG	MDV6/75.3 /6.1/75
cvi988 g44	GCAGTGATTATTCTTTCTGG	MDV6.3/75.5
cvi988 g45	ACCTGACTTAGTAAGTGTGT	MDV6.3/75.5
cvi988 g46	CTATAACTTGATTATATCAG	MDV6.4/75.4
cvi988 g47	TGTACAAAGACGGAAGCGCG	MDV6.4/75.4
cvi988 g48	CGTGTGAAGAACCCTAGCAA	MDV6.5(132BP)/75.2
cvi988 g49	TATCGGAGGAATGAGCGACG	MDV6.6/75.1
cvi988 g50	TTGATGATGAAGCGGTTTCGT	MDV6.6/75.1
cvi988 g51	GGCTCTTGTGTATAAAAGAG	MDV7/74
cvi988 g52	ACTCAAGTGCGAATTTGGGG	MDV7/74
cvi988 g53	CATCACGTGATAGTTTCGAGT	MDV9
cvi988 g54	GATCGGTGGTGTAAACCGTGT	MDV9
cvi988 g55	CCCTGCCGATCTTGACGCG	MDV9.5/73.4
cvi988 g56	GCGCTTTGGATCGGGTCCAG	MDV9.5/73.4
cvi988 g57	ACTGGAACGTTGATTGGGAA	MDV8
cvi988 g58	AATTACTCGGATATGAAAGC	MDV-10

cvi988 g59	GGATCATCATGCTGGTTACG	MDV-10
cvi988 g60	GTATGTGGGATGAACTTGCA	MDV8.4
cvi988 g61	AGATACCATACGCTTGGACG	MDV8.8
cvi988 g62	GTTTCGCACAATAATCCAAT	MDV8.4
cvi988 g62-1	TACCTTTCATGGGAATCCCA	MDV8.4
cvi988 g63	TTTGGCGTACAAATTCACGA	MDV-11
cvi988 g64	ACCACCGTCAAAGAAATAGA	MDV-11
cvi988 g65	TAACCCATCAACGATTGCTG	MDV-11.5
cvi988 g66	TCGCGGCATAAGCTATCGAG	MDV-12
cvi988 g67	TGTATTCTGTAGTAATGACA	MDV-12
cvi988 g68	TTGAACCCGAGACCACGCCC	MDV-12.4
cvi988 g69	TGGGTGATCTCGAACGACAA	MDV-12.4
cvi988 g70	CACGAGTTAGGCCTGGGCGT	MDV-12.8
cvi988 g71	ACCATCACATAGTGAAGTTG	MDV-12.8
cvi988 g72	AGACCAATACGGTTCTCGCA	MDV-13/13.5
cvi988 g73	TTGGAGAATTCTGTGAAGGT	MDV-13/13.5
cvi988 g74	GCGTTCGCTATAATATCCCA	MDV-14
cvi988 g75	TCTGTTAAAGCAGTAATCGT	MDV-14
cvi988 g76	CCTGGATCTCACCATTCTGGT	MDV-14
cvi988 g77	ATTCACATGTACAAAAGACC	MDV-14.5
cvi988 g78	ATAATCGCAACCTTGTGCTG	MDV-14.5
cvi988 g79	TCTTCCCAATTCATCGATAG	MDV-15
cvi988 g80	GCAAACCTCATATACAAACAC	MDV-15
cvi988 g81	AACATTATTATATGCAAGCG	MDV-15.5
cvi988 g82	ATATGCTTGCTGGATTTCAGG	MDV-15.5
cvi988 g83	GGGTGATGGATAATTGACGG	MDV-16
cvi988 g84	TACGTGTATAACAATTCTGG	MDV-16
cvi988 g85	AACTCATTGTTGTGACAG	MDV-16
cvi988 g86	GTACTCCACAATATAAAAGA	MDV-17
cvi988 g87	TGGAATATAGTGTTTATATG	MDV-17
cvi988 g88	AGATTGTATTCCATGCATGG	MDV-17
cvi988 g89	TATATCCTAATCGACCTGCG	MDV-18
cvi988 g90	TAACAACCTGGAACGATACCA	MDV-18
cvi988 g91	TGGAGGCCAGTAATGAACGA	MDV-18
cvi988 g92	GGAGACACCTGTAACTGGG	MDV-19
cvi988 g93	TGTTTGTATATAATGTAGTG	MDV-19

cvi988 g94	GTGATATAATATGTGATTGG	MDV-19
cvi988 g95	AATAAGCATGATATCCCAGA	MDV-20
cvi988 g96	CTACTTTTTTCGAAATACATG	MDV-20
cvi988 g97	CTAAACAGTCAAAAGACGTG	MDV-20
cvi988 g98	TCTACAGCACCTTCTACGCG	MDV-20.5
cvi988 g99	GCAACAAAATGTGCCTTGAG	MDV-20.5
cvi988 g100	CGACGTAATTGATAGTGATG	MDV-20.5
cvi988 g101	AAACCGAATCCGTGTATGCG	MDV-21
cvi988 g102	GATATGTTAGCCATCATGAG	MDV-21
cvi988 g103	GTTCTCGGGTACAAAGGTGA	MDV-21
cvi988 g104	CGCGTCTATAAATTTAACTG	MDV-22
cvi988 g105	TCATCTGCTAAGGATATCGA	MDV-22
cvi988 g106	TAATTGGGTATGGTATGCTG	MDV-22
cvi988 g107	GGATGATGAACATTATGATA	MDV-23
cvi988 g108	TTACTAAAAAATGGCCGTAG	MDV-23
cvi988 g109	TGGCAACGAAAACAGTGCAA	MDV-24
cvi988 g110	TTACCAATGACCTACCATCG	MDV-24
cvi988 g111	GAGTCCCGAAAACGAAAGAG	MDV-24
cvi988 g112	GATTACTGGAAATCCCGAAT	MDV-25
cvi988 g113	TATCTTTGTTAACGTCACCG	MDV-25
cvi988 g114	AAGAGATGCCAATATATGCT	MDV-25
cvi988 g115	TGAATTAGATGAAGAATCCG	MDV-26
cvi988 g116	AGAAGCTATTCTCGAAAAGG	MDV-26
cvi988 g117	GAGAAGCTGTTTATAAAGAA	MDV-26
cvi988 g118	GCGCGAGTCATAGAAATGAA	MDV-27
cvi988 g119	AGTTCCGTGAAGTTTATCAT	MDV-27
cvi988 g120	CATCGCGTGGATCATGTCAA	MDV-27
cvi988 g121	CAGAAACACGCATTACAGAG	MDV-28
cvi988 g122	CAGGTTCTATTATCTCAGAA	MDV-28
cvi988 g123	CACTCAGGAAGAGATTGTGA	MDV-28
cvi988 g124	GTTGTATCTGGATTGAAGCA	MDV-29
cvi988 g125	CCGGTGCGGATATCAGAACA	MDV-29
cvi988 g126	CATTATCTAACTTTGATCGG	MDV-29
cvi988 g127	GAGGGTCATAGTAATCCACG	MDV30
cvi988 g128	ACGGCTTGAATCTATTGACA	MDV30
cvi988 g129	TCGCATGTAGATAACTTGCA	MDV30

cvi988 g130	TGTATTAGTGACAACTACGA	MDV31
cvi988 g131	GGCTCTCATTCGGAGATGAG	MDV31
cvi988 g132	TACAAACACATGCAACAGAA	MDV31
cvi988 g133	GGAAATAGAACTGCATGACA	MDV31.5
cvi988 g134	AATACTTGGAGATCGAGTCG	MDV31.5
cvi988 g135	ACTTGCTGGAATTTACTCAG	MDV32
cvi988 g136	ACATACATCCCAAATTTACT	MDV32
cvi988 g137	GATACAACTTGAAGACAAG	MDV32
cvi988 g138	TATAAAGCGTATTTCTTCTG	MDV33
cvi988 g139	CATCAAATAATCCTTCAACG	MDV33
cvi988 g140	AATCTTCTGGAAAGTAGACT	MDV33
cvi988 g141	AGGACCACATATGAACGCAT	MDV34
cvi988 g142	ACGAAATGTGCAAATTCCT	MDV34
cvi988 g143	GATGGAAGCGCGAATAACCA	MDV34
cvi988 g144	GGGGATTATTAATGAACTG	MDV36
cvi988 g145	ATCACCAAGTTGCATCCTGG	MDV36
cvi988 g146	CTATACCCATTGATCCATCG	MDV36
cvi988 g147	TAGGATCTCAGGTGTATGAT	MDV35
cvi988 g148	ATTGGGCAAATTGCGCCTCA	MDV35
cvi988 g149	AAAGGCGTCTGCTAACGTAA	MDV35
cvi988 g150	TGGGACGCGAGAATATTGAC	MDV37
cvi988 g151	AATAGGAATGTAGTGAAGTC	MDV37
cvi988 g152	CCGTGTGGAAAATACGAACG	MDV37
cvi988 g153	AGGAGCTGGTGTGCGGAATCG	MDV38
cvi988 g154	TGTTACATATGATCTCACCC	MDV38
cvi988 g155	ATTTGTGAAAACGCAAACCC	MDV38
cvi988 g156	GGTCAACACTCCATCCCAAG	MDV39
cvi988 g157	CATACTACTAGGGAAGAACG	MDV39
cvi988 g158	CCCTACAAAAAGAAGTCGAA	MDV39
cvi988 g159	ATAGGGCGTTCTGACTCCCAT	MDV38.5
cvi988 g160	AGGGTATGAGTAATAAACTG	MDV38.5
cvi988 g161	CAGGTGGTCCGATAGAACGG	MDV39.5
cvi988 g162	AACGCCACGAGAAAAAACTG	MDV39.5
cvi988 g163	CATCACACTTCACAGTTGGG	MDV40
cvi988 g164	TATCCATGGTGATCCCCACA	MDV40
cvi988 g165	ACGGTTGAACCCACTGGTGG	MDV40

cvi988 g166	GATAGCATATAAATCACCCG	MDV41
cvi988 g167	TCGAGTTGGATACTTATGGT	MDV41
cvi988 g168	AATAGCGAATAGTTTCTGAG	MDV41
cvi988 g169	GCTTCGATGAAAATCACGAG	MDV42
cvi988 g170	TTGCATAAAACCTTGTACGA	MDV42
cvi988 g171	TCCAACAACAACATTATGGA	MDV42
cvi988 g172	GAAAGAGGAGTCTTGAAGCA	MDV43
cvi988 g173	GTCGATACTGATACCATCTG	MDV43
cvi988 g174	CATGACGTACAATGATGACA	MDV43
cvi988 g175	GCCATACATGAGCAATTATG	MDV44
cvi988 g176	TTGTCTTGGGAGATCGACTG	MDV44
cvi988 g177	GCGATAAAAGGCTAGTTGGA	MDV44
cvi988 g178	ATAATGATTATGAACACGAG	MDV46
cvi988 g179	TATATATACGAATTCAAACG	MDV46
cvi988 g180	GTCCGGATTAAATGCCACAT	MDV46
cvi988 g181	AATCACTTCATCCAAACGTG	MDV45
cvi988 g182	TCAAAATTTGATGTAAATCG	MDV45
cvi988 g182-1	TTTGATCCGACCATACCGAA	MDV45
cvi988 g183	TACCAGTTGTTAAATCTCCA	MDV47
cvi988 g184	TGGGCGACAATCTACACCGA	MDV47
cvi988 g185	CACAGCATGACTATGTAGCG	MDV47
cvi988 g186	AGAGGGATCAAAGGTTTGCG	MDV48
cvi988 g187	GGATGGTCGTATCCGCGAAG	MDV48
cvi988 g188	CTTGAAACGCACATTCCCAA	MDV48
cvi988 g189	CGAAAATACACCCTGCCACG	MDV49
cvi988 g190	CAAATCTAAACTATATCGG	MDV49
cvi988 g191	TTCAGAAGTCGCTATTGTCC	MDV49
cvi988 g192	GCAAATTATGGATAACCCTG	MDV49.5
cvi988 g192-1	AAACATGAACTGAAGACGC	MDV49.5
cvi988 g193	TTAGTGCTGATTCATAAGCA	MDV50
cvi988 g194	GTAAAGAGTATCCGTGTAAG	MDV50
cvi988 g195	CTGGCGCATATCTGTCAGGT	MDV50
cvi988 g196	TCGAGGTGTATAAACAAACG	MDV50.5
cvi988 g197	ATCGCATCTAGGGAGAACAG	MDV50.5
cvi988 g198	AGATTGAACAGAATTTCCGA	MDV51
cvi988 g199	CATACAGTTTCCATGACTCG	MDV51

cvi988 g200	TTTGTGTATATACGAACACA	MDV51
cvi988 g201	ACGAACCATATACTTTCTCA	MDV52
cvi988 g202	CTAACCGTTTCAGCCAGGGA	MDV52
cvi988 g203	GTACCCCATCTTCTGGCGAG	MDV52
cvi988 g204	AGCATGCCGATCTCATGTGA	MDV53
cvi988 g205	CTAGTTTAGACTGGATAGCG	MDV53
cvi988 g206	TAGTTACGTACGTTACAGCG	MDV53
cvi988 g207	ACGCTTCCCTATAATGCAAG	MDV54
cvi988 g208	TATACGAAGAGCAAGATCGG	MDV54
cvi988 g209	GTACGGATGTTGGAGAGGCG	MDV54
cvi988 g210	AGAGCTAGGAATATTGCTGT	MDV55
cvi988 g211	CAGCCCTCATCGTGGCATGT	MDV55
cvi988 g212	GCACGTGTTTGGAAGTCCCA	MDV55
cvi988 g213	CCAGTGGTTGTATAAGACGG	MDV56
cvi988 g214	AAATAGAAATCCGAATAGGG	MDV56
cvi988 g215	GATATGTTACAGGGAGTCTG	MDV56
cvi988 g216	GTGTGTTACGAGCTTTGGGG	MDV57
cvi988 g217	CCACGCCAACGAAAAGAATG	MDV57
cvi988 g218	ACTCCTCGGAAAGATCGCGA	MDV57
cvi988 g219	AATAATCTATCACTTTGTCA	MDV57.4
cvi988 g220	TTAAGAGGAAATATCTCCGT	MDV57.4
cvi988 g221	AATAATCTATCACTTTGTCA	MDV57.4
cvi988 g222	CCTATGACAAAAGAGTTGCA	MDV57.8
cvi988 g223	AATGATGTCGCCTACACCCG	MDV57.8
cvi988 g224	AGGGTACAGTTGCATGCGCG	MDV58
cvi988 g225	GAAGCATTCGTGGAGCCGAA	MDV58
cvi988225-1	CCGCGGTACTTAATGGTCGG	MDV58
cvi988 g226	CAATGGCGCGAAACCAAACA	MDV59
cvi988 g227	TGGATAAAATAGCAACGCAA	MDV59
cvi988 g228	GTCTGCATTAAGAGCTCTTG	MDV59
cvi988 g229	AACTCAGATACATCGTCTCG	MDV60
cvi988 g230	TCACTACCGCGACGACCCGA	MDV60
cvi988 g231	ATCCTGGTCAAAATCAACGA	MDV60
cvi988 g232	TCGCTGTGATACATCCCGCG	MDV61
cvi988 g233	TAGTGGCAACAGGATCACCA	MDV61
cvi988 g234	CTAGTGTAGAGAGAATTGGG	MDV61

cvi988 g235	TCTGAACGTACAAGACGCGG	MDV62
cvi988 g236	GACGTTTCGTCTACCACCCG	MDV62
cvi988 g237	ATATGCAGAGGGATATCCAA	MDV62
cvi988 g238	CTCCTCTTTAAACATATCTG	MDV64
cvi988 g239	GGAAGTTCAATCACGAGTAT	MDV64
cvi988 g240	GACAAACGTCGCTTGCGAAG	MDV64
cvi988 g241	TGGAAGCACTAACAGTTCCC	MDV63
cvi988 g242	TGCCGTTCCCTCAGAATTACG	MDV63
cvi988 g243	CATATCCCACCTCCACTTGG	MDV63
cvi988 g244	CGAAGATATTAGGCGATCGA	MDV63.5
cvi988 g244-1	TACTCCGGCTGTAACCCCAA	MDV63.5
cvi988 g245	CATATACATATGCAAGAGTG	MDV65
cvi988 g246	AGCTATGCATCGTCTCAGGG	MDV65
cvi988 g247	ATAGACATTCTAAATTGCGA	MDV65
cvi988 g248	ACGCTCGAAAGTGATGACTC	MDV66
cvi988 g249	ATGCAAAACATACGCAGTGT	MDV66
cvi988 g250	CATAGCATTTCATCATATG	MDV66
cvi988 g251	ATGTATCTATGCTACCTTGG	MDV67
cvi988 g252	TAAACACTAGATATCACTC	MDV67
cvi988 g253	TTGCTTTGTTTCTCCCAACA	MDV67
cvi988 g254	TCCCAAACATAACGGCCGCA	MDV68
cvi988 g255	CATTTGAAATAGAGGCCAGA	MDV68
cvi988 g256	TGGAACAGAGAAACGCTCCA	MDV68
cvi988 g257	TGAAATTGCACGAACAGCTG	MDV69
cvi988 g258	CATGCTTGGTTCTTAGCGAT	MDV69
cvi988 g259	ACAATCTTTATTATTCGATG	MDV69
cvi988 g260	GATGTAATGGATCCTATGTG	MDV70
cvi988 g261	TCTGGGAATATGTCTCAGCA	MDV70
cvi988 g262	ACGAATCCAGAATAGTAGCG	MDV70
cvi988 g263	GATCGACAGATATGCCACGT	MDV71
cvi988 g264	GTGACAATGGGTACATTTGG	MDV71
cvi988 g265	GGACGGAAACATATATGGAG	MDV71.4
cvi988 g266	TCCATACAAATGTATAGGGT	MDV71.4
cvi988 g267	GTCAATTGTATCATCGTATG	MDV71.8
cvi988 g268	GAATTGTTGACATTATGTAT	MDV71.8
cvi988 g269	TAGCAGTATCAGGAATACGT	MDV72.2

cvi988 g270	ACCTTAAACATAAATCCTG	MDV72.2
cvi988 g271	GCATCTCGACACATGTAACG	MDV72.2
cvi988 g272	CCTGGTATAACGATAACAGG	MDV72.4
cvi988 g273	CGATATACTCCAACCCGTAG	MDV72.4
cvi988 g274	GTATGATTCGGCAACTACGG	MDV72.4
cvi988 g275	CTTCCCAATAATGACCATGG	MDV72
cvi988 g276	ACCAAGACAACGATCCTGAA	MDV72
cvi988 g277	AGCAAATGACAACATCCGAG	MDV72.6 / MDV72.8
cvi988 g278	TTTCTGTGATAATAGTTACA	MDV72.6 / MDV72.8
cvi988 g279	ATTGTTAACATTATGTATCG	MDV72.6 / MDV72.8
cvi988 g280	TCCACGGATAGAATAGTACG	MDV73
cvi988 g281	AGATGGTATGAAGCAGATGG	MDV73
cvi988 g282	TACATGACCGCGCATGCGCG	MDV81/103
cvi988 g283	CAGAAAAAGGCGCCAAACAA	MDV81.5/102.5
cvi988 g284	ATAGCGTGTTGGCCAAATGG	MDV81.5/102.5
cvi988 g285	CAATTAAAGAAGATCATGCA	MDV81.5/102.5
cvi988 g286	ATTGTTCGTTCCAGGTGCATG	MDV82/102
cvi988 g287	CTAGGCTTCCGACATAGACG	MDV82/102
cvi988 g288	TCGTGGCGTAAAAACGAACA	MDV82/102
cvi988 g289	CGAGGGGGAACAAGAACTGG	MDV84/100
cvi988 g290	GCCAGGATCTCCAGTAGAGG	MDV84/100
cvi988 g291	AGCGTGGGCTATGGGCAGCG	MDV84/100
cvi988 g292	GAGGGGCGCAAATAGATGGT	MDV83/101
cvi988 g293	ATTTCCACGAGACCCCCAAA	MDV83/101
cvi988 g294	GCTAGACCACAAGATGTGGG	MDV83/101
cvi988 g295	AGATACGTATAACAATGCTG	MDV84.5/99.5
cvi988 g296	CAGCAGGACACAATTTGACT	MDV84.5/99.5
cvi988 g297	GAAGTAGATGAGCGATTGTG	MDV85/99
cvi988 g298	CACATCAAAAATCGCCATGT	MDV85.3/98.9
cvi988 g298-1	TCGTAGTATCCATAAGCGCG	MDV85.3/98.9
cvi988 g299	AGTCCCGTTGATGAATATGG	85.6/85.9/98.3/98.6
cvi988 g300	CCCGCAAATGTATCCGGTA	85.6/85.9/98.3/98.6
cvi988 g301	GGGAGGGGGAGAATATGGTG	MDV86/98
cvi988 g302	AGAATTGTATAAGAGAACGT	MDV86/98
cvi988 g303	TTCAGCCCCAAGCACAACCG	MDV86.1
cvi988 g304	CCGACATAGCTCGAGCCAAA	MDV86.1

cvi988 g305	GTGCCTTTCTCTACGCAGAT	MDV86.2/86.4/97.3/97.6
cvi988 g306	TCGTGGGGCACCTATTTGCG	MDV86.2/86.4/97.3/97.6
cvi988 g307	AGGACCGGCAGAACTTCTCA	MDV86.6
cvi988 g308	AATAAGTCGAGTAGATTTGA	MDV86.6
cvi988 g309	GGTCCCCTGCGATTCCAAAC	MDV87
cvi988 g310	GGCCAGCTTTCATAAAGGGC	MDV87
cvi988 g311	TGGAGTGAAATCTTTAGGGA	MDV87
cvi988 g312	TCAACTGCCACATTACATC	MDV88
cvi988 g313	GTGCTCTCCGAAGAGCATCG	MDV88
cvi988 g314	GATTGGGTGAAAAATGCTAT	MDV88
cvi988 g315	TCACCCACAAACGAGACGA	MDV89/89.5
cvi988 g316	ATGATGTATTGGTGTGTCT	MDV89/89.5
cvi988 g317	GGAATCGCCAACAGGAGACG	MDV89/89.5
cvi988 g318	GCGGACGTAAATGGAGATTG	MDV90
cvi988 g319	GGTGTCTATACTCGCACAC	MDV90
cvi988 g320	GATGATATGGATATACCAAG	MDV90
cvi988 g321	CCTCAAATGAGTAGTACACC	MDV91
cvi988 g322	TATGTGTATACAAAATCCTC	MDV91
cvi988 g323	CCAACTATCCACAAGCTATG	MDV91.5
cvi988 g324	GAACAAATACAGGATGATGT	MDV91.5
cvi988 g325	TGGAAATGAATCCCCCGAAA	MDV92.4
cvi988 g325-1	GCTGTTATGTATAATTCCGT	MDV92.4
cvi988 g326	CGTTACCGCCCGGATCTGAA	MDV92
cvi988 g327	GAACATAATCTAATTATGGAG	MDV92
cvi988 g328	CAAATAATTACGATAGAACG	MDV92.8
cvi988 g329	ATAAACCCAAATGTTATGGA	MDV92.8
cvi988 g330	GAATATGGCGTAGGTCCCGA	MDV93
cvi988 g331	CATGTCTGTAATGAACGATT	MDV93
cvi988 g332	ACGTAGACCACTATTGCCAT	MDV93
cvi988 g333	ATTCCCACATTACATCCGAA	MDV94
cvi988 g334	ACTTGTAATTAAAGTCCCT	MDV94
cvi988 g334-1	GATTATAAGAATCATTCTCG	MDV94
cvi988 g335	AATCCACTTAGCTCACGACT	MDV94.5
cvi988 g336	AAGCAGTGGCTTGAACGAAA	MDV94.5
cvi988 g337	TAGACCAGATGCCTCGAAAG	MDV95
cvi988 g338	ATATCTGTGGAAATGGATTG	MDV95

cvi988 g339	ATATCCTACGATGGGGAGAG	MDV95
cvi988 g340	GATATCCACCAGTTGTTCGAT	MDV95.5
cvi988 g341	AGTATCCTGCATGAGGTATG	MDV95.5
cvi988 g342	TCCCAATCACTGCAACGAAA	MDV95.5
cvi988 g343	TAGGACTGGACATTTGGGGT	MDV96
cvi988 g344	TCGTAGATGTATTTATGACA	MDV96
cvi988 g345	ACAATGGACGTCCAAGTGCA	MDV96.5
cvi988 g346	ACGAAAATGAGGCTAGCGAT	MDV96.5
gB-C-sgRNA	TACGTCTGCCCACCACCGGT	MDV40
gB-N-sgRNA	TGTTTGAAAGATGGTTCGGAC	MDV40
Meq-C-sgRNA	ATACTCAGTCTACGGTCTGG	MDV5/76
Meq-N-sgRNA	TCGACCCGAGAGAAAGATCG	MDV5/76
pp38-C-sgRNA	CCTCGTCGGCGACCCCTGCG	MDV73
pp38-N-sgRNA	TGGTATGTTAGTCGGTAGAA	MDV73
US2-HVT-C-sgRNA	GTACAGCAGACCTGTGTCGG	NA
US2-HVT-N-sgRNA	AGATATCTGCAGCCCCGAGT	NA

8.1.4 CVI988 screen *P-values*

Sorted in terms of *p-value* from smallest to largest.

Dunnett's multiple comparisons test	Mean Diff.	Significance Summary	Adjusted <i>p-value</i>	MDV gene
gb vs. MDV81.5/102.5	-69240754	***	0.0005	MDV81.5/102.5
gb vs. MDV3/78	-60907421	**	0.0047	MDV3/78
gb vs. MDV-2/79	-60240754	**	0.0055	MDV-2/79
gb vs. MDV84.5/99.5	-57240754	*	0.0114	MDV84.5/99.5
gb vs. MDV3.4/78.3	-56574088	*	0.0133	MDV3.4/78.3
gb vs. MDV3.8/78.1	-54240754	*	0.0223	MDV3.8/78.1
gb vs. MDV85/99	-54240754	*	0.0223	MDV85/99
gb vs. MDV56	-53907421	*	0.024	MDV56
gb vs. MDV-2.6/78.5	-53574088	*	0.0258	MDV-2.6/78.5
gb vs. MDV57.4	-52907421	*	0.0296	MDV57.4
gb vs. MDV91	-51907421	*	0.0365	MDV91

gb vs. positive control	-51240754	*	0.0417	positive control
gb vs. MDV3.2/78.4	-50240754	ns	0.0508	MDV3.2/78.4
gb vs. MDV81/103	-50240754	ns	0.0508	MDV81/103
gb vs. MDV64	-48252584	ns	0.0739	MDV64
gb vs. MDV57.8	-48240754	ns	0.0742	MDV57.8
gb vs. MDV72.2	-48240754	ns	0.0742	MDV72.2
gb vs. MDV52	-47907421	ns	0.0788	MDV52
gb vs. MDV54	-47574088	ns	0.0837	MDV54
gb vs. HVT US2	-47240754	ns	0.0888	HVT US2
gb vs. MDV-2.3/78.6	-46240754	ns	0.1058	MDV-2.3/78.6
gb vs. MDV72	-46240754	ns	0.1058	MDV72
gb vs. MDV85.3/98.9	-46240754	ns	0.1058	MDV85.3/98.9
gb vs. MDV57	-45240754	ns	0.1253	MDV57
gb vs. MDV9	-44907421	ns	0.1324	MDV9
gb vs. MDV50	-44574088	ns	0.1398	MDV50
gb vs. MDV71.4	-43907421	ns	0.1556	MDV71.4
gb vs. MDV86/98	-43574088	ns	0.1641	MDV86/98
gb vs. MDV58	-42907421	ns	0.1819	MDV58
gb vs. MDV71	-42907421	ns	0.1819	MDV71
gb vs. MDV86.1	-41574088	ns	0.222	MDV86.1
gb vs. MDV62	-41513974	ns	0.2239	MDV62
gb vs. MDV71.8	-40907421	ns	0.2444	MDV71.8
gb vs. MDV61	-40181614	ns	0.2706	MDV61
gb vs. MDV60	-39907421	ns	0.2808	MDV60
gb vs. MDV8.4	-39574088	ns	0.294	MDV8.4
gb vs. MDV51	-39574088	ns	0.294	MDV51
gb vs. MDV94	-39240754	ns	0.3072	MDV94
gb vs. MDV8.8	-38774588	ns	0.3265	MDV8.8
gb vs. MDV-14	-37240754	ns	0.3958	MDV-14
gb vs. MDV93	-36922821	ns	0.4112	MDV93
gb vs. MDV95	-36907421	ns	0.4119	MDV95
gb vs. 85.6/85.9/98.3/98.6	-36574088	ns	0.4282	85.6/85.9/98.3/98.6
gb vs. MDV91.5	-36115111	ns	0.4517	MDV91.5
gb vs. MDV63	-35907421	ns	0.4624	MDV63
gb vs. MDV69	-35574088	ns	0.48	MDV69
gb vs. MDV3.6/78.2	-34240754	ns	0.5527	MDV3.6/78.2
gb vs. MDV96.5	-34240754	ns	0.5527	MDV96.5

gb vs. MDV46	-33560874	ns	0.5913	MDV46
gb vs. MDV82/102	-33240754	ns	0.6098	MDV82/102
gb vs. MDV72.6 / MDV72.8	-33200214	ns	0.6123	MDV72.6 / MDV72.8
gb vs. MDV72.4	-33102878	ns	0.6177	MDV72.4
gb vs. meq	-33037861	ns	0.6216	meq
gb vs. MDV53	-32574088	ns	0.6484	MDV53
gb vs. MDV65	-31761358	ns	0.6958	MDV65
gb vs. MDV67	-31752251	ns	0.6962	MDV67
gb vs. MDV9.5/73.4	-31574088	ns	0.7067	MDV9.5/73.4
gb vs. MDV0.5/80.5	31010046	ns	0.739	MDV0.5/80.5
gb vs. MDV94.5	-30907421	ns	0.7446	MDV94.5
gb vs. MDV68	-30658294	ns	0.7587	MDV68
gb vs. MDV66	-30574088	ns	0.7634	MDV66
gb vs. MDV70	-30266861	ns	0.7807	MDV70
gb vs. MDV59	-29867078	ns	0.8019	MDV59
gb vs. MDV95.5	-28965401	ns	0.8473	MDV95.5
gb vs. MDV96	-28754181	ns	0.8574	MDV96
gb vs. MDV63.5	-28639914	ns	0.8627	MDV63.5
gb vs. MDV86.2/86.4/97.3/97.6	-26907421	ns	0.9298	MDV86.2/86.4/97.3/97.6
gb vs. MDV-14.5	-26574088	ns	0.9402	MDV-14.5
gb vs. MDV-1/80	-25588001	ns	0.9615	MDV-1/80
gb vs. MDV-11	-25356184	ns	0.9641	MDV-11
gb vs. MDV5/76	24209946	ns	0.9781	MDV5/76
gb vs. MDV-15.5	-23515328	ns	0.9797	MDV-15.5
gb vs. MDV36	-22936908	ns	0.9809	MDV36
gb vs. pp38	-22607371	ns	0.9816	pp38
gb vs. MDV89/89.5	-22574088	ns	0.9817	MDV89/89.5
gb vs. MDV-16	-22511731	ns	0.9818	MDV-16
gb vs. MDV7/74	-21569054	ns	0.9841	MDV7/74
gb vs. MDV-20	20955286	ns	0.991	MDV-20
gb vs. MDV49	-20879554	ns	0.9911	MDV49
gb vs. MDV-21	20713266	ns	0.9912	MDV-21
gb vs. MDV55	-20664634	ns	0.9913	MDV55
gb vs. MDV45	-20505048	ns	0.9914	MDV45
gb vs. MDV50.5	-20510038	ns	0.9914	MDV50.5
gb vs. MDV-15	-20211628	ns	0.9916	MDV-15
gb vs. MDV87	-20040814	ns	0.9918	MDV87

gb vs. MDV83/101	-19685788	ns	0.9921	MDV83/101
gb vs. MDV49.5	-19585514	ns	0.9979	MDV49.5
gb vs. MDV5.8/75.7	-18688654	ns	0.998	MDV5.8/75.7
gb vs. MDV6.4/75.4	-18583174	ns	0.998	MDV6.4/75.4
gb vs. MDV6.6/75.1	-18778998	ns	0.998	MDV6.6/75.1
gb vs. MDV47	-19163534	ns	0.998	MDV47
gb vs. MDV5.4/75.92	-17391151	ns	0.9982	MDV5.4/75.92
gb vs. MDV5.5/75.91	-17299934	ns	0.9982	MDV5.5/75.91
gb vs. MDV-10	-17100591	ns	0.9983	MDV-10
gb vs. MDV35	-16806484	ns	0.9983	MDV35
gb vs. MDV88	-15121938	ns	0.9985	MDV88
gb vs. MDV5.7/75.8	-14891888	ns	0.9986	MDV5.7/75.8
gb vs. MDV6/75.3 /6.1/75	-14513924	ns	0.9986	MDV6/75.3 /6.1/75
gb vs. MDV40	-15050694	ns	0.9986	MDV40
gb vs. MDV48	-14495038	ns	0.9986	MDV48
gb vs. MDV6.5(132BP)/ 75.2	12946602	ns	0.9988	MDV6.5(132BP)/ 75.2
gb vs. MDV-27	13505509	ns	0.9988	MDV-27
gb vs. MDV43	-12272858	ns	0.9989	MDV43
gb vs. MDV86.6	-12120881	ns	0.9989	MDV86.6
gb vs. MDV31.5	-11731488	ns	0.999	MDV31.5
gb vs. MDV- 13/13.5	10119446	ns	0.9991	MDV-13/13.5
gb vs. MDV-28	9821176	ns	0.9991	MDV-28
gb vs. MDV-29	-10284644	ns	0.9991	MDV-29
gb vs. MDV44	-10728528	ns	0.9991	MDV44
gb vs. MDV34	-9245141	ns	0.9992	MDV34
gb vs. MDV-23	7970579	ns	0.9993	MDV-23
gb vs. MDV31	8318036	ns	0.9993	MDV31
gb vs. MDV37	8136829	ns	0.9993	MDV37
gb vs. MDV41	-8002884	ns	0.9993	MDV41
gb vs. MDV90	-8433921	ns	0.9993	MDV90
gb vs. MDV-12	-6828511	ns	0.9994	MDV-12
gb vs. MDV-18	7554356	ns	0.9994	MDV-18
gb vs. MDV38	6630892	ns	0.9994	MDV38
gb vs. MDV39.5	6685536	ns	0.9994	MDV39.5
gb vs. MDV92.4	-7381631	ns	0.9994	MDV92.4

gb vs. MDV6.3/75.5	-6127644	ns	0.9995	MDV6.3/75.5
gb vs. MDV39	6087842	ns	0.9995	MDV39
gb vs. MDV4/77 /5.1/77.5	5831252	ns	0.9996	MDV4/77 /5.1/77.5
gb vs. MDV8	-4586514	ns	0.9996	MDV8
gb vs. MDV-12.8	-5219108	ns	0.9996	MDV-12.8
gb vs. MDV-20.5	5410756	ns	0.9996	MDV-20.5
gb vs. MDV84/100	-5792208	ns	0.9996	MDV84/100
gb vs. MDV5.3/76.4	-4353181	ns	0.9997	MDV5.3/76.4
gb vs. MDV5.6/75.9	-3753598	ns	0.9997	MDV5.6/75.9
gb vs. MDV-11.5	3626619	ns	0.9997	MDV-11.5
gb vs. MDV-22	4085412	ns	0.9997	MDV-22
gb vs. MDV73	-4409901	ns	0.9997	MDV73
gb vs. MDV-12.4	-2797368	ns	0.9998	MDV-12.4
gb vs. MDV-19	2788106	ns	0.9998	MDV-19
gb vs. MDV-26	2557329	ns	0.9998	MDV-26
gb vs. MDV30	-2267918	ns	0.9998	MDV30
gb vs. MDV42	2549789	ns	0.9998	MDV42
gb vs. MDV92	-2519958	ns	0.9998	MDV92
gb vs. MDV5.2/76.8	-1519114	ns	0.9999	MDV5.2/76.8
gb vs. MDV-25	2137506	ns	0.9999	MDV-25
gb vs. MDV92.8	-1949768	ns	0.9999	MDV92.8
gb vs. negative control	85748293	****	<0.0001	negative control
gb vs. MDV-17	-186041	ns	>0.9999	MDV-17
gb vs. MDV-24	-406978	ns	>0.9999	MDV-24
gb vs. MDV32	1323362	ns	>0.9999	MDV32
gb vs. MDV33	-1160291	ns	>0.9999	MDV33
gb vs. MDV38.5	1094712	ns	>0.9999	MDV38.5

Dunnett's multiple comparisons test	Mean Diff.	Significance Summary	Adjusted <i>p</i> -value	MDV gene
HVT US2 vs. MDV5/76	71450700	***	0.0003	MDV5/76
HVT US2 vs. MDV-20	68196040	***	0.0007	MDV-20
HVT US2 vs. MDV-21	67954020	***	0.0007	MDV-21

HVT US2 vs. MDV-27	60746263	**	0.0049	MDV-27
HVT US2 vs. MDV6.5(132BP)/75.2	60187357	**	0.0056	MDV6.5(132BP)/75.2
HVT US2 vs. MDV-13/13.5	57360200	*	0.0111	MDV-13/13.5
HVT US2 vs. MDV-28	57061930	*	0.0119	MDV-28
HVT US2 vs. MDV31	55558790	*	0.0167	MDV31
HVT US2 vs. MDV37	55377583	*	0.0174	MDV37
HVT US2 vs. MDV-23	55211333	*	0.018	MDV-23
HVT US2 vs. MDV-18	54795110	*	0.0198	MDV-18
HVT US2 vs. MDV39.5	53926290	*	0.0239	MDV39.5
HVT US2 vs. MDV38	53871647	*	0.0242	MDV38
HVT US2 vs. MDV39	53328597	*	0.0271	MDV39
HVT US2 vs. MDV4/77 /5.1/77.5	53072007	*	0.0287	MDV4/77 /5.1/77.5
HVT US2 vs. MDV-20.5	52651510	*	0.0313	MDV-20.5
HVT US2 vs. MDV-22	51326167	*	0.041	MDV-22
HVT US2 vs. MDV-11.5	50867373	*	0.045	MDV-11.5
HVT US2 vs. MDV-19	50028860	ns	0.0529	MDV-19
HVT US2 vs. MDV-26	49798083	ns	0.0553	MDV-26
HVT US2 vs. MDV42	49790543	ns	0.0554	MDV42
HVT US2 vs. MDV-25	49378260	ns	0.0599	MDV-25
HVT US2 vs. MDV32	48564117	ns	0.0698	MDV32
HVT US2 vs. MDV38.5	48335467	ns	0.0728	MDV38.5
HVT US2 vs. gb	47240754	ns	0.0888	gb
HVT US2 vs. MDV-17	47054713	ns	0.0918	MDV-17

HVT US2 vs. MDV-24	46833777	ns	0.0954	MDV-24
HVT US2 vs. MDV33	46080463	ns	0.1087	MDV33
HVT US2 vs. MDV5.2/76.8	45721640	ns	0.1156	MDV5.2/76.8
HVT US2 vs. MDV92.8	45290987	ns	0.1242	MDV92.8
HVT US2 vs. MDV30	44972837	ns	0.1309	MDV30
HVT US2 vs. MDV92	44720797	ns	0.1365	MDV92
HVT US2 vs. MDV-12.4	44443387	ns	0.1429	MDV-12.4
HVT US2 vs. MDV5.6/75.9	43487157	ns	0.1663	MDV5.6/75.9
HVT US2 vs. MDV5.3/76.4	42887573	ns	0.1824	MDV5.3/76.4
HVT US2 vs. MDV73	42830853	ns	0.1839	MDV73
HVT US2 vs. MDV8	42654240	ns	0.189	MDV8
HVT US2 vs. MDV-12.8	42021647	ns	0.2078	MDV-12.8
HVT US2 vs. MDV84/100	41448547	ns	0.2261	MDV84/100
HVT US2 vs. MDV6.3/75.5	41113110	ns	0.2374	MDV6.3/75.5
HVT US2 vs. MDV-12	40412243	ns	0.262	MDV-12
HVT US2 vs. MDV92.4	39859123	ns	0.2828	MDV92.4
HVT US2 vs. MDV41	39237870	ns	0.3074	MDV41
HVT US2 vs. MDV90	38806833	ns	0.325	MDV90
HVT US2 vs. MDV34	37995613	ns	0.3606	MDV34
HVT US2 vs. MDV-29	36956110	ns	0.4094	MDV-29
HVT US2 vs. MDV44	36512227	ns	0.4314	MDV44
HVT US2 vs. MDV31.5	35509267	ns	0.4834	MDV31.5
HVT US2 vs. MDV86.6	35119873	ns	0.5041	MDV86.6

HVT US2 vs. MDV43	34967897	ns	0.5124	MDV43
HVT US2 vs. MDV48	32745717	ns	0.6382	MDV48
HVT US2 vs. MDV6/75.3 /6.1/75	32726830	ns	0.6394	MDV6/75.3 /6.1/75
HVT US2 vs. MDV5.7/75.8	32348867	ns	0.6616	MDV5.7/75.8
HVT US2 vs. MDV40	32190060	ns	0.6709	MDV40
HVT US2 vs. MDV88	32118817	ns	0.6753	MDV88
HVT US2 vs. MDV35	30434270	ns	0.7712	MDV35
HVT US2 vs. MDV-10	30140163	ns	0.7871	MDV-10
HVT US2 vs. MDV5.5/75.91	29940820	ns	0.7978	MDV5.5/75.91
HVT US2 vs. MDV5.4/75.92	29849603	ns	0.8029	MDV5.4/75.92
HVT US2 vs. MDV6.4/75.4	28657580	ns	0.8617	MDV6.4/75.4
HVT US2 vs. MDV5.8/75.7	28552100	ns	0.8666	MDV5.8/75.7
HVT US2 vs. MDV6.6/75.1	28461757	ns	0.8708	MDV6.6/75.1
HVT US2 vs. MDV47	28077220	ns	0.8874	MDV47
HVT US2 vs. MDV49.5	27655240	ns	0.9042	MDV49.5
HVT US2 vs. MDV83/101	27554967	ns	0.9081	MDV83/101
HVT US2 vs. MDV87	27199940	ns	0.9206	MDV87
HVT US2 vs. MDV-15	27029127	ns	0.9261	MDV-15
HVT US2 vs. MDV45	26735707	ns	0.9352	MDV45
HVT US2 vs. MDV50.5	26730717	ns	0.9353	MDV50.5
HVT US2 vs. MDV55	26576120	ns	0.9401	MDV55
HVT US2 vs. MDV49	26361200	ns	0.9469	MDV49
HVT US2 vs. MDV7/74	25671700	ns	0.9605	MDV7/74

HVT US2 vs. MDV-16	24729023	ns	0.9767	MDV-16
HVT US2 vs. MDV89/89.5	24666667	ns	0.9769	MDV89/89.5
HVT US2 vs. pp38	24633383	ns	0.977	pp38
HVT US2 vs. MDV36	24303847	ns	0.9779	MDV36
HVT US2 vs. MDV-15.5	23725427	ns	0.9792	MDV-15.5
HVT US2 vs. MDV81.5/102.5	- 22000000	ns	0.9829	MDV81.5/102.5
HVT US2 vs. MDV-11	21884570	ns	0.9832	MDV-11
HVT US2 vs. MDV-1/80	21652753	ns	0.9838	MDV-1/80
HVT US2 vs. MDV-14.5	20666667	ns	0.9913	MDV-14.5
HVT US2 vs. MDV86.2/86.4/97.3/97.6	20333333	ns	0.9915	MDV86.2/86.4/ 97.3/97.6
HVT US2 vs. MDV63.5	18600840	ns	0.998	MDV63.5
HVT US2 vs. MDV96	18486573	ns	0.998	MDV96
HVT US2 vs. MDV95.5	18275353	ns	0.9981	MDV95.5
HVT US2 vs. MDV59	17373677	ns	0.9982	MDV59
HVT US2 vs. MDV66	16666667	ns	0.9983	MDV66
HVT US2 vs. MDV68	16582460	ns	0.9983	MDV68
HVT US2 vs. MDV70	16973893	ns	0.9983	MDV70
HVT US2 vs. MDV94.5	16333333	ns	0.9984	MDV94.5
HVT US2 vs. MDV9.5/73.4	15666667	ns	0.9985	MDV9.5/73.4
HVT US2 vs. MDV65	15479397	ns	0.9985	MDV65
HVT US2 vs. MDV67	15488503	ns	0.9985	MDV67
HVT US2 vs. MDV53	14666667	ns	0.9986	MDV53
HVT US2 vs. MDV72.4	14137877	ns	0.9986	MDV72.4
HVT US2 vs. meq	14202893	ns	0.9986	meq

HVT US2 vs. MDV3/78	- 13666667	ns	0.9987	MDV3/78
HVT US2 vs. MDV46	13679880	ns	0.9987	MDV46
HVT US2 vs. MDV72.6 / MDV72.8	14040540	ns	0.9987	MDV72.6 / MDV72.8
HVT US2 vs. MDV82/102	14000000	ns	0.9987	MDV82/102
HVT US2 vs. MDV-2/79	- 13000000	ns	0.9988	MDV-2/79
HVT US2 vs. MDV3.6/78.2	13000000	ns	0.9988	MDV3.6/78.2
HVT US2 vs. MDV96.5	13000000	ns	0.9988	MDV96.5
HVT US2 vs. MDV63	11333333	ns	0.999	MDV63
HVT US2 vs. MDV69	11666667	ns	0.999	MDV69
HVT US2 vs. MDV91.5	11125643	ns	0.999	MDV91.5
HVT US2 vs. MDV-14	10000000	ns	0.9991	MDV-14
HVT US2 vs. MDV84.5/99.5	- 10000000	ns	0.9991	MDV84.5/99.5
HVT US2 vs. 85.6/85.9/98.3/98.6	10666667	ns	0.9991	85.6/85.9/98.3/98.6
HVT US2 vs. MDV93	10317933	ns	0.9991	MDV93
HVT US2 vs. MDV95	10333333	ns	0.9991	MDV95
HVT US2 vs. MDV3.4/78.3	-9333333	ns	0.9992	MDV3.4/78.3
HVT US2 vs. MDV8.8	8466167	ns	0.9993	MDV8.8
HVT US2 vs. MDV94	8000000	ns	0.9993	MDV94
HVT US2 vs. MDV3.8/78.1	-7000000	ns	0.9994	MDV3.8/78.1
HVT US2 vs. MDV8.4	7666667	ns	0.9994	MDV8.4
HVT US2 vs. MDV51	7666667	ns	0.9994	MDV51
HVT US2 vs. MDV56	-6666667	ns	0.9994	MDV56
HVT US2 vs. MDV60	7333333	ns	0.9994	MDV60

HVT US2 vs. MDV61	7059140	ns	0.9994	MDV61
HVT US2 vs. MDV85/99	-7000000	ns	0.9994	MDV85/99
HVT US2 vs. MDV-2.6/78.5	-6333333	ns	0.9995	MDV-2.6/78.5
HVT US2 vs. MDV71.8	6333333	ns	0.9995	MDV71.8
HVT US2 vs. MDV57.4	-5666667	ns	0.9996	MDV57.4
HVT US2 vs. MDV62	5726780	ns	0.9996	MDV62
HVT US2 vs. MDV86.1	5666667	ns	0.9996	MDV86.1
HVT US2 vs. MDV91	-4666667	ns	0.9996	MDV91
HVT US2 vs. MDV3.2/78.4	-3000000	ns	0.9997	MDV3.2/78.4
HVT US2 vs. MDV58	4333333	ns	0.9997	MDV58
HVT US2 vs. MDV71	4333333	ns	0.9997	MDV71
HVT US2 vs. MDV71.4	3333333	ns	0.9997	MDV71.4
HVT US2 vs. MDV81/103	-3000000	ns	0.9997	MDV81/103
HVT US2 vs. MDV86/98	3666667	ns	0.9997	MDV86/98
HVT US2 vs. positive control	-4000000	ns	0.9997	positive control
HVT US2 vs. MDV9	2333333	ns	0.9998	MDV9
HVT US2 vs. MDV50	2666667	ns	0.9998	MDV50
HVT US2 vs. MDV57	2000000	ns	0.9999	MDV57
HVT US2 vs. MDV0.5/80.5	78250800	****	<0.0001	MDV0.5/80.5
HVT US2 vs. negative control	132989047	****	<0.0001	negative control
HVT US2 vs. MDV-2.3/78.6	1000000	ns	>0.9999	MDV-2.3/78.6
HVT US2 vs. MDV52	-666667	ns	>0.9999	MDV52
HVT US2 vs. MDV54	-333333	ns	>0.9999	MDV54

HVT US2 vs. MDV57.8	-1000000	ns	>0.9999	MDV57.8
HVT US2 vs. MDV64	-1011830	ns	>0.9999	MDV64
HVT US2 vs. MDV72.2	-1000000	ns	>0.9999	MDV72.2
HVT US2 vs. MDV72	1000000	ns	>0.9999	MDV72
HVT US2 vs. MDV85.3/98.9	1000000	ns	>0.9999	MDV85.3/98.9

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