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SINGAPORE

**FUNCTIONAL STUDIES OF VIRAL AND HOST CELL
FACTORS INVOLVED IN THE REGULATION OF
CORONAVIRUSES REPLICATION AND PATHOGENESIS**

NG YAN LING

SCHOOL OF BIOLOGICAL SCIENCES

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FACTORS INVOLVED IN THE REGULATION OF
CORONAVIRUS REPLICATION AND PATHOGENESIS**

NG YAN LING

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfillment of the requirement for
the degree of Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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PROF JAMES P TAM

Authorship Attribution Statement

This thesis contains material from 2 paper(s) published in the following peer-reviewed journal(s) where I was the second author.

Chapter 1 was published as Liu, D.X., Ng, Y.L., & Fung, T.S. (2019). Coronaviruses as Vaccine Vector for Veterinary Pathogens. In Vanniasinkam, Tikoo & Samal (Ed.), *Viral Vectors in Veterinary Vaccine Development*. Springer Nature. (In press) and Liu, D.X., Ng, Y.L., & Fung, T.S. (2019). Infectious Bronchitis Virus. In S. K. Samal (Ed.), *Avian Virology: Current Research and Future Trends*. Caister Academic Press. <https://doi.org/10.21775/9781912530106.05> (In press)

The contributions of the co-authors are as follows:

- Prof Liu Ding Xiang provided the initial book chapter direction and edited the manuscript drafts.
- Ng Yan Ling prepared the manuscript drafts. The manuscript was revised by Prof Liu Ding Xiang and Dr. Fung To-Sing.

... /6.08.2019 ...
Date

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NG YAN LING

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ABBREVIATIONS

2'-O-MTase	2'-O-methyltransferase
3CLPro	3C-like protease
3Ecto	zinc-finger domain
5-FU	5-fluorouracil
aa	amino acids
ACE2	angiotensin-converting enzyme 2
AnCoV	Sable antelope coronavirus
APN	aminopeptidase N
ATM	ataxia-telangiectasia mutated
ATR	Rad3-related
BACs	bacterial artificial chromosomes
BCoV	Bovine CoV
BGH	bovine growth hormone
BL	blocking loop
BSL	bulged stem-loop
BtCoV	Bat coronavirus
BuCoV	Bulbul coronavirus
CRCoV	Canine respiratory coronavirus
CAM	chorioallantoic membrane
CEACAM1	carcinoembryonic antigen-related adhesion molecule 1
CiCoV	Civet Severe acute respiratory syndrome CoV
CMCoV	Common moorhen coronavirus
CMV	cytomegalovirus
COVID-19	Coronavirus Disease 2019
CoVs	Coronaviruses
CPD	cyclic phosphodiesterase
CPSF	Cleavage and polyadenylation specificity factor
CstF	Cleavage Stimulatory Factor
CTD	C-terminal domain
DC-SIGN	dendritic cell-specific intracellular adhesion molecule-3-grabbing nonintegrin
DI	defective interfering
DMVs	double-membrane vesicles

DPP4	dipeptidyl peptidase 4
DrCoV	Dromedary camel coronavirus
E	envelope
ECoV	Equine coronavirus
EF1 α	elongation factor 1 α
eIF3F	eukaryotic initiation factor 3f
eIF4F	eukaryotic initiation factor 4f
EM	electron microscopy
ER	endoplasmic reticulum
ERGIC	endoplasmic reticulum-Golgi intermediate compartment
ExoN	exoribonuclease
FCoV	Feline CoV
FECV	feline enteric CoV
FIPV	feline infectious peritonitis virus
FPV	fowlpox virus
GCoV	Giraffe coronavirus
gRNAs	genomic RNAs
HCoV	Human coronavirus
HDV	hepatitis delta virus
HE	hemagglutinin-esterase
HeCoV	Hedgehog coronavirus
HEF	HE fusion protein
Hel1	Superfamily 1 helicase
HMW	high molecular weight
hnRNP-A1	heteronuclear ribonucleoprotein
HR	heptad repeat regions
HR	Homologous RNA Recombination
HVR	hypervariable region
H δ R	hepatitis delta virus ribozyme
IB	Infectious bronchitis
IBV	Infectious bronchitis virus
ICTV	International Committee on Taxonomy of Viruses
IFN	interferon
ISG15	interferon-stimulated gene 15
ISGs	IFN-stimulated genes

kb	kilobases
LC3	light chain 3
LKR	linker region
LMW	low molecular weight
L-SIGN	liver/lymph node-specific intracellular adhesion molecule-3-grabbing nonintegrin
M	membrane
MAVS	mitochondrial activator of virus signaling
MCS	multiple cloning site
MDA-5	melanoma differentiated-associated gene
MERS-CoV	Middle East respiratory syndrome CoV
MHV	Mouse hepatitis virus
mIBV	murinized IBV intermediate
MiCoV	Mink coronavirus
M _{pro}	main protease
MuCoV	Munia coronavirus
N7-MTase	N7-methyltransferase
NendoU	nidoviral uridylate-specific endoribonuclease
NHCoV	Night heron coronavirus
NS6	non-structural 6
NS7	non-structural 7
NSP3	non-structural protein 3
NSP	non-structural protein
NTD	N-terminal domain
OB	oligosaccharide binding
oct	conserved coronavirus octanucleotide motif
ORFs	Open reading frames
PAS	Polyadenylation signal
PCoV	Porcine coronavirus
PdCoV	porcine deltacoronavirus
pDI-C	plasmid containing a cDNA encoding TGEV-derived DI
PEDV	Porcine epidemic diarrhea virus
PHB	prohibitin
PHEV	Porcine hemagglutinating encephalomyelitis virus
PK	pseudoknot

PL2 _{pro}	papain-like protease 2
PL _{pro}	papain-like protease
pp	polyproteins
PP1	protein phosphatase 1
PRCV	Porcine respiratory coronavirus
PRRs	pattern recognition receptors
RabCoV	Rabbit coronavirus
RatCoV	Rat coronavirus
RBD	receptor binding domain
RdRP	RNA-dependent RNA polymerase
RES	restriction enzyme site
rIBV	recombinant IBV
RIG-I	retinoic acid-inducible gene I
RLRs	(RIG-I)-like receptors
RNP	ribonucleoprotein
RTC	replicase-transcriptase complex
RT-PCR	reverse transcription PCR
SARS-CoV	Severe acute respiratory syndrome CoV
SdCoV	Sambar deer coronavirus
SER	smooth endoplasmic reticulum
SF	superfamilies
sgRNAs	sub-genomic RNAs
SL	stem-loop
TGEV	Transmissible gastroenteritis virus
ThCoV	Thrush coronavirus
TM	transmembrane
tRNAs	transfer RNAs
TRS	transcription regulatory sequence
TRS-L	leader TRS
ts	temperature-sensitive
Ubl1	ubiquitin-like domain 1
Ubl2	ubiquitin-like domain 2
Upf1	Up-frameshift 1
UTRs	untranslated regions
VLPs	virus-like particles

VPg	viral protein genome-linked
WBkCoV	Waterbuck coronavirus
WCL	whole cell lysate
WECov	Wigeon coronavirus
WiCoV	White-eye coronavirus
WtDCoV	White-tailed deer coronavirus
XPB1	X box-binding protein 1
XPB1S	spliced XPB1
XPB1U	unspliced XPB1
ZBD	Zinc Binding Domain

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PUBLICATIONS

1. Liu, D.X., **Ng, Y.L.**, & Fung, T.S. (2019). Coronaviruses as Vaccine Vector for Veterinary Pathogens. In Vanniasinkam, Tikoo & Samal (Ed.), *Viral Vectors in Veterinary Vaccine Development*. Springer Nature. (In press)
2. Liu, D.X., **Ng, Y.L.**, & Fung, T.S. (2019). Infectious Bronchitis Virus. In S. K. Samal (Ed.), *Avian Virology: Current Research and Future Trends*. 133-178. Caister Academic Press. <https://doi.org/10.21775/9781912530106.05>
3. Lim, X.Y., **Ng, Y.L.**, Tam, J.P., Liu, D.X. (2016). Human Coronaviruses: A Review of Virus-Host interactions. *Diseases*. 4(3), 26. <https://doi.org/10.3390/diseases4030026>

ABSTRACT

Upper respiratory tract infections can be caused by a variety of viruses, such as rhinoviruses, influenza viruses, and respiratory syncytial virus. Of these, coronavirus (CoV, *Coronaviridae*, *Nidovirales*) is an important upper respiratory tract pathogen that infects both humans and livestock. It is responsible for the Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), Middle East Respiratory Syndrome coronavirus (MERS-CoV) and Coronavirus Disease 2019 (COVID-19) outbreaks that resulted in human cases with varying clinical outcomes, from minor upper respiratory tract infections to fatalities. Despite its medical importance, Coronavirus-host interactions that lead to increased mammalian infectivity and pathogenicity remain mostly enigmatic. In this study, two host factors identified from siRNA screening: RNA (Guanine-7-) Methyltransferase (RNMT) and Cleavage and Polyadenylation Factor I Subunit 1 (CLP1) are functionally characterized to better understand their contribution to Infectious Bronchitis Virus (IBV) replication and pathogenesis, through the generation of CRISPR/Cas9 mediated gene knockout (KO) cell lines. Here, we show that RNMT and CLP1 are enhancers in IBV infection. Total IBV genomic and subgenomic RNA expressed across multiple post-infection time points over 24 hours were under-expressed by at least 50% in RNMT^{-/-} and CLP1^{-/-} cells, compared to wild-type H1299 cells. Wild-type RNMT and CLP1^{-/-} overexpression further confirmed its role as an enhancer in H1299 cells, with an average 2-fold increase in viral subgenomic RNA, protein expression, and viral titer within 24 hours (RNMT p-value: 0.0526; CLP1 p-value: 0.014). Continuous passage of wild-type IBV in RNMT- and CLP1- KO cells showed adaptation of the virus to the mutant cells. Sequencing of RNMT- and CLP1-adapted IBV revealed several point mutations in IBV non-structural proteins (NSPs) 3, 5, 15, and 16, with a unique tri-nucleotide deletion in the NSP5 of CLP1-adapted IBV. Our findings are a promising start to co-immunoprecipitation assays to determine the virus-host interface in CoV replication. These findings would shed light on the signaling pathways and immunoevasion mechanisms utilized by CoVs to interact with these host factors, which may initiate new research directions in understanding CoV-host interactions.

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION TO CORONAVIRUSES

1.1.1 History of Coronaviruses

Early records of diseases caused by coronaviruses (CoVs) date back to 1931 in North Dakota, United States. Known as Infectious Bronchitis (IB), it was described as “an apparently new respiratory disease of baby chicks” when baby chicks between two days to three weeks old infected with the disease were found to be listless and gasping (1). IB was contagious and mortality rates were as high as 90%. Mucus exudate found in the trachea and bronchi of infected chicks’ during post-mortem suggested that the disease was transmissible through contact exposure or bronchial exudate transfer to healthy chicks. However, the viral nature of IB was established two years later, when Bushnell and Brandly reported on similar cases in chickens (2). The prevailing dogma had been bronchitis in chickens was caused by bacteria, but bacterial and toxin origins of the disease were ruled out as the causative agent responsible for this new disease was filterable. This was a paradigm-shifting discovery.

Since then, different CoVs have been discovered, and the symptoms they cause can extend beyond the respiratory system (**Table 1-1**). Following the isolation of prototype murine CoV strain JHM in 1949 (3, 4), diverse CoVs have been identified, highlighting the wide distribution of CoVs across mammals and birds. Certain animal CoVs, such as bovine CoV (BCoV) and infectious bronchitis virus (IBV), continue to persist in the poultry industry today.

In the 1960s, virologists working on human and animal viruses began to perform electron microscopy on organ culture fluids infected with IBV, MHV, Transmissible gastroenteritis virus (TGEV), and human CoVs. In all cases, the virion particles were pleomorphic, found to be between 80-150nm and decorated with crown-like projections (5-9). Later, this new group of viruses became known as CoVs (corona denoting the reminiscence of a solar corona), and was officially recognized as a new genus in 1971 (10).

Over the next thirty years, CoVs have been intensively studied mainly due to the economic burden of disease in domestic animals and its function as a viral pathogenesis disease model. CoV research surged in 2002, following the emergence of the severe acute respiratory syndrome CoV (SARS-CoV) outbreak. Started in the Guangdong province of China, SARS-CoV eventually infected 8,096 people and resulted in a 10% mortality worldwide (11). A decade later in 2012, the Middle East respiratory syndrome CoV (MERS-CoV) resulted in an outbreak with mortality rate of 35%. In 2020, the Coronavirus Disease 2019 (COVID-19) epidemic caused by the 2019 novel coronavirus (2019-nCoV) has resulted in over 75,000 confirmed cases, surpassing that of SARS and MERS. These high mortality rates highlighted the prospect for CoVs circulating among animal species to be transmitted to humans. This prompted the need to understand their replication mechanisms to identify novel drug targets that can be developed against CoVs. Today, new CoV species continue to be discovered in rats (12), bats (13, 14), and birds (15, 16), although most of them are only known as genomic sequences and have yet to be isolated or propagated *in vitro*.

1.1.2 Coronavirus Taxonomy

CoVs are taxonomically classified under the *Nidovirales* order, one of the biggest group of positive-sense single-stranded (ssRNA+) viruses. The order *Nidovirales* comes from the Latin word *nidus*, for nest (17), as nidoviruses produce a 3' co-terminal nested set of subgenomic mRNAs (sgRNAs) during infection (18). Nidoviruses can be distinguished from other ssRNA+ viruses based on two characteristics: 1) the organization and composition of the multidomain replicase gene located 5' two-thirds of the viral RNA genome and 2) the presence of multinuclear zinc-binding domain (ZBD) and the uridylyate-specific endoribonuclease (NendoU) domain in replicase ORF1b (19).

CoVs were traditionally classified into four genera based on phylogenetic clustering inferred from concatenated multiple sequence alignments of 3C-like protease (3CLPro) (19). However, extensive changes were made to the order in the 2018 International Committee on Taxonomy of Viruses (ICTV) taxonomy, in which nidoviruses were reorganized into seven suborders (**Figure 1-1**). The *Coronaviridae* family is now under

the suborder of *Cornidovirineae* and is further divided into two subfamilies, *Letovirinae* and *Orthocoronavirinae*. The introduction of a new subgenus rank also redistributes the *alpha*-, *beta*-, *delta*- and *gamma*CoVs across 12, 5, 4 and 2 subgenera respectively. As ICTV established stringent criteria in defining CoV species in a manner consistent with other families, feline CoV, canine CoV, and TGEV were merged as one single species, known as *alphacoronavirus 1* in 2009 (20).

Almost all *alpha*- and *beta*CoVs have mammalian hosts, except for *gamma*- and *delta*CoVs, which are isolated mainly from avian hosts. Several CoVs listed in **Figure 1-1** have been studied extensively for decades, such as murine CoV, avian CoV, alphaCoV 1, and betaCoV 1 as they are amenable to isolation and growth in tissue culture.

Table 1-1: Clinical characteristics of CoVs.

Virus	Host Species	Transmission	Clinical Disease
<i>AlphaCoVs</i>			
FCoV	Cat	Fecal-oral route	Gastroenteritis
TGEV	Pig	Fecal-oral route	Gastroenteritis, diarrhea, vomiting, dehydration
HCoV-229E	Human	Aerosols Fomite	Common cold, pneumonia, bronchiolitis
<i>BetaCoVs</i>			
HCoV-OC43	Human	Aerosols Fomite	Common cold, pneumonia
MERS-CoV	Human	Aerosols Oronasal secretions	Pneumonia
MHV	Mouse, Rat	Aerosols Direct contact	Enteritis, hepatitis, demyelination, encephalitis
SARS-CoV	Human	Aerosols Oronasal secretions	Pneumonia
<i>GammaCoVs</i>			
IBV	Chicken	Aerosols Fecal-oral route	Bronchitis, nephritis
TuCoV	Turkey	Aerosols Fecal-oral route	Gastroenteritis
<i>DeltaCoVs</i>			
PDCoV	Pig	Fecal-oral route	Gastroenteritis

The following abbreviations are used: FCoV, Feline coronavirus; TGEV, Transmissible gastroenteritis virus; HCoV-229E, Human coronavirus 229E, HCoV-OC43, Human coronavirus organ culture 43; MERS-CoV, Middle East respiratory syndrome coronavirus; MHV, Mouse hepatitis virus; SARS-CoV, Severe acute respiratory syndrome coronavirus; IBV, infectious bronchitis virus; TuCoV, Turkey coronavirus; PDCoV, Porcine deltacoronavirus

1.1.3 Nomenclature and Morphology

Under negative contrast electron microscopy (EM), CoV virions appear roughly spherical and pleiomorphic (**Figure 1-2**). Virions are reported to have an average diameter of 80 to 150nm, although extreme sizes from 50nm to 200nm are noted in older literature (21, 22). The virion surface is covered with a fringe of 20nm crown-like surface projections consisting of trimers of spike (S) glycoprotein. In some *beta*CoV, there exists a second protein projection on the surface, the homodimeric hemagglutinin-esterase (HE) glycoprotein, which measures about 5-7nm in length (23, 24). When studied using electron cryotomography, coronavirus virions are homogenous in size, with an envelope diameter of $85 \pm 5\text{nm}$ (25). In the same study, it is reported that the viral envelope is almost twice in thickness ($7.8 \pm 0.7\text{nm}$) compared to biomembranes (25). On the virion interior, the nucleocapsid (N) protein is extensively folded onto itself to form a compact structure that follows the envelope curvature at 4nm (25).

Figure 1-2 Coronavirus morphology. A representative electron micrograph image of Middle East respiratory syndrome coronaviruses (MERS-CoV). The structural proteins of coronaviruses comprise of spike, membrane, nucleocapsid, and envelope protein. The molecular weight of each structural protein monomer is shown. An infectious bronchitis virus virion is 82nm. Image: Cynthia Goldsmith/Maureen Metcalfe/Azaibi Tamin, from <https://www.cdc.gov/coronavirus/mers/photos.html>.

1.1.4 Genome Structure and Organization

CoV possess the most complex and the largest replicating RNA genome among known RNA viruses, rivaled only by the okavirus of the *Roniviridae* family. With genome sizes typically ranging between 26 to 41 kb, this large RNA genome poses a significant burden on the virus as it is more likely to accumulate replication errors when compared to its ssRNA+ counterparts with a smaller RNA genome. To circumvent deleterious effects brought about by RNA replication, RNA viruses, including CoVs, adopt several mechanisms to preserve the integrity of its genome. These can include 1) RNA recombination events (26), 2) relying on the proofreading capability of its 3'-to-5' exoribonuclease activity (ExoN) (found in non-structural protein 14 (NSP14) of CoVs) (27, 28), 3) promoting replicase fidelity and 4) mechanisms to repair the genome termini (29). Despite its high frequency of RNA recombination events (~25% across the entire genome) (30, 31), CoVs' genome organization remains well conserved, occurring in the invariant order 5'-*replicase*-S-E-M-N-3' (**Figure 1-3**). Also found at the distal ends of the CoV genome are 5' (~200-600 nt) and 3' (~200-500 nt) untranslated regions (UTRs) (32-34). These UTRs fold into a set of secondary RNA structures that direct virus RNA synthesis and translation (**Figure 1-4**) (35).

Figure 1-3 The genome organization of infectious bronchitis virus (IBV). The IBV viral RNA genome is 27.6 kilobases (kb) long and is arranged in an invariant order of 5'-rep 1a-rep 1b-S-3a-3b-E-M-5a-5b-N-3'.

The CoV genome functions as 1) a mRNA template for the initial translation of *replicase* gene upon entry into the host cell; 2) a substrate for assembly and packaging into a virion particle and 3) a genomic RNA (gRNA) for replication and transcription. As with all positive-sense RNA virus, CoV mRNAs contain a cap at the 5' termini of its viral genomic and sgRNAs (36) and a 3' termini polyadenylated tail (34). The *replicase-transcriptase* constitutes 5' two-thirds of the virus genome and is the only gene directly

translated from the genomic RNA upon virus entry, while downstream open reading frames (ORFs) products are derived from sgRNAs.

In the last third of the CoV genome, clusters of group-specific accessory genes can be found dispersed between the structural genes. These genes are hypothesized to be acquired through horizontal gene transfers from cellular or heterologous viral RNAs and can be highly diverged among various subgroups of CoVs, particularly within the S-E intergenic region (37). Interestingly, some accessory genes are partially embedded within another gene with an alternate reading frame, such as the *internal* (*I*) gene found in MHV (38). These accessory genes are generally dispensable for replication, but they are retained in the virus genome under selective pressure, indicating an essential role for *I* gene in viral virulence and pathogenesis (39-42).

1.1.5 Coronavirus Structural Proteins

CoVs all share a canonical set of four structural proteins: spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins. Except for the N protein, which is found in the ribonucleoprotein core, all structural proteins can be found on the virion surface. Also, some *BetaCoVs* also possess a fifth structural protein known as hemagglutinin-esterase (HE). The function of each structural protein and accessory genes are summarized in **Table 1-2**.

Table 1-2: Functions of structural and accessory proteins of IBV Beaudette.

	Function	PDB ID	CoV	Ref
Structural Proteins				
S	Receptor binding and membrane fusion	6CV0	IBV	(43)
M	Virus Assembly			
E	Virus Assembly	5X29	SARS-CoV	(44)
N	Complexes with viral genomic RNA	2CA1	IBV	(45, 46)
	Interacts with the viral membrane protein during virion assembly	2BXX		
	Enhancing the efficiency of virus transcription and assembly			
Accessory Proteins				
3a	Unknown	-	-	
3b	Unknown	-	-	
5a	Unknown	-	-	
5b	Delay interferon activation	-	-	

1.1.5.1 S Protein

The CoV S protein is a class I fusion protein composed of three segments: a sizeable N-terminal ectodomain, a single-pass transmembrane anchor, and a small C-terminal endodomain (47) (**Figure 1-5**). These S proteins are synthesized as a single-chain

precursor and trimerize upon folding to form the crown structure on the CoV surface and mediates host receptor binding and subsequent membrane fusion events (48). The ectodomain is composed of two subunits - the receptor binding S1 and a membrane fusion S2, which are roughly similar in size. The IBV S monomer is 200 kDa and is heavily N-glycosylated (47, 49). This glycosylation occurs co-translationally and plays a significant role in assisting S monomer folding and oligomerization before trimerization (50). In IBV, there are as many as 29 putative N-glycosylation sites, of which 8 of them have been confirmed by mass spectrometry (51). Glycosylation of the S protein at different residues may differentially affect folding, cleavage and fusogenicity of the protein. Substitution of asparagine to aspartic acid or glutamine at N212 and N276 has been shown to abrogate fusogenicity of S protein, decreasing the infectivity of the recombinant viruses (51). N283, other the other hand, plays a role in IBV replication (51). Adjacent to the transmembrane region is a highly conserved cytoplasmic cysteine-rich region motif. In TGEV, this region is found to be palmitoylated (52). Although palmitoylation is dispensable for S-M interaction, it is imperative in virus-like particles (VLPs) formation (52).

S protein is a critical determinant of CoV tropism, pathogenicity, and host range (53). It adopts a convoluted receptor recognition pattern upon binding to its cognate host receptor following furin cleavage into S1 and S2 domains (54). There are two furin consensus motif in IBV, RRFRR(537)/S and RRRR(690)/S (55). Time-course experiments using a peptide furin inhibitor showed that RRRR/S motif is associated with the viral entry and syncytium formation (55). Other cellular proteases are also known to induce S protein cleavage, such as endosomal cathepsin proteases L during SARS-CoV entry (56). Recently, teicoplanin, a glycopeptide antibiotic, has been reported to inhibit cathepsin L of SARS- and MERS-CoV in the late endosome/lysosome, providing a novel target for antivirals and vaccines aimed at inhibiting virus entry (57).

Within the S1 domain are two functional subdomains: the N-terminal domain (NTD) which contains the receptor-binding domain (RBD) and a C-terminal domain (CTD), both of which are capable of binding to carbohydrate moieties (58-61) and

proteinaceous receptors respectively (61-63). Crystal structures for S1 NTD and CTD domain complexed with their respective receptor have been resolved for porcine respiratory coronavirus (PRCoV) (64), SARS-CoV (65), MERS-CoV (66), MHV (67) and human coronavirus (HCoV-NL63) (68), providing answers to many intriguing questions surrounding receptor recognition by CoVs. Recently, a cryo-EM study of IBV S1 domain confirmed the unique structural features of spikes among different CoV genera, thereby proposing that the evolutionary relationship of CoV spikes be in the order of alpha-, delta-, gamma-, and beta genera based on structural and functional comparisons (43). Although cryo-EM structures of porcine deltacoronavirus (PdCoV) and IBV spikes have also been reported, structural information reporting on the receptor-ligand complex is unavailable (43, 69, 70). In IBV M41 strain, the RBD of the S1 protein is mapped to N-terminal 253 residues, of which N38, H43, P63, and T69 are critical for binding to the respiratory tract of chickens (60). Surprisingly, cold-adapted IBV Beaudette strain to Vero cells is attributed to a single amino acid mutation, Q294L. Q294L is sufficient to obliterate cell-cell fusion and virion incorporation at non-permissive temperature (71). These results demonstrate how virus-host interaction may affect the infection outcome in chickens.

Within the S protein, S1 plays a significant role in viral attachment. Studies have shown that S1 alone is insufficient for binding to host tissues (72), but S2 contributes to the overall avidity and specificity of virus attachment and host range (73). In multiple sequence analysis of several CoV S1 sequences, while the S1 domain exhibited wide diversity among CoVs, the S2 domain remains relatively well conserved (43). CoV spike is formed by the association of heptad repeat regions (HR1 and HR2) of the S2 monomers to distance the bulb away from the viral membrane. Collectively, the fusion peptide, HR1, and HR2 are essential for viral envelope fusion upon receptor binding. Recombinant IBVs expressing chimeric Beaudette and M41-CK S proteins showed that S2 conferred IBV growth in Vero cells and is a major cellular tropism determinant (74). Specifically, the adaptation of IBV Beaudette in Vero cells was subsequently narrowed to a Beaudette-specific motif, (686)SRRKRS^{SL}IE(694) surrounding the S2' cleavage site (74).

At the cytoplasmic tail of IBV S lies a dilysine endoplasmic reticulum (ER) retrieval signal sequence (-KKXX-COOH) that functions to retain the climatic reporter protein (VSVG) in the ERGIC during assembly (75). Remarkably, a dibasic motif (-KKHXX-COOH) can also be found in the cytoplasmic tails of SARS-CoV and *alpha*CoVs, suggesting that intracellular targeting signaling may be involved to localize the S proteins near the virus assembly site (75). Despite the importance of this ER signal sequence, overexpressed S proteins were still retained intracellularly even though the dilysine motif was lacking (76). Instead, Y1143 was shown to be a crucial component of the retention signal (76). While a tyrosine independent signal is retained in TGEV S, it is obliterated in SARS-CoV through reverse genetics (77). Infectious cDNA clones lacking the tyrosine motif were irrecoverable, indicating that this endocytosis signal is indispensable for productive virus infection (78). S protein can also inhibit host translation in SARS-CoV and IBV through interactions with eukaryotic initiation factor 3f (eIF3F), suggesting that this interaction may regulate the expression of host genes induced during CoV infection (79).

Figure 1-5 Linear and folded representations of the coronavirus spike protein. The following abbreviations were used: SP, signal peptide; RBD, receptor-binding domain; FP, fusion peptide; HR, Heptad Repeat; TM, transmembrane domain.

1.1.5.2 M Protein

The CoV M (218-263 aa; 25-30 kDa) is a triple spanning, type III integral membrane protein that adopts either the N^{exo}/C^{endo} or the N^{exo}/C^{exo} topology in the viral envelope (**Figure 1-6**) (80). While N^{exo}/C^{endo} topology is adopted in most CoVs (81, 82), the N^{exo}/C^{exo} topology is also detected in a significant number of TGEV M molecules, suggesting the coexistence of both topologies may have relevance for viral function (83). The M protein N-terminal ectodomain is glycosylated in the Golgi apparatus by either N-linked glycans (in alpha- and gammaCoVs) (84, 85) or O-linked glycans (in betaCoVs) (86). Notably, M protein glycosylation status has resounding effects on tissue tropism and immunoevasion. In one study, recombinant MHV viruses carrying either an unglycosylated or N-linked glycan containing M protein is reported to have altered interferogenic capacity, with higher interferon (IFN) levels measured in viruses carrying an N-linked glycan while the unglycosylated M proteins induced IFN poorly and differentially affects their ability to infect the liver (87).

Figure 1-6 Linear and folded representations of the coronavirus membrane protein. TM, transmembrane domain.

Despite its small size, the M protein is present in large quantities on the virion surface, accounting for ~40% of the overall mass of a CoV virion (84). The M monomer is comprised of an N-terminal ectodomain (25 aa) and a C-terminal endodomain (81). Most M proteins, such as M proteins from IBV and BCoV, do not contain signal sequences (88). Structural analysis revealed that two M conformations give rise to membrane curvature. The first conformer, elongated M, displays a low range of membrane curvature and is associated with spike clustering, regulating the formation of a convex rigidified viral envelope while the second conformer, compact M, showed flexibility and low spike density (89).

On the other hand, the M protein endodomain organizes into a lattice formation through homotypic interactions (90, 91). This lattice-like matrix helped to ensure proper S and HE protein incorporation into the vacancies of the lattice to result in the regular positioning of the proteins in the lattice (92). M-N interactions were also found occurring near or at the MHV budding site, suggesting that M-N complex may be necessary for virus assembly (93).

Additionally, CoV M also cooperates with the E protein to drive VLP formation (94, 95). Besides its interactions with fellow M, N and E proteins, M proteins also interact with cellular factors to progress through its replication cycle. In one study, co-immunoprecipitation and immunofluorescence studies identified A159-K160 on the M protein to be essential for M's interaction to beta-actin, and mutations of these amino acids abolished the interaction in an infectious clone system (96). This observation is later supported by the enrichment of beta-actin in IBV virions (97). Recently, MERS-CoV M is reported to interact with TRAF3 adapter to suppress type I IFN expression, leading to diminished IRF3 activation (98).

1.1.5.3 E Protein

The CoV E is a small (74-109 aa; 8-12 kDa) homopentameric transmembrane found in limited quantities (~20 copies per virion) in the virion envelope (99). It is mainly hydrophobic, consisting of a hydrophobic domain (HD) and a charged cytoplasmic tail

(**Figure 1-7**) (100, 101). While the membrane topology of E protein remains unknown, two studies postulated E protein exists as either a single-pass transmembrane protein or a membrane hairpin (102, 103). The E protein is palmitoylated at key cysteine residues to act as a morphogenetic protein in CoV assembly (104-106). In IBV, a fully conserved proline residue can be found in its C-terminal tail, in addition to the 6-residue RDKLYS ER retention signal (94). This proline residue serves as a Golgi complex-targeting signal, evident from mutagenesis studies made to the proline residue and its predicted beta-strand (107, 108). Despite sharing structural similarities, the protein sequence of E protein diverges widely among CoVs genera with only 30% identity (99).

E proteins oligomerize to form cation-selective ion channels upon translation (109, 110). In a pivotal study, T16A and A26F mutation abrogate ion channel activity, as is the case with corresponding SARS-CoV N15A and V25F mutations (111). Surprisingly, E protein overexpression, and not the T16A mutant, have revealed to disrupt the intracellular trafficking of VSV-G and alters the Golgi-complex morphology (112). This phenomenon is later explained by the presence of IBV E protein in both low and high molecular weight (LMW/HMW) pools in infected cells and virions (113). At steady state, the virion-associated E protein resides mainly in the HMW pool and formed homo-oligomers. While the T16A mutant exists almost exclusively in the HMW pool, the A26F enrich in the LWM pool, explaining why A26F mutant does not support the formation of virus-like particles (112) nor abolished oligomer formation (111).

Figure 1-7 Linear and folded representations of the coronavirus envelope protein. TM, transmembrane domain.

The function of the E protein is to aid in the assembly, release, and morphogenesis of CoV virions. Its ability to function in multiple facets of the replication cycle relies on the physical interaction between its E and M proteins (94, 114). Their co-expression in TGEV (115), IBV (94, 95, 116) and MHV (117, 118) are sufficient to permit nucleocapsid independent VLP formation. However, the absolute requirement of E proteins during these processes vary depending on the virus genus, and its precise function remains to be defined. In one study, engineered E protein deletions from TGEV and MERS-CoV results in a progeny of replication-competent, propagation-defective virions (119, 120). Additionally, recombinant MHV virus lacking the E gene displayed low virus titer and small plaque formation though replication is permitted, implying that MHV E is dispensable for its replication (121). E gene depletion from SARS-CoV also resulted in diminished virus growth and particle formation (122). In contrast, recombinant IBV containing E gene deletions could not be recovered. Mutant IBVs containing a heterologous hydrophobic domain displays defective virus release and appears to accumulate intracellularly, exemplifying E protein's critical role in IBV replication and as a potential target in generating live attenuated vaccines (123). Additionally, MHV E mutants are shown to have aberrant morphology, demonstrating another pivotal role of E protein in CoV morphogenesis (124).

1.1.5.4 N Protein

The CoV N protein (349-470 aa; 43-50kDa) is the only structural protein that resides in the interior of the virion (**Figure 1-8**). N protein sequence comparisons in various CoVs revealed three distinct and highly conserved domains: the NTD, an RNA-binding domain, and a CTD (125).

All three domains have been reported by several studies to bind with viral RNA via different mechanisms (126-128). The NTD is both widely divergent in terms of sequences and length. In IBV, the NTD is found in amino acids 19-162 (46) while MHV NTD is mapped to amino acids 60-197 (129). In both cases, the N-termini of the NTD is associated with the 3' end of the gRNA through electrostatic interactions (45, 130). So far, the N protein structure has been resolved in IBV (45), SARS-CoV (131) and MHV (132). In IBV, X-ray structures of the NTD domain are U-shaped and are rich in

aromatic and basic residues for its interaction with RNA (45, 46). In particular, the finger-like β -hairpin is postulated to grip RNA through neutralizing its phosphate backbone with its positively charged residues, while the basic moieties from the hydrophobic arm make contact with the aromatic residues (45, 127). Additionally, Tyr94 and Arg76 have been identified through mutagenesis studies as the key residues critical for RNA-binding within the NTD of IBV N (133).

Connecting the NTD and CTD is an intrinsically disordered linker region (LKR) known as the SR region, owing to its serine- and arginine-rich tract (134). Despite being found in the N protein of all CoVs, the core-motif of the SR region is relatively variable (135). Transferring SR region within the same genus, such as from MHV to BCoV, is not allowed, indicating that this SR region participates in protein-protein interaction specific only for each virus (136). Additionally, the C-terminus of the SR region is implicated in protein-protein interaction and nuclear localization (137). Its localization to the nucleolus plays a significant role in promoting virus translation and assembly by sequestration of the ribosome and interrupting cell division (138). The SR proteins are essential for binding to RNA (139) and regulating RNA splicing and alternative splice site selection, indirectly playing a role in cell signaling through phosphorylation and

dephosphorylation (137, 140). So far, several putative N protein phosphorylation sites have been identified in SARS-CoV (141-143) and IBV (144). They have been shown to bind various viral and host proteins, including M proteins and heteronuclear ribonucleoproteins (hnRNP-A1) (126, 145).

Conversely, CTD is a helix rich domain comprised of mostly hydrophobic residues (45). This domain is reportedly conserved among CoVs, suggesting a crucial role for this domain (131). Notably, the CTD domain is also known as a dimerization domain, owing to its ability to self-associate into homo- and oligomers through domain-swapping mechanisms to exist in a more stable conformation (131, 146-150).

The N protein wraps CoV gRNA into nucleocapsids. Under electron microscopy, each nucleocapsid is 10-15nm in diameter and can be up to several 100nm in length (151). Nucleocapsid formation is critical for three purposes: 1) to protect the genome integrity, 2) to ensure timely replication of its viral genome, and 3) for transmission. However, this requires sequence-specific and non-specific forms of RNA binding. To this end, several RNA-binding substrates, such as the transcription regulatory sequence (TRS), acts as a binding site for the N protein, (130, 152, 153).

Besides its integral role in viral core formation, the N protein participates in genomic mRNA replication, viral RNA synthesis, assembly, and budding. In MHV, N proteins interact with non-structural protein 3 (NSP3) to mediate genomic RNA localization to the replicase-transcriptase complex (154). In virus assembly, the SARS-CoV N undergoes dimerization (147, 155) and associates with viral gRNA (153, 156, 157), in addition to other viral proteins and cellular membranes (90, 158). Following assembly, the N protein is shown to localize in the ER-Golgi complex in virus budding by microscopic techniques (159, 160). The accumulation of N proteins in SARS-CoV, IBV, and MHV have all resulted in an increase in VLPs and virion formation (106, 123, 161).

CoV N can induce changes in signal transduction and viral pathogenesis. In two separate studies, SARS-CoV and MHV N both reportedly interact with hnRNP-A1 to

direct a change from RNA synthesis to ribonucleoprotein complex formation (145, 162). The interaction between N protein CTD with elongation factor 1 α (EF-1 α) inhibits F-actin bundling to suppresses host protein translation and cytokinesis (163). Further, the N protein CTD is a critical IFN antagonist, as the N deletion mutant containing impaired RNA-binding resulted in a complete lost in IFN- β antagonist activity (164). Likewise, IBV N protein can activate host kinases ataxia-telangiectasia mutated (ATM) and Rad3-related (ATR) to phosphorylate the N protein at Thr378 and Ser379 (144).

1.1.5.5 HE Protein

The CoV HE (48-65kDa) is the fifth structural protein found only in a small group of *beta*CoVs. Under the electron microscope, HE forms a secondary set of 5-7nm inner projections beneath the canopy of S proteins peplomers (165-167). Like the M proteins, HE is an N-glycosylated class I membrane protein and adopts a N^{exo}/C^{endo} membrane topology. Comprised of an N-terminal signal peptide, ectodomain, transmembrane anchor, and an endodomain at the C-terminal, the mature HE protein is a homodimer stabilized by intra- and interchain disulfide bonds (168, 169). Intriguingly, the HE protein is not unique to CoVs – as sequence and structural pieces of evidence have reported a 30% sequence identity to the HE fusion protein (HEF) of influenza C viruses (170, 171), suggesting that heterologous RNA recombination may have occurred between CoVs and influenza C viruses during evolution. Based on phylogenetic data, the acquisition of HE by the *beta*CoV predecessor is likely to have taken place recently, following the split-off between the group 2 CoV and SARS-CoV (172, 173).

HE is dispensable for MHV replication. In one study, HE gene is lost in MHV strains after virus adaptation to tissue culture to genetic mutations, suggesting HE may only be an accessory protein not essential for viral replication (174). However, mice experimentally infected with HE-deficient MHV strains developed hepatitis and meningitis (175-177). However, upon intracranial inoculation of HE-expressing MHV strains, the HE-defective variants can be selected and isolated from the brain and spinal cord (178, 179).

While the function of HE remains enigmatic, HE expression appeared to provide a compelling advantage during infection. First, HE can promote virus spread and entry by serving as a cofactor of S protein to facilitate virus attachment to O-acetylated sialic acids, which are known co-receptors for several group 2 viruses (180-182). Furthermore, HE could prevent virion aggregation and virus travel through the extracellular mucosa (183). However, HE expression may come at a fitness cost, as demonstrated by Lissenberg and colleagues (184). In this study, three isogenic recombinant MHVs differing in their expression of HE was generated via targeted RNA recombination. Following cell culture adaptation, it was found that the HE genes were rapidly lost in wild type HE virus. Competition experiments with mixed infections showed that the loss of these HE genes is not due to enzyme inactivity as wild-type and enzymatically inactive HE both propagated with equal efficiencies. On examination using esterase-negative mutants arising from the wild-type HE virus, it showed that the loss of HE incorporation into virions correlated well with growth advantage of HE-deficient viruses (184). By contrast, in another study, the presence of HE greatly enhanced neurovirulence in mice regardless of its enzymatic status (185). These results imply that sialic-containing co-receptors can function to influence the course of MHV infection. Hence the expression of HE protein is a double-edge sword – it can be an advantage to the virus *in vivo*, but a burden *in vitro*.

1.1.6 Coronavirus Non-Structural Proteins

1.1.6.1 Accessory Proteins

Besides structural genes, CoV also encodes several other genes that have homologous versions within each genus but have no similarities in different groups, termed accessory genes. While the relevance of these proteins has not yet been fully established, these group-specific genes are not expressed by all CoVs and have been demonstrated to be expendable for virus growth *in vitro*. Nonetheless, many of these accessory proteins encoded by these genes could be vital for viral replication and virulence in their natural hosts.

1.1.6.1.1 *Alphacoronaviruses*

In *alpha*CoVs such as TGEV and feline infectious peritonitis virus (FIPV), an accessory gene cluster can be found in the S-E intergenic region and downstream of the N gene. TGEV contains ORF 3a, 3b, and 7, while FIPV contains 3a, 3b, 3c, 7a and 7b. Amino acid sequence analysis indicates that TGEV ORF 3a and 3b are highly similar to FIPV ORF 3a and 3c. In the TGEV Purdue strain, gene 3b is shown to produce a 31 kDa integral membrane protein implicated in viral pathogenesis (186). Conversely, gene 7 is reported to counteract host cell defenses and modulate virus virulence (187). Similarly, FIPV gene 3c is the first accessory gene associated with feline enteric CoV (FECV) to FIPV conversion, although the function of 3c remains enigmatic (188-190). In one study, transient expression of a full-length 3c gene localizes in the perinuclear region in macrophage-like Fcwf-4 cells (191). However, the 3c-defective FCoV replicates poorly in 3c-stable expression cells, indicating that 3c protein might have a virulence-suppressing effect as neither autophagy activation or inhibition managed to rescue viral replication in FCoV infected cats (191). Despite the important roles of these genes, ORF 3 and 7 deletions from the virulent FIPV strain 79-1146 produced attenuated viruses, implying that directed deletion of these group-specific genes may be a novel direction in live, attenuated vaccine development (39).

1.1.6.1.2 *Betacoronaviruses*

*Beta*CoVs have two accessory ORFs, 2a, 2b (encoding for HE) and two group-specific ORFs, 4 and 5a genes, located between ORF1b and S gene, and between the S and E gene respectively. In addition to these ORFs, there is also an *I* gene embedded within the N gene of several *beta*CoVs (192). In MHV, the hydrophobic 23 kDa *I* protein is incorporated into virus particles (38). As many *beta*CoVs carry the *I* gene, it is postulated that the *I* gene may play important biological roles in viral replication, exemplified through a study by Fischer and colleagues. In this study, the *I* protein is reported to be expendable for MHV replication, although its *I* gene deletion mutants generally formed smaller plaques (38). On the other hand, the SARS-CoV *I* gene, known as 9b protein, is found to mediate host immunoevasion by targeting a mitochondrial-associated adaptor molecule, MAVS (193). Deletion of SARS-CoV ORF 6-9 is found to have little effect on virus pathogenicity in a mouse model (194).

Conversely, the MHV and BCoV 2a proteins are expressed in the cytoplasm of infected cells (195). In BCoV, the 2a protein is a phosphoprotein and is believed to possess cyclic phosphodiesterase (CPD) activity based on structural homology of an enzyme involved in pre-tRNA splicing (172). Meanwhile, there is now compelling evidence that MHV 2a has CPD activity and cleaves the second messenger 2'5'-oligoadenylate synthetase (OAS) to counteract the OAS RNaseL pathway (196).

ORF 2b encodes for HE, an accessory structural protein (see **Section 1.1.5.5 HE Protein**). The ORF 4 is variable among members of *beta*CoVs. In MHV strains JHM and MHV-S, the gene 4 region consist of a single ORF (MHV-JHM: 15 kDa) (197) while MHV-A59, this is made up of two ORFs, 4a (MHV-A59: 2.2kDa) and 4b (MHV-A59: 11.7 kDa) (198). While the precise functions of ORF 4 gene products remain enigmatic, they are predicted to be an integral membrane protein.

1.1.6.1.3 *Gammacoronaviruses*

The prototypic *gamma*CoV, IBV, expresses two accessory genes termed ORF 3 and 5. Each ORF gives rise to two gene products: 3a and 3b, and 5a and 5b (199, 200). Among the four proteins, 3a and 3b proteins were derived from a functionally polycistronic sgRNA3 through leaky ribosome scanning, similar to the E proteins which are also generated through an internal ribosome entry site (199, 200). Amino acid sequence analysis showed that IBV 3a and 3b are well-conserved within *gamma*CoVs as similarities among field isolates are estimated to be 82.2% and 95% respectively (201). Generally, 3a protein becomes localized in the cytoplasm of infected or transfected cells either through a diffused pattern or in sharp puncta along the smooth endoplasmic reticulum (SER) (202). The transfected 3b protein is found to be localized in the nucleus instead of the cytoplasm, where most replication events take place in the mammalian cell (203).

Accumulating evidence demonstrates that viral replication in cultured cells does not require these IBV accessory proteins. Deletions in IBV ORF 3 and ORF 5, and the subsequent recovery of their resultant rIBVs have shown that 5b could delay IFN

activation and exhibit attenuation *in vitro* and *in vivo* (204). A recombinant live attenuated vaccine harboring IBV mutants with 3ab and 5ab deletions has been reported to show protection against IB in chickens (205), supporting previous reverse genetics system studies that also indicate 3b and 5 genes as non-essential for replication (206, 207). Shen and co-workers adapted an IBV Beaudette strain to monkey kidney Vero cells and found the emergence of an IBV mutant with a truncated 3b gene, suggesting that 3b is not evolutionarily conserved and is functionally dispensable for viral propagation (208).

1.1.6.1.4 Deltacoronaviruses

DeltaCoVs, which mainly infects domestic pigs, contain three accessory genes, known as non-structural 6 (NS6), non-structural 7 (NS7) (209) and a recently discovered NS7a (210). Ectopic expression of NS6 has significantly inhibited the production of Sendai virus-induced IFN- β and activation of IRF3 and NF- κ B (211). Surprisingly, NS6 interacts with RIG-I/MDA5 to elicit a reduction in RLR-mediated IFN- β production, weakening the binding of dsRNA (211).

Taken together, the precise role for CoV accessory proteins in the viral replication cycle, as evidenced in this section, is still a conundrum. A detailed analysis of the mechanism of action is warranted to provide an insight into the virus-host interactions at the molecular level and give clues to help elucidate viral pathogenesis.

1.1.6.2 Non-structural Proteins

The CoV non-structural proteins (NSPs) are composed of two huge polyproteins (pp), pp1a and 1ab, that are processed auto-proteolytically into 15 or 16 distinct NSPs by a virus-encoded protease (**Figure 1-9**). Collectively, these NSPs assemble to form the *replicase-transcriptase* complex (RTC) that is responsible for gRNA replication and transcription within the infected host cell.

1.1.6.2.1 NSP1

The CoV NSP1 is an IFN antagonist protein that ranges from 110 aa residues in *alpha*CoVs to 245 aa residues long in *beta*CoVs (212, 213). In contrast to *alpha*- and *beta*CoVs, the *gamma*- and *delta*CoVs genomes do not encode NSP1 (214). Nonetheless, studies aimed at exploring the biological functions of NSP1 have converged on its role as a chief pathogenicity factor (215). Experiments have shown that SARS-CoV NSP1 interferes with host transcription and translation by promoting mRNA degradation, including the expression of type I IFN (216, 217). Moreover, SARS-CoV NSP1 can circumvent the activation of the IFN-beta promoter involved in the host immune response (218). On the other hand, while TGEV NSP1 can also inhibit host mRNA translation, they are incapable of modifying or degrading host mRNAs (219). Recently, IBV is reported to induce host shutoff using accessory protein 5b, a functional equivalent of NSP1 (220).

1.1.6.2.2 NSP2

Unlike most CoVs that encode the IFN antagonist NSP1, the N-terminal of IBV begins with NSP2, an 87kDa protein of 673 residues long (221-223). While its exact function in IBV remains poorly defined, studies have shown that NSP1, NSP2, and NSP3 processing are regulated by PL1_{pro} (224). NSP2 deletion is expendable for MHV and SARS-CoV replication, although the mutant viruses exhibit an attenuated growth and RNA synthesis (225). While NSP2 is not necessary for coronaviral replication, genome-wide analysis for intraviral-protein interactions has revealed multiple interactions between NSP2 and other viral proteins in SARS-CoV, implying that NSP2 may be involved in virus-host interactions (226, 227).

1.1.6.2.3 NSP3

The CoV NSP3 is the largest protein encoded by the CoV genome. Averaging around 200 kDa, it comprises eight domains in all CoVs: the ubiquitin-like domain 1 (Ubl1), Glu-rich acidic domain (the “hypervariable region”), X domain (macrodomain), ubiquitin-like domain 2 (Ubl2), papain-like protease 2 (PL2_{pro}), zinc-finger domain (3Ecto), Y1 and CoV-Y, in addition to two transmembrane (TM) domains TM1 and

TM2 (228). The NSP3-TM domain mediates PL_{2pro} tethering for processing at the cleavage site and facilitates ER membrane association of cytosolic proteins (229). IBV genomic RNA is reported only to encode one PL_{pro}, which is similar to PL_{2pro} in other CoVs (230, 231), and is 1592 residues long (232). The structures of the papain-like protease proteins of MERS-CoV (233), SARS-CoV (234), TGEV (235) and IBV (236) have been resolved. The IBV PL_{pro} is largely reminiscent of SARS-CoV PL_{pro}, apart from the blocking loop (BL) 1 which is present in IBV and absent in SARS-CoV PL_{pro} (236). The catalytic triad of NSP3, namely Cys101, His264, Asp275, is conserved among CoVs and is regulated by the flexible BL 2 (236). NSP3 plays multiple roles in the viral life cycle. PL_{pro} auto-proteolytically cleaves at sites between NSP1/2, 2/3, and 3/4 (231, 232, 237). Besides its primary protease function, PL_{pro} also deubiquitinates and removes interferon-stimulated gene 15 (ISG15) in host cells to prevent activation of innate immune responses (238, 239).

1.1.6.2.4 NSP4

NSP4 is an integral membrane protein composed of four TM helices and a cytosolic C-terminal domain (240). At 514 aa residues long, the IBV NSP4 is the only NSP that is released upon cleavage by PL_{pro} at the N-terminal and main protease (M_{pro}) at the C-terminal. Engineered deletion and mutation on different helices and C-terminal of MHV NSP4 have reported that only the NSP4 TM1, -2 and -3 are essential, while TM4 and C-terminal aa K398 to T492 are dispensable (241). When expressed alone, NSP4 localizes to the ER and is then recruited to the RTC in infected cells (240), implying that NSP4 may be correlated with early secretory pathway in viral replication. Together with NSP3 and NSP6, the NSP3-4-6 complex is shown to promote double-membrane vesicles (DMVs) formation through homotypic and heterotypic interactions (242, 243), and with NSP2 in the formation of replicative structures (226, 243).

1.1.6.2.5 NSP5

Known as the main protease (M_{pro}), the CoV NSP5 is a ~33kDa chymotrypsin-like protease analogous to picornaviruses (244, 245). IBV M_{pro} is 307 aa residues long (232, 246). Based on the crystal structure of IBV NSP5, M_{pro} is composed of domains I, II, and III (247). Domains I and II both have a chymotrypsin-like, two β -barrel fold

similar to TGEV and SARS-CoV (245, 248). Structurally, the active sites of IBV NSP5 are found at the centers of domain I and II with the catalytic dyad contributed by His41 and Cys143 (247). Domain III, on the other hand, comprises of five α helices that assemble into a globular structure (247). Belonging to the C30 endopeptidases, M_{pro} is responsible for processing pp1a and pp1ab to produce 12 NSPs (172, 249-251). The resultant protein products (NSP4 to NSP16) will eventually assemble into components of the RTC (249, 252). Along with NSP12 and NSP13, the M_{pro} constitutes one of three highly-conserved proteins throughout the *nidovirales* (253). The M_{pro} of IBV nsp5 is shown to be auto-proteolytic at two Gln-Ser dipeptides, namely Gln2779-Ser2780 and Gln3086-Ser3087 (254, 255). Substitutions at the P1 position of IBV M_{pro} cleavage sites yielded diverse effects, from being well-tolerated to nonrecoverable (256), indicating that processing intermediates might be involved in negative-strand genomic RNA replication, while the mature protein is essential for sgRNA transcription. The first seven N-terminal residues of NSP5 play a critical role in dimerization (245). Deletion of the first five amino acids in the N-terminal could lead to the abolishment of NSP5 activity, although modification to the N-terminal permits the formation of a super-active octamer (257, 258). NSP10, NSP12, and NSP14 also interact with NSP5 directly through yeast two-hybrid screens and interactome analysis (227, 259), and indirectly with NSP16 as part of the NSP10-14-16 complex (227, 259-261).

1.1.6.2.6 NSP6

Contrary to NSP4, NSP6 contains 6 TM domains and adopts a N^{endo}/C^{endo} topology (262). In IBV, NSP6 is 293 aa residues long and together with SARS-CoV, are thought to cause autophagy induction through the generation of autophagosome from the ER through omegasome intermediate containing Atg5 and LC3II (263). Additionally, the overexpression of NSP6 can disrupt intracellular membrane trafficking and induce microtubule organization complex vesiculation (242, 263). When IBV BeauR or M41 NSP6 is expressed alone, piled, disordered membranes were observed in infected cells (264). Interestingly, the colocalization of NSP4 and NSP6 revealed in a co-expression study implies that the two NSPs may interact in the formation of replicative structures (265).

Table 1-3: Non-structural proteins (NSP) of IBV Beaudette: Functions, cleavage sites and size (in amino acid residues)

NSP	Function	Start (nt)	Stop (nt)	Size (aa)	PDB ID	CoV	Ref
1	IFN Antagonist	-	-	-	2HSX	SARS-CoV	(212)
2	IFN Antagonist	529	2547	673	3LD1	IBV	(266)
3	Papain-like protease (PLpro)	2548	7323	1592	2FE8	SARS-CoV	(234)
	Deubiquitinase				4X2Z	IBV	(236)
	IFN antagonist						
4	Transmembrane scaffold	7324	8865	514	3GZF	FCoV	(267)
5	Main protease (Mpro, 3CLpro)	8866	9786	307	2BX3	SARS-CoV	(268)
	IFN Antagonist				2Q6D	IBV	(247)
6	Transmembrane scaffold	9787	10665	293			
7	Hexadecameric complex	10666	10914	83	2KYS	SARS-CoV	(269)
					3UB0	FCoV	(270)
8	Hexadecameric complex	10915	11544	210	2AHM	SARS-CoV	(271)
	Primase				3UB0	FCoV	(270)
9	RNA-binding protein	11545	11877	111	2J97	HCoV-229E	(272)
					5C94	IBV	(273)
10	Zinc-binding Domain (ZBD)	11878	12312	145	2FYG	SARS-CoV	(274)
	2'-O-Methyltransferase (2'-O-MTase)						
	cofactor						
11	Unknown	12313	12351	13			
12	RNA-dependent RNA polymerase (RdRP)	12313	15131	932			
13	ZBD	15132	16931	601			
	RNA 5'triphosphatase						
	RNA helicase						
14	3'-5' exoribonuclease (ExoN)	16932	18494	521	5C8U	SARS-CoV	(275)
	N7-methyltransferase						
15	Endoribonuclease (NendoU)	18495	19508	338	2H85	SARS-CoV	(276)
					2GTH	MHV	(277)
16	2'-O-MTase	19509	20414	302	2XYR	SARS-CoV	(261)

Figure 1-9 Infectious Bronchitis Virus (IBV) replicase gene and processing scheme of replicase protein products.

(A) Ribosomal frameshifting elements of IBV replicase gene. Pseudoknot stems are indicated as S1 and S2, and loops are indicated as L1 and L3.

(B) Translation of the *replicase* gene, ORF1a, and ORF1b, starts after the release of the viral genome into host cytoplasm via ribosomal frameshifting, producing polyprotein (pp) 1a and 1ab. Pp1a and pp1ab will then be autoproteolytically cleaved at cleavage sites by papain-like protease (PL_{pro}) (in red triangles) and main protease (M_{pro}) (in orange triangles) into 15 non-structural proteins.

1.1.6.2.7 NSP7

The 83 aa SARS-CoV NSP7 (73 aa residue for IBV) protein is determined by X-ray crystallography (271) and NMR methods (278), but its precise role remains to be defined. In structural studies, NSP7 is found to be part of a hexadecameric complex composed of recombinant NSP7 and NSP8. When expressed alone, the NSP7 structure is composed of three antiparallel helix bundles, with an α -helix at the C-termini. However, when in interaction with NSP8, the positions and spatial orientations of the helices differ from the original state, suggesting that NSP7's protein conformation is strongly influenced by its interaction with NSP8 (269). In SARS-CoV, it was reported that NSP7 and NSP8 activation conferred NSP12 processivity, although several mutations on NSP7 only affected viral replication to a minor extent (279).

1.1.6.2.8 NSP8

In many well-studied (+)RNA viruses, a viral protein genome-linked (VPg) is often linked to the 5' end of its gRNA (280). VPg oligonucleotides in poliovirus, for example, acts as a primer for RdRP to catalyze RNA synthesis (281). Since CoV gRNA is 5' capped instead of Vpg, and RNA synthesis is template-dependent (282), CoVs uses an alternate method to generate primers. Under these circumstances, the 210 aa long NSP8 serves multiple functions in the replicase-transcriptase complex. Firstly, as a subunit for the hexadecameric complex (271) and secondly, to function as a second, non-canonical RNA polymerase in RNA synthesis (283, 284). To synthesize primers for RdRP RNA synthesis, NSP8 relies on Mn^{2+} or Mg^{2+} to generate short 5'-[G/U]CC-3' sites on the template RNA (283). IBV NSP8 is also capable of forming stable structures with NSP12 at the N-termini and C-termini independent of the 5' and 3' UTRs of the gRNA (285).

1.1.6.2.9 NSP9

IBV NSP9 is 111 aa protein that has no designated function at the moment. However, various studies have postulated NSP9 to be involved in ssRNA binding through biochemical and reverse genetics approaches (286, 287). A number of studies have

described the structure for SARS-CoV NSP9, with one study describing the protein as a single β -barrel with an oligonucleotide-oligosaccharide binding (OB)-fold which exists as a dimer that can bind RNA in a non-sequence-specific manner (272, 286, 288). Recently, the dimeric nature of NSP9 is also confirmed in IBV, indicating that dimerization is conserved among all CoVs (273). However, more studies are required to find out how NSP9 dimerization and mutagenesis may affect its interacting partners, NSP8 and NSP12 (226).

1.1.6.2.10 NSP10

The IBV NSP10 is a 145 aa long protein thought to serve as a stimulatory subunit of NSP16 2'-O-methyltransferase (2'-O-MTase) (289) and a zinc-binding domain (ZBD) in CoV replication (274). SARS-CoV NSP10 monomer is composed of two anti-parallel N-terminal helices, a β -sheet, two zinc-binding motifs, and a C-terminal (274). It is thought to play a role in viral replication, as temperature-sensitive (ts) MHV NSP10 mutants exhibited defects in minus-strand RNA synthesis (290). Moreover, MHV NSP10 double mutants (Asp47 and His48 to Ala) showed reductions in viral growth, RNA synthesis and processing of NSP4-11, suggesting that NSP10 is a crucial cofactor in both positive- or negative-strand synthesis (291). Since NSP10 binding promotes NSP14's ExoN activity (292), this indicates that NSP10 regulates NSP14-ExoN activity to safeguard the replication fidelity of CoVs.

1.1.6.2.11 NSP11

In pp1a, NSP11 is the last NSP produced in the C-terminal should ribosomal frameshift not occur. Only 13 aa long in IBV, most of its RNA sequence coincides with the RNA frameshifting sequence and NSP12. So far, NSP11 has not been detected in CoV infection; hence, no functional role for this protein has been assigned. The cleavage site at NSP10, 11 and 12 of IBV is expendable in a CoV infectious clone system (293).

1.1.6.2.12 NSP12

In CoV, NSP12 encodes for the RNA-dependent RNA polymerase (RdRp) (932 aa long in IBV). The primer-dependent activity of SARS-CoV NSP12 is confirmed through

the extension of poly(A) templates by NSP12 using oligo(U) primers (282). Although no crystal structures have been reported, a 3D structural model of the SARS-CoV NSP12 catalytic domain revealed conserved sequence motifs in the palm subdomain, finger domain and a structural motif between the palm and thumb in both *de novo* and primer-dependent RdRps (294). CoV RdRp RNA synthesis follows a two-step mechanism. First, the addition of primers to the 3' end of the RNA template by NSP8 primase (283) synthesizes short oligonucleotides complementary to the 3' end (295, 296). Next, during the elongation phase, the nucleotidyl transfer reaction occurs to generate the complementary RNA product. For many years, the CoV RdRp is thought to be primer-dependent until recently, when SARS-CoV NSP12 is shown to perform both primer-dependent and *de novo* RNA synthesis (297).

1.1.6.2.13 NSP13

ss(+)RNA viruses whose genomes are larger than 7kb encode a helicase for multiple purposes, such as resolving nucleic acid duplexes, displacing proteins found in polynucleotide-protein complexes and translocating along dsDNA or RNA without unwinding. In CoV, the helicase is encoded by NSP13, whose function was initially identified through genome sequence analysis (298). It comprises a Cys/His rich N-terminal domain, a β -barrel followed by the enzymatic core of HEL1 that mediates nucleic acid binding through stacking interactions (299, 300). NSP13 is closely related to the eukaryotic up-frameshift 1 (Upf1) helicases, which moves in the 5' to 3' direction upon binding to the nucleic acid (299), as demonstrated in HCoV-229E (301, 302) and subsequently in SARS-CoV on dsDNA using a 5' single-strand overhang (303). Biochemical studies of SARS-CoV NSP13 have reported that the unwinding process occurs in discrete steps of 9.3 bases with a catalytic rate of 30 steps per second, and it does not discriminate between DNA and RNA substrates (304). In IBV, a point mutation on NSP13 (R132P) obviates sgRNA synthesis (305).

1.1.6.2.14 NSP14

The CoV NSP14 (521 aa, ~60kDa in IBV) is a dual-function enzyme comprising of the N-terminal exonuclease (ExoN) domain (28, 172) and the C-terminal guanosine N7-methyltransferase (N7-MTase) activity (306, 307). The N-terminal ExoN domain

consists of three subdomains: I (DE), II (E) and III (D), making NSP14 part of the DEDD exonuclease superfamily that performs DNA proofreading (308). NSP14 hydrolyzes ss- or dsRNA in a 3' to 5' direction into short oligonucleotides of 8 to 12 nt and 5 to 7 nt, respectively (28, 292), and remove mismatched 3' end nucleotides that mimic RdRp misincorporated products (292). In mutants lacking ExoN activity, the CoV exhibited higher sensitivity to 5-FU, and hence are more susceptible to lethal mutagenesis (309). On the other hand, the C-terminal N7-MTase activity completes the 5' cap of CoV RNA a methyl group addition to the guanosine cap at the N7 position (307, 310). N7 methylation is required for its subsequent methylation at the O-2' position that is essential to evade host restriction (260, 311, 312).

1.1.6.2.15 NSP15

NSP15 (338 aa in IBV) encodes for a nidoviral NendoU that plays an essential role in RNA processing. Although NendoU is unique to the *nidovirales*, no viral homologs of NendoU and its substrates have been reported to date. Through phylogenetic studies, Snijder and coworkers hypothesized that NSP15 highly resembles XendoU (172), an Mn^{2+} dependent endoribonuclease employed during the synthesis of U16 snoRNAs (313, 314). This was later supported by a study that confirmed the preference of NSP15 for manganese as a co-factor (315). Additionally, expression of recombinant CoV NSP15 in SARS-CoV, MHV, IBV, and HCoV-229E all revealed that NSP15 is stimulated by Mn^{2+} to cleave preferentially at the 3' ends of uridines to generate a 2', 3' cyclic phosphodiester cleavage product and 5' OH ends (315-318). Structural information has been published for SARS-CoV NSP15 (276), MHV (277) and most recently, for MERS-CoV (319). Comparison of each crystal showed that the NSP15 monomers formed homohexamers of a dimer of trimers. Each monomer exists in three parts: a small N-terminal, an intermediate section and a large-C terminal. Although the overall architecture is conserved among CoVs NSP15, MERS contained a serine substitution of F285 that disrupts RNA binding to increase its catalytic activity. NSP15 of HCoV-229E, MHV and SARS-CoV are reported to form a hexameric complex, as NSP15 with 28 and 11 amino acid residue deletions in the N-terminal and C-terminal completely destructs the active site (320). Early evidence for NSP15 function was obtained from SARS-CoV, where NSP15 has been shown to counteract MAVS-induced apoptosis (321). Alanine substitutions at MHV NSP15 enzymatic site (H262,

H277, and G275) only resulted in minor defects on RNA synthesis (318). However, a point mutation (D6408A) in HCoV-229E prevented viral RNA accumulation. This suggested that NSP15 is required for viral replication (317).

1.1.6.2.16 NSP16

The completion of an mRNA cap is performed by NSP16, the last NSP at the C-terminal of pp1ab. At 302 aa long, IBV NSP16 function is initially inferred from bioinformatics analyses that revealed a catalytic K-D-K-E tetrad distinctive to AdoMet-dependent 2'-O-MTases and an AdoMet-binding site (172, 322). Later, NSP16's 2'-O-MTase activity was verified using recombinant FCoV NSP16 (323). In this study, NSP16 is shown to be capable of recognizing short, cap-0-containing RNAs and subsequently methylates at the 2'-O position of the first nucleotide of the N7-methylated substrate. NSP16's activity relies on a viral co-factor, NSP10 (227, 259), as revealed by yeast-two hybrid, pull-down studies, and crystal structures of NSP10-16 complex (289). Further, NSP16 regulation by NSP10 is demonstrated by Bouvet and colleagues, where purified NSP10/ NSP16 interaction resulted in an efficient 2'-O-MTase activity (260). Conversely, RNA without a methylated cap structure (Gppp-RNA) could not be served as a substrate.

mRNA capping of SARS-CoV occurs in a stepwise fashion involving multiple viral co-factors. In the first step, NSP13 acts as a GTase to generate a diphosphate 5'-ppN at the first nucleotide of the mRNA. In the second step, the cap core structure (GpppN) is formed by an RNA guanylyltransferase. While the source of RNA guanylyltransferase remains unclear, it is hypothesized that CoVs may use cellular enzymes in this step. Next, NSP14 then methylates the guanosine cap at the N7 position to form a cap-0 structure. Finally, the cap-0 structure is converted into a cap-1 structure by NSP16/NSP10 complex. Mutations on the 2'O-MTase "hot spots" (Val42, Met44, Ala71, Lys93, Gly94, and Tyr96) can disrupt its interaction with NSP10 and abrogate 2'O-MTase activity (261, 324). Reverse genetics studies using CoV mutants lacking 2'O-MTase activity has supported the functional relevance of NSP16 in evading host immune response (312, 325-327). SARS-CoV with mutations inserted

into the catalytic tetra has led to virus attenuation (328), while stop codon insertion at the 5' end of NSP16 coding sequence abolished CoV RNA synthesis (329).

1.1.7 Coronavirus Replication Cycle

1.1.7.1 Attachment and Entry

Viral infection of host cells begins with receptor recognition by CoVs, that is initiated by the binding interaction between the S protein and its cognate receptors (**Figure 1-10**). The locations of RBDs within the S1 region of the CoV vary depending on the genera. For instance, MHV and IBV RBDs are located on the N-terminus of S1 subunit (60, 330), while they are located at the C-terminus of SARS-CoV and TGEV (331, 332). Host range and tissue tropism are mainly determined by the interaction between the S protein and its receptor. In the case of CoVs, it is the preference to utilize peptidases as their receptor. For instance, *alpha*CoVs such TGEV, PRCoV and FCoV uses aminopeptidase N (APN); SARS-CoV and HCoV-229E have an affinity for angiotensin-converting enzyme 2 (ACE2); MHV uses carcinoembryonic antigen-related adhesion molecule 1 (CEACAM1); MERS-CoV engages dipeptidyl-peptidase 4 (DPP4). The cellular receptor for *gamma*CoVs is currently unknown, although sialic acids are thought to serve as non-specific attachment factors (333-335). CoV can also bind to dendritic cell-specific intracellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) or liver/lymph node-specific intracellular adhesion molecule-3-grabbing nonintegrin (L-SIGN) as attachment factors with the primary receptor (336).

The receptor binding induces conformational changes of the S protein to mediate membrane mixing and fusion between the virus and the host. S protein cleavage occurs at the S1/S2 and S2' sites to result in the exposure of the viral fusion peptide (337). This is catalyzed by host proteases derived from different stages of its viral replication cycle: 1) proprotein convertases (e.g., furin) during virus assembly in infected cells; 2) extracellular proteases (e.g., elastase) upon virus egress; 3) cell surface proteases (e.g., TMPRSS2) upon virus attachment and entry; and 4) lysosomal proteases (e.g. endosomal cathepsins) after virus endocytosis in virus-infected cells (62). Fusion peptide insertion into the host membrane upon S2' cleavage triggers HR1 and HR2 binding to form an antiparallel six-helix bundle (338). This

formation permits membrane mixing to deliver the viral genome into the cytoplasm eventually.

Figure 1-10 Coronavirus replication cycle. Coronavirus infection begins with the attachment of the S1 domain of the spike protein (S) with its cognate receptor. This receptor binding process drives the conformational change in the S2 subunit in S, promoting the fusion of the viral and cell plasma membrane. Following the release of the nucleocapsid to the cytoplasm, the viral gRNA is translated through ribosomal frameshifting to produce polyproteins pp1a and pp1ab. pp1a and pp1ab are autoproteolytically processed by the host and viral proteases to generate 16 non-structural proteins (NSPs) that will then be assembled to form the replicase-polymerase. The replicase-polymerase is involved in the coronaviral replication, a process in which the genomic RNA is replicated, and the subgenomic RNA will be transcribed and translated to form the structural proteins. The viral products produced will be assembled in the ERGIC and bud out as a smooth-wall vesicle to the plasma membrane to egress via exocytosis. ERGIC, Endoplasmic reticulum Golgi intermediate compartment.

1.1.7.2 Replicase gene translation and processing

The next step of the CoV life cycle involves the translation of the replicase gene that requires the use of ribosomal frameshifting, an alternate translation mechanism where a translating ribosome shifts -1 position from rep1a into rep1b reading frame with a fixed probability (339). Ribosomal frameshifting is governed by two RNA elements, the universal slippery sequence 5'-UUUAAAC-3' and an RNA pseudoknot. Recently, RNA dimerization located in stem 3 of the SARS-CoV is also found to play a role in this process (340). During elongation, the ribosome usually unwinds the pseudoknot and

terminates the translation of ORF1a at the stop codon to yield pp1a. Occasionally, the pseudoknot blocks the mRNA entrance channel of the ribosome and causes the ribosome to stall and change the reading frame by -1 nucleotide (341, 342). The consequent pause required for the ribosome to melt out the mRNA structure permits transfer RNAs (tRNAs) to enter the P and A site of the ORF1b reading frame to synthesize pp1ab as translation resumes (341). While studies show that the frequency of ribosomal frameshifting could occur up to 25% of the time, it is unclear how frequent this phenomenon occurs *in vivo*. Although the rationale of using ribosomal frameshifting by CoV remains enigmatic, investigators hypothesized that this alternate translation mechanism could help to regulate the proportion of pp1a and pp1ab required for RNA replication (343).

Following the production of pp1a and pp1ab, the polyproteins are autoproteolytically processed by its encoded viral protease to form mature protein products, termed NSP1-16 (except *gamma*- and *delta*CoVs which do not encode NSP1). The detailed descriptions of each NSP are described (see **Section 1.1.6.2 Non-Structural Proteins**). These processed NSPs will assemble to form the CoV RTC. The RTC creates an environment suitable for the generation of gRNA and sgRNAs.

1.1.7.3 Replication and transcription

The expression and assembly of the RTC facilitate gRNAs replication and the transcription of multiple sgRNAs that expresses the structural and accessory genes located at the last-third of the CoV genome. Both the gRNA and sgRNA are produced via negative-strand intermediates. The anti-leader, containing 5' oligouridylate tracts and 3' anti-leader sequences, is only 1% in abundance as compared to their positive-sense counterparts (344). Each sgRNA contains a leader RNA of 70-100 nts identical to the 5' end of the genome fused to the body RNA at the 3' end. The fusion of the leader and body RNA happens at short motifs on the genome, known as transcriptional regulatory site (TRS). All positive-sense sgRNAs share the same 3'-terminal with the genomic RNA, creating a set of nested RNAs characteristic to *nidovirales*.

The *cis*-acting sequences located at the 5' and 3' ends of the CoV genome are also crucial for viral RNA replication. The presence of *cis*-acting elements was established in studies using defective interfering (DI) RNAs that compete for the viral RNA synthesis machinery (345). In the 5' UTR of the genome of various CoVs, there are several stem-loop structures that may extend into the replicase 1a gene (346-350). On the 3' UTR of the genome, a bulged stem-loop, pseudoknot, and hypervariable region can be found (33, 351-353). Together, these structures help regulate the different stages of RNA synthesis, although the regulation mechanism of each stage remains unknown.

The most critical aspect in CoV RNA synthesis is the fusion between the leader and body TRS segments during the production of sgRNAs. Initially, this leader-body TRS fusion was postulated to occur by leader-primed transcription, whereby genomic mRNA is transcribed directly from genome-length negative-strands (354, 355). However, there is now a consensus that this fusion takes place through discontinuous negative-strand RNA extension (356). However, this model does not explain how RdRP decides on the number of TRS-B to cross to produce different sgRNAs. How the TRS-B sequences get directed to TRS-L is also unclear.

In addition to gRNA and sgRNA production, CoVs can also undergo homologous and non-homologous RNA recombination (26, 357). RNA recombination in CoVs is initially thought to be due to the template-switching ability of the RdRP during RNA replication (358). Evidence demonstrating RNA recombination in IBV has been observed both *in vitro* and in the field (359-364). The ability of these CoVs to recombine may play a prominent role in viral evolution and to prevent the accumulation of deleterious mutations.

1.1.7.4 Assembly and egress

With the transcription of gRNA and sgRNA, the next step is to enable the translation of the structural and accessory proteins that help to direct the assembly of progeny viruses. Upon translation, CoV S, M, and E proteins are inserted into the ER, where

they transit into the site of virion assembly – the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) – via the secretory pathway (159, 365). Here, the viral genomes encapsulated by the N protein coalesce, besides the envelope components, will bud into the ERGIC to form mature virions (151).

The assembly of CoV requires cooperative protein-protein interactions mostly involving the M protein. However, as mentioned earlier, the sole expression of M protein is insufficient for VLP formation and requires the co-expression of E protein to produce CoV envelopes (117). The expression of N protein can further enhance VLP formation and compensate for mutational defects in M protein (161). The S protein is the last structural protein incorporated into virions, but studies have shown that it is not required for assembly (118). Since E protein is found in limited quantities, M protein likely provides the impetus for envelope maturation. However, some studies postulated that the E protein plays a role in membrane curvature (82, 115, 124) and preventing M protein aggregation (106). Additionally, virus egress may also be promoted by the E protein by altering the host secretory pathway (366).

The condensation of the nucleocapsid and the envelope components completes the virion assembly. This interaction is brought about by the interaction between the M and N protein (367). Since nucleocapsid formation is concomitant with genome replication, it is unclear how the nucleocapsid traffics to the ERGIC and selectively package only the positive-sense full-length CoV genomes. Through DI RNA analyses, the genomic packaging signal is mapped to NSP15 in MHV (368). The mechanism of how this signal works is undetermined, although virus formation is not affected by mutation of this signal. Additionally, packaging signals for other CoVs such as TGEV is located at the 5' end of the virus genome, suggesting that the location of these packaging signals may be virus-specific (369).

Following assembly and budding, the virions are exported from the infected cells in vesicles and are released by exocytosis. Whether CoV uses the constitutive pathway for post-Golgi transport or adopts a unique pathway for its exit remains to be defined. In some CoVs, a portion of the S protein that is not assembled into virions will transit

to the plasma membrane, where it mediates cell-cell fusion between neighboring uninfected cells, promoting undetected virus spread.

1.2 CORONAVIRUS GENETICS AND REVERSE GENETICS TO STUDY VIRUS-HOST INTERACTIONS

In the past, CoV genetics were studied using three types of mutants. The first was naturally arising deletion mutants that offered clues to some of the phenotypic changes found in various pathogenic strains. The second was defective RNA templates, of which the RTC was provided by a helper virus (33, 370-372). The last was temperature-sensitive (*ts*) mutants isolated from MHV and IBV using chemical mutagenesis (373-376). Reversible and easy to use, *ts* mutants quickly became powerful tools to study gene function after natural mutants. However, due to difficulty in their generation, and production of random mutants that result in conditional-lethal RNA-negative phenotypes, the use of *ts* mutants in CoV genetics has been limited. Despite these challenges, there is value in the use of *ts* mutants. As *ts* mutants are unable to synthesize viral RNA after initial infection and must be maintained at the non-permissive temperature, this suggests that they are likely to be defective in different aspects of viral RNA synthesis. A detailed characterization into their genotype and phenotype can provide early insights into the mechanisms of RNA synthesis as well as the protein-RNA and protein-protein interactions that govern the activities of the replication-transcription complex (290). Besides, these conditional-lethal mutants may also be used to define the number of complementation groups that contribute to a specific phenotype, providing valuable information into putative pathways the viral proteins must travel to achieve their functional configurations, and this been used with success with polioviruses (377). In recent years, there has been a revival of classical coronaviral genetics for replicase *ts* mutants, as they can now be sorted into complementation groups using reverse genetics (290).

Reverse genetics techniques for CoVs were developed as early as the 1990s when the large size of the CoV genome and expression of specific CoV cDNA sequences in bacterial cloning systems became major impediments in the generation of recombinant CoVs using classical genetic techniques (378). Here, we review

approaches developed to study the function of viral genes using IBV, TGEV and FIPV as CoV models.

1.2.1 Homologous RNA Recombination (HR)

Targeted RNA recombination was the first reverse genetics method devised by Masters and colleagues (379). Initially designed for MHV, this system harnesses on the intrinsic property of the CoV replication machinery to recombine RNA molecules. It involves a chimeric RNA donor bearing the desired mutations being transfected into cells that have been infected with a parental virus presenting specific characteristics (i.e., *ts* or host range-based selection) that can be selected against (31, 359, 380). Recombinant CoVs generated by targeted RNA recombination can then be isolated by parental virus counterselection and purified.

Despite the existence of HR since the 1990s, attempts at recombining IBV RNA molecules had proven to be unsuccessful until recently, when van Beurden and colleagues established a targeted RNA recombination reverse genetics system on IBV H52 (**Figure 1-11**) (381). Using this approach, the IBV donor plasmid, p-IBV, was assembled from five fragments: p-IBV-5, p-IBV-1b, p-IBV-S, p-IBV-SIR, and p-IBV-3 T, in a directed fashion. Each of these five plasmids contained gene fragments of the various IBV structural and accessory genes that, when ligated together, formed the last third of the IBV genome. On the other hand, the mIBV donor plasmid, p-MHV-S, was constructed using the ectodomain of MHV A59 spike gene from pTUG. Subsequently, the *EcoRI* and *XhoI* RES were silently removed, and the MHV spike ectodomain was ligated into p-IBV-5-1b and further cloned, resulting in the final plasmid p-IBV-5-1b-mhvS-SIR-3 T, termed p-mIBV. (382-386).

Targeted RNA recombination proceeds in two steps. In the first step, the genomic IBV H52 viral RNA and its chimeric synthetic modified RNA, p-mIBV were co-transfected along with p-IBV-N into non-susceptible BHK-21 cells. Next, a recombinant chimeric murinized (m) IBV intermediate (mIBV) was generated by seeding transduced BHK-21 cells onto LR7 cells for syncytia formation. mIBV was rescued by harvesting the

cell culture supernatant and subjecting to two cycles of virus plaque purification on LR7 cells. Here, the isolation and propagation of mIBV were made possible by the replacement of the IBV S glycoprotein ectodomain with that of the MHV. In the next step, the recombinant IBV (rIBV) was generated by exchanging the IBV S ectodomain back into the mIBV genome through HR between p-IBV donor RNA and recipient virus mIBV.

Since the development of this method, a more sophisticated protocol has since been established based on a switch in species specificity (378, 387, 388). With this method, manipulating the last one-third of the 3' CoV genome has been successful in FIPV (389), TGEV (390) and most recently, in IBV (381). Targeted RNA recombination with interspecies chimeric CoV is also possible by isolation based on their regained natural cell tropism (391). However, despite its value, there are apparent drawbacks to targeted RNA recombination. For technical reasons, this method cannot be expanded to the 5' two-thirds of the genome where the replicase is located. Hence, full-length cDNA-based reverse genetic techniques are required to overcome the limitations faced by HR. In order to bypass the barriers presented by the vast size of the replicase gene, three other methods were developed to overcome these challenges to allow the propagation of full-length CoV cDNAs, namely bacterial artificial chromosomes (BACs), *in vitro* ligation and vaccinia virus-based vectors.

1.2.2 Bacterial Artificial Chromosomes (BACs)

The BAC system is based on *Escherichia coli* and its single-copy plasmid F vector that follows a strictly controlled replication to produce only one or two plasmid copies per cell. Amongst bacterial cloning systems, BAC has been chosen for reverse genetics as it is capable of maintaining large DNA fragments from various genomic sources with high structural stability even after generations of continual growth (392). The first full-length cDNA-based reverse genetics system for CoVs using the BAC approach was developed for TGEV in 2000 (**Figure 1-12**) (393, 394).

In this system, the full-length cDNA copy of the TGEV genome is assembled in the BAC plasmid *pBeloBAC11*, a synthetic low-copy-number plasmid, downstream of a cytomegalovirus (CMV) promoter (395). The infection is initiated from the transfected BAC DNA through transcription of infectious CoV RNA using the host RNA polymerase II (396). This method is advantageous in avoiding the potential limitations in *in vitro* capping and synthesis of genomic RNA. Recently, infectious clones of SARS-CoV Frankfurt-1 have also been reported to use modified BACs under the control of T7 RNA polymerase promoter to prevent splicing by CMV promoter after transcription in the nucleus (397).

BAC clones can also be modified into *E. coli* by homologous recombination in two steps, using the lambda red system and *I-SceI* homing endonuclease, for counterselection (398). This method employs a sequence bearing the desired modifications and the insertion of an *I-SceI* recognition site in the first recombination step, followed by the induction of *I-SceI* to cleave at the recognition site *in vivo*, generating a DNA double-stranded break at the second recombination step. This strategy confers a precise and efficient method to modify a BAC clone in a “scarless” fashion.

Figure 1-12 Assembly of full-length TGEV cDNA clone using Bacterial Artificial Chromosome (BAC) method. A plasmid containing a cDNA encoding TGEV-derived DI (pDI-C) was used to construct the complete cDNA of the TGEV genome. As pDI-C RNA contains three deletions across the genome, a set of cDNAs encoding the fragments of the missing regions were generated by RT-PCR (highlighted in yellow boxes). The final TGEV cDNA was flanked by a CMV promoter at the 5' end and a 24bp poly(A) tail at the 3' end, followed by the hepatitis delta virus ribozyme (HδR) and bovine GH termination (BGH) and polyadenylation sequences. Figure adapted from (393).

The BAC system poses several advantages. Manipulation of the BAC clones follows standard cloning protocols such as selecting suitable restriction sites and ligating cDNA fragments into the vector, making it simple to construct them, although slight modifications have to be made to BAC clones (392). Likewise, the high stability of the exogenous sequences provided by the BACs permitted infinite cDNA production of the viral RNA in the cell. Together with BACs' high transfection efficiency in mammalian cells, BACs quickly became an attractive tool for CoV reverse genetics (399) and has been successfully employed in SARS-CoV (400-402) and MERS-CoV (119).

1.2.3 *in vitro* Ligation

The third system constructs a full-length genomic cDNA through a systematic assembly of smaller cloned cDNA fragments via *in vitro* ligation (382). In this approach, each smaller clone is ligated in an orderly manner using asymmetric restriction sites and is then transfected to permissive host cells. Since the assembled full-length cDNA contains a T7 RNA polymerase promoter and poly(A) tail, this full-length cDNA can be transcribed *in vitro* into a mature, capped mRNA transcript that can generate infectious viruses after co-transfection to susceptible cells with a capped N gene RNA transcript. Initially applied to TGEV (382), this approach has been expanded to MERS-CoV (403), MHV (385), SARS-CoV (384) and IBV (42, 133, 305).

The *in vitro* cDNA assembly approach has proven simple and straight forward, as conventional techniques can be used to perform mutagenesis of independent cDNA fragments in parallel, and is compatible with other reverse genetics methods, such as BAC and vaccinia vectors. However, this approach presents two potential caveats - premature transcription termination and the lack of restriction enzymes that provide a unique overhang to direct individual fragment ligation. As such, several mutations must be inserted to the genome to avoid premature transcription termination, as exemplified in the engineering of MHV infectious cDNA by *BsaI* and *SapI*, which leaves behind 1-4nt overhangs that can only be annealed with complementary overhangs generated at an identical site (385).

Following this strategy, the construction of a full-length cDNA clone derived from a Vero cell-adapted IBV Beaudette strain was obtained by *in vitro* assembly of five cDNA fragments spanning the entire IBV genome (**Figure 1-13**) (305). *BsmBI* or *BsaI* were introduced into the 5' and 3' ends of each fragment using specific primers by RT-PCR. These cDNA fragments were then digested and ligated into a full-length cDNA. The infectious viruses were then recovered by *in vitro* transcription. An expanded version of this method has been reported, where an entire CoV genome was constructed using synthetic cDNAs (403, 404). This approach has been used with success at identifying and evaluating promising zoonotic vaccine candidate strains for SARS-CoV (405).

Figure 1-13 Assembly of an IBV full-length cDNA clone by *in vitro* ligation. A full-length cDNA of Vero cell adapted IBV Beaudette strain was assembled by *in vitro* ligation of five contiguous cDNA fragments (A to E) spanning the entire viral genome, that was flanked by native or engineered *BsmBI* and *BsaI* restriction sites. The assembled full-length cDNA contained a T7 RNA polymerase promoter (T7) at the 5' end and poly(A) tail at the 3' end, allowing for the *in vitro* transcription of a full-length, capped polyadenylated transcripts. The viral genes and relevant restriction sites are indicated. Figure adapted from (305).

1.2.4 Vaccinia Virus Vectors

The vaccinia virus reverse genetics system involves the use of vaccinia virus cloning vectors to generate full-length CoV genome using long-range reverse transcription-polymerase chain reaction (RT-PCR) (406, 407). This system represented a fundamental approach to CoV genetics and was first reported for the generation for human CoV 229E (HCoV-229E), and soon after, this system was extended to FIPV (408, 409), IBV (383) and MHV-A59 (410). Vaccinia virus vectors were attractive reverse genetic tools for several reasons. Firstly, the vaccinia virus can accommodate at least 25kb of foreign DNA sequence, allowing recombinant vaccinia viruses to replicate in cell culture without compromising on viral titer when compared to a non-recombinant virus (411). Secondly, vaccinia virus vectors have been designed for foreign DNA insertion by *in vitro* ligation, negating the need to generate plasmid intermediates to carry the entire cDNA insert (412). Finally, the cloned cDNA insert is open to mutagenesis through vaccinia virus-mediated homologous recombination (413, 414).

Here, the vaccinia virus-based system for IBV will be illustrated (**Figure 1-14**) (383). In principle, the procedure is a multistep process that can be divided into two parts: generation of a full-length IBV cDNA and the recovery of the infectious rIBV. In the former, large amounts of subgenomic cDNA fragments are generated either by RT-PCR or by amplification using bacterial plasmid DNA. The selection of enzyme restriction sites for the construction of the full-length cDNA was strictly limited to those found in the IBV genome to prevent potential modifications made to the IBV infectious clone, (415). Eventually, the plasmids containing the adjoining sequences of the IBV genome were ligated to create the full-length cDNA downstream of the T7 promoter. Here, the ligation strategy is to dephosphorylate the ends of various cDNA fragments using calf intestinal alkaline phosphatase to regulate the ligation reaction.

Next, the full-length IBV cDNA is transduced into CK cells previously infected with a helper virus to recover the recombinant vaccinia virus containing the IBV genome, termed rIBV (416). A second plasmid, pCi-Nuc, is also co-transfected to express IBV N, under the control of both the CMV and T7 RNA polymerase promoter (417). FPV is

selected as a helper virus for two reasons. FPV infection in the mammalian cells is abortive, and together with the rarity of recombination events between two poxviruses, the resulting virus collected from the cell culture contains exclusively recombinant vaccinia viruses (250, 406). In the case of IBV, seven out of eighteen clones contained an insert of the expected size.

Figure 1-14 Assembly of an IBV full-length cDNA clone in the vaccinia virus genome. Three contiguous cDNA fragments (pFRAG1 to pFRAG3) spanning the entire viral genome of IBV Beaudette CK strain was cloned in vaccinia virus DNA (V) by *in vitro* ligation. pFRAG1 contained a T7 RNA polymerase promoter (T7) at the 5' end and pFRAG3 fragment a 28 nt poly(A) tail at the 3' end. The assembly of the full-length IBV cDNA and its insertion into the vaccinia virus genome is shown with the final orientation of the cDNA in the vaccinia genome. Figure adapted from (383).

1.2.5 Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated protein 9 (Cas9)

In addition to the use of reverse genetic systems to study virus-host interactions, the use of forward genetics has also provided an unbiased and comprehensive strategy to uncover host factors that promote or restrict virus replication. Initially, the use of forward genetics was limited to organisms such as yeasts and often relied on the use of radiation and chemical mutagens to introduce random mutations (418). While these methods have markedly improved our understanding of many fundamental biological processes, their application to cultured mammalian cells proved to be challenging. In the early 1990s, technological advances in RNAi made it possible to disrupt gene expression in mammalian cell culture through gene knockdown (419).

Recently, the prokaryotic CRISPR-Cas9 adaptive immune system revolutionized biological research by efficiently inducing gene knockouts in almost every cell type (420-422). In contrast to gene knockdown approaches, CRISPR-Cas9 generated gene knockouts displayed a more pronounced phenotype, higher signal-to-noise ratio, and fewer false-positives (423, 424). To date, CRISPR-Cas9 has been used to identify host factors required for virus replication, such as Zika virus (425) and CoVs (426). The relative ease of use and reproducibility of CRISPR-Cas9 has made it a powerful tool to study virus-host interactions and to identify new antiviral targets and therapeutics.

Initially, the CRISPR-Cas9 system is an adaptive immune system that protects the prokaryotes and archaea against invading foreign pathogens. The CRISPR locus is made up of an array of short repeated sequences separated by spacers with unique sequences derived from the nucleic acid of viruses and plasmids from invading pathogens. These spacers are used as recognition elements to find matching virus genomes and induce cleavage of invading genetic elements. CRISPR activity requires the presence of a distinct set of CRISPR-associated genes adjacent to the CRISPR locus, that codes for proteins essential for adaptive immune response. Since new spacers are directionally integrated to one side of the CRISPR locus, a chronological record of past virus encounters was created and passed down to the offspring.

The CRISPR-Cas mediated defense can be divided into three stages. In the first stage known as adaptation, the bacterium inserts a new spacer into the CRISPR locus. These spacers are typically 23-44 bp in length, copied from phage or plasmid DNA of the invading pathogen. In the second stage, expression, the CRISPR system expresses the Cas genes and transcribe CRISPR into a long precursor CRISPR RNA (pre-crRNA). Later, this pre-crRNA is processed into mature crRNA by Cas proteins and accessory factors. Then, trans-activating crRNA (tracrRNA) base-pairs crRNA to guide a Cas protein to the target site to induce nucleic acid cleavage 3 nt upstream of a short protospacer adjacent motif (PAM) sequence in the final stage known as interference.

Among the three CRISPR-Cas systems, type II systems are most commonly used for gene editing. The major advantage of this system lies in the presence of a single protein, Cas9, that is used for targeting and cleavage of desired sequences. In commercial gene editing, a single guide RNA is formed by the fusion of TracrRNA and crRNA. Upon targeting, the Cas9 cleaves the DNA strand and introduces a double-strand break (DSB) at a specific location 3 nt upstream of a PAM sequence (**Figure 1-15**).

With the advancement in CRISPR-Cas9 genetic engineering technology, it is now possible to study the function of genes through the generation of functional gene knockouts (**Figure 1-16**), achieved in part by the non-homologous end-joining (NHEJ) processes induced by DSBs in the absence of a repair template. Since NHEJ is error-prone and frequently introduces frameshift insertions, deletion and stop codon mutations, the expression of the encoded protein may be disrupted, resulting in functional knockouts.

Figure 1-15 Overview of CRISPR-Cas9. (A) The overview of the CRISPR locus is shown. The major elements of the CRISPR locus include the tracrRNA, Cas operon, and the CRISPR repeat-spacer array. (B) The CRISPR repeat-spacer array is transcribed into pre-crRNA that contains the archived foreign DNA sequenced. The pre-crRNA transcript is processed by tracrRNA, Cas9, and RNaseIII to become a mature crRNA. (C, D) TracrRNA forms a complex with crRNA and guides Cas9 to bind DNA through complementary protospacer RNA-targeting sequence. (E) Cas9 RuvC and HNH nuclease domains introduce a double-strand break in the crRNA-paired sequence. The figure is taken from (427).

Figure 1-16 CRISPR-Cas9 gene editing mechanism through the introduction of double-strand breaks (DSBs). DSBs induced by Cas9 can be repaired via the nonhomologous end-joining (NHEJ) or homology-directed repair (HDR). Repair via NHEJ is often error-prone, as the ends of the DSB are re-joined by an endogenous DNA repair machinery. At times, this re-joining of DNA strands introduces random indel mutations at the joining site, resulting in gene knockout. Alternatively, should a repair template be supplied, HDR can be employed to permit precise gene editing. The figure is taken from (428).

1.3 OBJECTIVES AND SIGNIFICANCE

As obligate intracellular parasites with an enormous potential to cause worldwide epidemics, CoVs are known to harness the use of cellular factors and remodelling intracellular structures to advance their infection. With the constant emergence and re-emergence of new and old CoVs posing a significant health threat, establishing the virus-host interaction becomes a crucial frontier in understanding CoV pathogenesis. Not only does it provide an insight into understanding how cellular targets may be manipulated in the viral life cycle, but studies on virus-host interactions can also provide a wealth of information on the role of individual proteins and cellular pathways implicated in the replication of human and animal RNA viruses. In the past, the establishment of virus-host interactions is performed mainly via small-interfering RNA (siRNA), RNA interference (RNAi) screens and Stable Isotope Labelling with Amino acids in Cell culture (SILAC) techniques. While these methods usually generate a multitude of potential cellular targets depending on investigator's research interest, these methods do not study each gene in isolation or functionally characterize the role of each cellular target in CoV infection. Hence, using IBV as a model coronavirus, this study aims to further investigate and functionally characterize two host genes: 1) RNA (Guanine-7-) Methyltransferase (RNMT) and 2) Cleavage and Polyadenylation Factor I Subunit 1 (CLP1) in CoV infection.

The rationale for selecting RNMT and CLP1 stems from the identification of 83 host factors that support IBV infection in an earlier siRNA screening study performed by our group (429). Among the 83 cellular factors that are required for IBV infection, close to 45% of these factors are enriched in the nucleus and nucleolus. This finding is peculiar as CoV replication and transcription occur almost exclusively in the cytoplasm, indicating that factors that are involved in eukaryotic RNA splicing and transcription may also be recruited during CoV replication. As such, functional study of genes that are involved in RNA splicing and transcription, such as RNMT and CLP1, could contribute new avenues for therapeutics targeting the nucleus and nucleolus cellular factors. The study of RNMT is important as it is involved in mRNA capping. RNA viruses, such as influenza viruses, are known to cap-snatch from eukaryotic transcripts (430). Since CoVs also contain a 5' cap, it may recruit RNMT to cap its genomic and subgenomic RNAs. The study of CLP1 is important as polyadenylation

may be pertinent in CoV infection. Although CoV is not known to encode any viral proteins that regulate polyadenylation, poly(A) tails of CoV has been reported to be regulated constantly throughout infection (431).

Therefore, the objectives of this study are:

1. To study the role of RNMT and CLP1 in CoV infection via CRISPR/Cas9-mediated gene knockout (KO);
2. To investigate the functional role of RNMT and CLP1 in IBV infection through comparison with wild-type (WT) infected cells;
3. To establish the relationship between RNMT and CLP1 with their viral counterparts;
4. To dissect the stages of the CoV life cycle at which RNMT and CLP1 are implicated.

It is hoped that the mechanism in which these host factors are exploited in viral infection can provide new avenues in identifying druggable antiviral targets.

CHAPTER 2

MATERIALS AND METHODS

2.1.1 Chemicals and Reagents

The chemicals and reagents utilized in this study are summarized in **Table 2-1**.

Table 2-1: List of reagents and chemicals

Chemical and Reagent	Brand
3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)	Sigma-Aldrich
30% Acrylamide/Bis Mixed Solution (29:1)	Naclai-Tesque
4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI)	Invitrogen
6X DNA Loading Dye	New England Biolabs
Agarose	Invitrogen
Ammonium Persulfate (APS)	Sigma-Aldrich
Ampicillin	Calbiochem
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Crystal Violet Solution	Sigma-Aldrich
Deoxyribonucleotides (dNTPs)	1 st Base Biochemicals
Dithiothreitol (DTT)	GE Healthcare
DNA Ladders – 100bp, 1kb	New England Biolabs Axygen
Dulbecco's Modified Eagle Medium (DMEM)	Naclai-Tesque
Fluorosafe DNA Stain	1 st Base Biochemicals
Fetal Bovine Serum (FBS)	Gibco
Paraformaldehyde (PFA)	Sigma-Aldrich
Kanamycin	Gold Biotechnology
LB Agar	BD, Difco
LB Broth (Miller)	BD, Difco
Lipofectamine 2000	Invitrogen
Luminol	Sigma-Aldrich
Opti-MEM Reduced Serum Medium	Gibco
M-PER Mammalian Protein Extraction Reagent	Thermo Scientific
p-Coumaric Acid	Sigma-Aldrich
Penicillin-Streptomycin Stock Solution	Naclai-Tesque
Protein Molecular Weight Ladder	Bio-Rad
Puromycin	Invivogen
Roswell Park Memorial Institute Medium (RPMI)	Naclai-Tesque
Sodium Chloride	Merck
Sodium Dodecyl Sulfate (SDS)	Merck
Sodium Hydroxide	Merck
Triton-X-100	BDH Laboratory Supplies
TRIzol LS Reagent	Ambion
TRIzol Reagent	Ambion
Trypsin	Gibco
Tween 20	Promega

2.1.2 Enzymes, Inhibitors and Master Mix

The enzymes used in this study are summarized in **Table 2-2**. Restriction enzymes, unless otherwise stated below, were purchased from New England Biolabs.

Table 2-2: List of enzymes and inhibitors

	Brand	Cat. #
ENZYMES		
Kapa HiFi PCR Kit	Kapa Biosystems	KK2102
Phusion High-Fidelity DNA Polymerase	ThermoFisher Scientific	F530S
Q5 High-Fidelity DNA Polymerase	New England Biolabs	M0491S
Restriction Enzymes - <i>Bam</i> HI	ThermoFisher Scientific	FD0054
Restriction Enzymes - <i>Kpn</i> I	ThermoFisher Scientific	FD0524
Restriction Enzymes - <i>Spe</i> I	ThermoFisher Scientific	FD1254
Restriction Enzymes - <i>Xba</i> I	ThermoFisher Scientific	FD0684
Reverse Transcriptase (ImProm-II)	Promega	M314A
Reverse Transcriptase (M-MLV RNase H ⁻)	Promega	M3681
T4 DNA Ligase	Promega	M1801
<i>Taq</i> DNA Polymerase	ThermoFisher Scientific	EP0402
INHIBITORS		
Protease Inhibitor Cocktail Tablets	Roche	11697498001
Ribonuclease Inhibitor	ThermoFisher Scientific	EO0381
RNase A	ThermoFisher Scientific	EN0531
MASTER MIX		
2X SYBR qPCR	Primerdesign Ltd	PFAST-LR-SY

2.1.3 Antibodies

The supplier, catalog number, and animal source of primary and secondary antibodies used in this study were summarized in **Table 2-3**.

Table 2-3: List of primary antibodies

Antibody	Animal Source	Brand	Cat. #
B-Actin	Goat	Santa Cruz Biotechnology	sc-1616
CLP1	Rabbit	Sigma-Aldrich	HPA057770
FLAG-tag	Mouse	Abcam	ab49763
GAPDH	Mouse	ThermoFisher Scientific	AM4300
RNMT	Rabbit	Sigma-Aldrich	SAB-2102036-100UL

Anti-serum against IBV M, N, and S proteins were raised in rabbits immunized with bacterially expressed fusion proteins as described (199, 432). Horseradish peroxidase (HRP)-conjugated secondary antibodies used for western blot analysis were purchased from Dako. Alexa-594-conjugated secondary antibodies (Cat #. A27016) used for immunostaining was a gift from Dr. Yoon Ho Sup.

2.1.4 Commercial kits

The commercial kits used in this study are summarized in **Table 2-4**.

Table 2-4: List of Commercial Kits

Commercial Kits	Brand	Cat. #
Dual-Luciferase Reporter Assay System	Promega	E1910
Gel Extraction Kit	Omega-Biotek Qiagen	D2500-02 28706
mMessage mMachine T7 Transcription Kit	Invitrogen	AM1344
PCR Cleanup Kit	Omega-Biotek	D6492-02
Plasmid Miniprep Kit	Axygen Bio Basic	AP-MN-P-250 BS88136
Western Enhanced Chemiluminescent (ECL) Kit	Bio-Rad	1705060

2.1.5 Buffer Preparation

The buffers prepared for this study and their respective recipes are summarized in **Table 2-5**. Unless otherwise stated, the quantities required for the preparation of each buffer are meant for one litre. Stripping buffer for western blot (Cat #. 46430) was purchased from ThermoFisher Scientific.

Table 2-5: List of buffer solutions prepared and respective recipes

Buffer	Recipe (per litre)
10X Phosphate Buffer Saline (PBS)	80 g NaCl (1.37M) 2g KCl (0.027M) 14.4g Na ₂ HPO ₄ (0.1M) 2.4g KH ₂ PO ₄ (0.018M) Adjust pH to 7.4 with HCl
10X SDS-PAGE Running Buffer	30.2g Tris Base (0.25M) 144g Glycine (1.92M) 10g SDS (1%)
10X Towbin Wet Transfer Buffer	30.2g Tris Base (2M) 144g Glycine (1.92M)
10X Tris Buffer Saline (TBS)	60.6g Tris Base (0.5M) 87.8g NaCl (1.5M) 1ml Tween 20 Adjust pH to 7.4 with HCl
1X Annealing Buffer (100ml)	1ml 1M Tris (pH7.5-8.0) (0.01M) 1ml 5M NaCl (0.05M) 0.2ml 0.5M EDTA (0.001M)
2X Laemmli Buffer (300ml)	78.9g Glycerol 63ml 10% SDS Solution 19.74ml 1M Tris-HCl (pH 6.8) 300ml DI water 0.03g Bromophenol Blue
50X Tris-Acetate-EDTA (TAE)	242g Tris Base (2M) 18.6g EDTA (0.05M) Adjust pH to 8.4 with Acetic Acid
ECL Solution A (500ml)	50ml 1M Tris-HCl (pH 8.5) (0.1M) 1.1ml 0.09M p-Coumaric Acid (0.2mM) 2.5ml 0.25M Luminol (1.25mM) 450ml DI Water
ECL Solution B (10ml)	1ml 30% H ₂ O ₂ 9ml DI Water
ECL Stock Solution (5ml/10ml)	74mg p-Coumaric Acid in 5ml DMSO (0.09M) 443mg luminol in 10ml DMSO (0.25M)
SOC Medium	20g Bacto Tryptone 5g Bacto Yeast Extract 0.584g NaCl (0.01M) 0.186g KCl (0.0025M) 0.952g Anhydrous MgCl ₂ (0.01M) 2.467g Heptahydrate MgSO ₄ (0.01M) 3.063g Glucose (0.02M)

2.2 CELLS AND VIRUSES

2.2.1 Cell Culture

Human non-small cell lung carcinoma H1299 cells (ATCC CRL-5803) were obtained from American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium supplemented with 5% FBS and 1% penicillin-streptomycin (PS). African green monkey Vero cells (ATCC CCL-81) were cultured in DMEM high glucose (4,500mg/L) medium supplemented with FBS and PS as above. All cells were grown and maintained in a 37°C incubator with humidified 5% CO₂.

All experiments used low-passage (<20 passage) cells. Adherent monolayer cells were sub-cultured every 2 to 3 days upon reaching ~90% confluency. Firstly, the spent culture medium was aspirated and washed with PBS once before trypsinization using 0.05% trypsin for 30 seconds under room temperature for H1299, and 45 seconds at 37°C for Vero cells. Next, trypsin was aspirated, and the cells were dislodged using four times the volume of fresh RPMI or DMEM supplemented with FBS per volume of trypsin added. The dislodged cells were collected and centrifuged at 1000rpm for 2 minutes. The supernatant was aspirated and the cell pellet was resuspended with fresh culture medium before plating to new culture dishes. For cell preservation, the centrifuged cell pellet was resuspended in freezing medium containing 50% DMEM High Glucose, 40% FBS and 10% DMSO and stored in a freezing container at -80°C.

2.2.2 Infectious Bronchitis Virus Stock Preparation

The Beaudette strain of IBV (ATCC VR-22) were obtained from ATCC and adapted to H1299 and Vero cells, as described (433). IBV Beaudette was amplified in ~90% confluent Vero cells at the multiplicity of infection (MOI) of 0.1 and cultured in plain DMEM at 37°C until >90% syncytium formation was observed. The infected cells were then frozen at -80°C and subsequently subjected to a series of three freeze/thaw cycles to mediate the further release of IBV from the cells. IBV was clarified by centrifugation at 4°C at 1,500rpm for 15 minutes. The resulting supernatant was aliquoted and stored at -80°C as virus stocks. The mock cell lysate was prepared by the same treatment using uninfected Vero cells. The virus titer of IBV was quantified by plaque assays and TCID₅₀ (Median Tissue Culture Infectious Dose).

2.2.3 Virus Infection

For experiments involving IBV infection, confluent monolayers of cells were washed twice with its respective plain culture medium before infection with IBV at the indicated MOI or incubated with the same volume of mock lysate. Following 2 hours of adsorption, the cells were washed twice with plain culture medium and covered with plain culture medium for incubation at 37°C until indicated time points to harvest RNA, protein, and supernatant.

2.2.4 Virus Titration

2.2.4.1 Plaque Assay

The viral supernatant was harvested and clarified by centrifugation at 1,500rpm. A 10-fold serial dilution was then performed using plain DMEM to determine the virus titer of IBV stocks and viral supernatants collected from infection experiments. Next, 200µl of each serially diluted supernatant was applied to 100% confluent H1299 or Vero cells in 6-well plates and agitated to ensure complete coverage of the monolayers. The plate is then incubated at 37°C and taken out every 10 minutes for agitation. After 2 hours of adsorption, the unbound viruses were removed, and the cells were washed twice with plain DMEM. 2ml of 0.5% agarose overlay medium was added to each well, and the plates were incubated at 37°C for two days to facilitate plaque formation. On the fifth day, the agarose overlay was removed, and the cells were fixed with 4% PFA for 1 hour before crystal violet staining. The plaque numbers were counted and the viral titer of individual samples was expressed in the unit of log plaque-forming units per ml (PFU/ml). To measure plaque size, 40 plaques were selected by freehand selection tool and its area was measured using NIH ImageJ software.

2.2.4.2 Median Tissue Culture Infectious Dose (TCID₅₀) Assay

96-well dishes were prepared before infection by seeding each well with 40,000 cells in 100µl full DMEM medium to quantify the viral titre one day before the infection. On the day of infection, a series of dilutions from 1:10 to 1:100000 of the original virus sample was prepared and applied to each well in octa-duplicates. The plates were then left to incubate at 37°C with CO₂ for three days. The viral titre was calculated

according to Reed and Muench method (434) by recording the number of infection positive and negative wells.

2.3 MOLECULAR CLONING

2.3.1 Preparation of Chemically Competent Cells

Glycerol stock of *E. coli* strain Top10 was streaked onto an antibiotic-free LB plate overnight at 37°C for colony formation. On day 2, a single colony of *E. coli* was picked and inoculated into a 10ml starter culture of LB and incubated at 37°C in shaker overnight. Following incubation, the starter culture was diluted into 1L of LB media and grown in 37°C shaker until the OD₆₀₀ reaches 0.4 using a spectrophotometer. After which, the bacterial culture was immediately chilled on ice for 30 minutes with occasional swirling and centrifuged at 3,000rpm for 15 minutes at 4°C. The resulting supernatant was decanted. All cell pellets were combined and gently re-suspended in 100ml of ice-cold MgCl₂ and centrifuged at 3,000rpm for 15 minutes at 4°C. Once again, the supernatant was decanted, and the pellet was resuspended in 200ml of CaCl₂ and kept on ice for 20 minutes. Next, the cells were harvested by centrifugation at 3,000rpm for 15 minutes at 4°C, and the supernatant was decanted. The cell pellet was then resuspended in 50ml of ice-cold 85mM CaCl₂ containing 15% glycerol and centrifuged at 2,100rpm for 15 minutes at 4°C to concentrate the competent cells. Finally, the supernatant was decanted, and the pellet was resuspended in 2ml of ice-cold 85mM CaCl₂ containing 15% glycerol and aliquoted into 1.5ml microfuge tubes, snap-frozen in liquid nitrogen and stored at -80°C.

2.3.2 Polymerase Chain Reaction (PCR) for molecular cloning

PCR was used to amplify the coding sequence of genes, as the second step of cloning into expression vectors following reverse transcription (See **Section 2.5.2 Reverse Transcription**). A typical setup of a PCR reaction and its thermocycler program are outlined in **Table 2-6** and **Table 2-7**, respectively. Following PCR amplification, the product was resolved using agarose gel electrophoresis for visualization and purification.

Table 2-6: PCR reaction set up

Components	20µl Reaction	50µl Reaction	Final Concentration
5X Taq Buffer	4µl	10µl	
10mM dNTPs	0.4µl	1µl	200µM each
Forward Primer	1µl	2.5µl	0.5µM
Reverse Primer	1µl	2.5µl	0.5µM
Template DNA ¹	Xµl	Xµl	
Taq Polymerase	0.2µl	0.5µl	0.02U/µl
H ₂ O	to 20µl	to 50µl	

¹For plasmid DNA, 150ng of template DNA was used. If the cDNA synthesis template were used as a template, an amount not exceeding 10% of the final PCR reaction volume was used (i.e., 5µl).

Table 2-7: 3-Step PCR Thermocycling conditions

Cycle Step	Temperature (°C)	Time (MM:SS)	Cycles
Initial Denaturation	98	00:30	1
Denaturation	98	00:10	
Annealing ¹	X	00:30	28-35
Extension	72	00:30/kb	
Final Extension	72	10:00	1
Hold	4	∞	1

¹A list of annealing temperatures used in this study can be found in **Table 2-14**.

2.3.3 Agarose Gel Electrophoresis

Agarose gel was prepared by mixing required amounts of agarose in 50ml of 1X TAE buffer and microwaved for 1 minute until the agarose has completely dissolved. The agarose solution was then left to cool to ~50°C, and 1.5µL of EtBr or 3µl of FluorSAFE was added to achieve a final concentration of 0.2-0.5µg/ml for visualization. Different agarose gel percentages were used based on the size of the DNA fragments to be resolved. As a rule, 1% agarose gel was cast for PCR products ranging between 500bp to 10kb, and 1.5% agarose gel for 1.5% for products between 200bp to 3kb. Before electrophoresis, DNA samples were mixed with 6X DNA loading dye and

loaded onto the lanes of the agarose gel. Electrophoresis was carried out at 110V and monitored following the migration of the indicator dyes. DNA bands were visualized using a gel imager under UV shadowing.

2.3.4 DNA Gel extraction and PCR purification

Following separation and visualization of the DNA bands by agarose gel electrophoresis, the fragments of interest were excised using a scalpel and placed in a 1.5ml microfuge tube. The protocol for agarose gel extraction was performed using a DNA gel extraction kit as per manufacturer's protocol. Briefly, each gel slice is weighed and dissolved in 100µl of Buffer CP per 100mg gel slice. The gel slices were melted on a 55°C heat block for 7 minutes with an occasional vortex. The dissolved DNA fragments in CP buffers were then applied to quick-spin columns and centrifuged at 13,000rpm for 1 minute, followed by two rounds of washing using the wash buffer. After decanting the flow-through, the column was subjected to another round of dry spin, and the DNA fragments were eluted with 30µl of nuclease-free water. The DNA concentration was measured using a Nanodrop 1000 spectrophotometer.

PCR purification is an alternate method to remove primers, dNTPs, enzymes, and other impurities from PCR reaction products following electrophoresis. The protocol for PCR purification was performed using a PCR purification kit as per the manufacturer's protocol. The PCR product was mixed with five times the volume of CP buffer, applied to quick-spin columns and centrifuged at 13,000rpm for 1 minute. After decanting the filtrate, the column was subjected to 2 rounds of washing using DNA wash buffer followed by one round of dry spin. The DNA product was then eluted with 30µl of nuclease-free water. The DNA concentration was measured using a Nanodrop 1000 spectrophotometer.

2.3.5 Plasmid Miniprep (Miniprep)

For small scale isolation of plasmid DNA from bacteria, the plasmid miniprep kit was used as per manufacturer's protocol. In the first step, 3ml of overnight bacteria culture were harvested by centrifugation at 13,000rpm for 1 minute. After the supernatant was

decanted, the cell pellet was resuspended in 100µl of Solution I (resuspension buffer) with vortex. Next, 200µl of Solution II (lysis buffer) was added to lyse the suspension by inverting the microfuge tube gently 6-8 times. The lysis reaction was neutralized by the addition for 400µl of Solution III (neutralization solution) by inverting the microfuge tube 6-8 times before centrifugation at 13,000rpm for 10 minutes. The clear supernatant was applied to the quick-spin columns and centrifuged at 13,000rpm for 1 minute. After two rounds of washing using 700µl of wash buffer and one round of dry spin, the plasmid DNA was eluted with 30µl of nuclease-free water and quantified using a spectrophotometer.

2.3.6 Cloning using Restriction Enzymes

PCR products and vectors were digested with one pair of compatible restriction enzymes to ensure the insert was incorporated in the right orientation into a cloning or expression vector. Standard restriction enzyme protocol was followed for all directional cloning, as outlined in **Table 2-8**. The digestion mixtures were incubated at 37°C or 55°C for 4 hours before DNA electrophoresis. Purified vector and inserts were quantified and ligated at a molar ratio of 1:3 using T4 DNA ligase at 16°C overnight as summarized in **Table 2-9**. To calculate the volume of vector and inserts required, the length of the vector and inserts and DNA amounts were converted into pmol using the formula $\mu g DNA \times \frac{pmol}{660pg} \times \frac{10^6}{1\mu g} \times \frac{1}{N} = pmol DNA$, where N refers to the number of nucleotides and $\frac{pmol}{660pg}$ refers to the average molecular weight of a nucleotide pair.

Table 2-8: Restriction enzyme digestion protocol

Components	Volume (µl)
Restriction Enzyme 1	3 (10 units)
Restriction Enzyme 2	3
DNA (3µg)	X
10X NEBuffer	3
Nuclease-Free H ₂ O	to 30
Total Volume	30

Table 2-9: Ligation Protocol

Components	Volume (μl)
Vector	X ng (0.02 pmol)
Insert	X ng (0.06 pmol)
10 Ligation Buffer	2
T4 DNA Ligase	1
Nuclease-Free H ₂ O	to 20
Total Volume	20

For bacterial transformation, 10μl of ligation mixture was added to 40μl of Top10 *E.coli* chemically competent cells and incubated on ice for 20 minutes, followed by heat shock at 42°C for 45 seconds and returned on ice for 5 minutes. Next, 200μl of SOC media was added, and the *E. coli* was recovered at 37°C with shaking for 1 hour. The recovered bacteria were spread on LB agar plates containing antibiotics such as ampicillin (100μg/μl) or kanamycin (50μg/μl) and incubated overnight at 37°C. On average, five colonies were picked and inoculated into LB broth to determine the presence of insert DNA in plasmid by colony PCR using a forward vector primer and a reverse insert primer.

2.3.7 Plasmids

The complete list of plasmids generated for this study is outlined in **Table 2-10**. The lentiviral vector *plentiCRISPR_V2* (AddGene #52961) was used in this study to enable CRISPR/Cas9 mediated genome editing in mammalian cells. Developed by Dr. F. Zhang, *plentiCRISPR_V2* contains two expression cassettes, hSpCas9 and the chimeric guide RNA. For overexpression studies, *pXJ40-FLAG* and *pcDNA3.1+N-DYK* (Genscript) were used. The *pXJ40-FLAG* vector was previously developed by J. H. Xiao, which contains CMV and T7 promoters followed by an N-terminal FLAG-tag (DYKDDDDK) coding sequence upstream of the multiple cloning site (MCS).

For co-localization studies, plasmids *pEGFP-C2* (Clontech) and *pCDH-CMV-mCherry-T2A-Puro* (AddGene #72264) were used. *pCDH-CMV-mCherry-T2A-Puro*

was digested using *BamHI* and *XbaI* restriction enzymes to generate N-terminal GFP fusion proteins. *pEGFP-C2* was digested with *KpnI* and *BamHI* to generate C-terminal mCherry fusion proteins.

Table 2-10: List of expression plasmids and primer sequence used for cloning

Plasmid Name	Vector Name	Restriction Enzyme Sites	Forward Primer Sequence (5' → 3')	Reverse Primer Sequence (5' → 3')
<i>plentiCRISPRv2-CLP1</i>	plentiCRISPRv2	<i>BsmBI</i>	CACCGAGGACTTCCGGCGGGAATG T	AAACACATTCCCGCCGGAAGTCCTC
<i>plentiCRISPRv2-RNMT</i>	plentiCRISPRv2	<i>BsmBI</i>	CACCGACAGTGTGAGCAGCGGTAT G	AAACCATACCGCTGCTGACACTGTC
<i>PXJ40-FLAG-RNMT_{wt}</i>	PXJ40-FLAG	<i>BamHI/BGIII</i>	CGGGATCCGCAAATTCTGCAAAAG CA	GAAGATCTTCACTGCTGTTTCTCAAAG GC
<i>pcDNA-FLAG-CLP1</i>	pcDNA3.1+N-DYK	<i>KpnI/BamHI</i>	GGGGTACCGGCGAGGAGGCCAAC GA	CGGATCCTCACTTCAGATCCATAAACC GG
<i>pEGFPC2-CLP1¹</i>	pEGFP-C2	<i>KpnI/BamHI</i>		
<i>pEGFPC2-RNMT</i>	pEGFP-C2	<i>KpnI/BamHI</i>	CGGATCCGCAAATTCTGCAAAAGCA	GGGGTACCTCAGAGGATAAACTGCAT GGCA
<i>pmCherry-NSP3</i>	pCDH-CMV-mCherry-T2A-Puro	<i>BamHI/XbaI</i>	GCTCTAGAGGTAAGACTGTCACCTT TGG	CGGATCCTCAACCTGCCTTTTTCTCTA CTAAC
<i>pmCherry-NSP5</i>	pCDH-CMV-mCherry-T2A-Puro	<i>BamHI/XbaI</i>	GCTCTAGATCTGGTTTTAAGAACT GGTTTCTCC	CGGATCCTCATTGTAATCTAACACCAC CAATCTG
<i>pmCherry-NSP15</i>	pCDH-CMV-mCherry-T2A-Puro	<i>BamHI/XbaI</i>	GCTCTAGATCTATCGACAATATTGC	CGGATCCTCATTGAAGCTGTG
<i>pmCherry-NSP16</i>	pCDH-CMV-mCherry-T2A-Puro	<i>BamHI/XbaI</i>	GCTCTAGATCAGCATGGACGTGTG GTTA	CGGATCCTCACATAGTGCACACAAAAG

¹CLP1 insert is derived from the digestion of pcDNA-FLAG-CLP1 and ligated to pEGFP-C2.

2.4 *In Vitro* Transcription

The *in vitro* transcription of luciferase RNA was performed using the mMesaage mMachine T7 transcription kit (Invitrogen). Briefly, the capped transcription is set up at room temperature as outlined in **Table 2-11** and incubated at 37°C for 1 hour, using linearized plasmid *pKTOLuc81A* as the template DNA, followed by the addition of TURBO DNase at 37°C for an additional 15 minutes to remove the template DNA. Finally, the RNA was precipitated using 30µl of lithium chloride and 30µl of nuclease-free water. The concentration of the resultant RNA is quantified using a Nanodrop spectrophotometer.

Table 2-11: Reaction set up for *in vitro* transcription

Components	Volume (µl)
2X NTP/CAP	20
10X Reaction Buffer	4
Linear template DNA	X (2µg)
Nuclease-Free H ₂ O	to 40
Total Volume	40

2.5 RNA Manipulation

2.5.1 Total RNA extraction from mammalian cells and IBV

Total RNA was harvested from cultured cells and viruses using TRIzol and TRIzol LS reagents (Ambion), respectively. In brief, the cells were washed twice with PBS and lysed with 1ml of TRIzol per 10cm² growth area. For viruses in suspension, 0.75ml of TRIzol was added per 0.25ml of the sample. The lysates were homogenized by pipetting up and down several times and incubated for 5 minutes to permit complete dissociation of the nucleoproteins complex. Next, 200µl of chloroform was added and shaken vigorously by hand for 15 seconds and incubated at room temperature for 3 minutes. The lysates were then subjected to phase separation by centrifugation at 12,000g at 4°C for 15 minutes. After removing the aqueous phase into a new microfuge tube, an equal volume of isopropanol was added to precipitate the RNA and centrifuged at 12,000g for 10 minutes. After washing the RNA pellet with 70% ethanol,

the pellet was air-dried and solubilized in 30µl of RNase-free water. The concentration of the total RNA was determined using a Nanodrop spectrophotometer.

2.5.2 Reverse Transcription (RT)

The extracted total RNA was reverse transcribed into cDNA using the MMLV reverse transcriptase (Promega) according to the manufacturer's protocol. For cDNA synthesis of long messages, the ImProm-II Reverse Transcription System (Promega) was used. In short, 5µg of total RNA was mixed with 1µl of oligo-dT/random primer/specific primer and topped up with RNase-free water to 12.5µl. The mixture was then incubated at 70°C for 5 minutes to denature RNA higher-order structures. Next, the pre-RT mixture was then cooled to 4°C to allow primer annealing. The reverse transcription reaction mix was then prepared by combining the components outlined in **Table 2-12** and incubated at suitable temperatures for 1 hour to synthesize the cDNA product. Lastly, the reverse transcriptase was inactivated by heating at 70°C for 15 minutes. The cDNA could now be used for downstream quantitative real-time PCR (qRT-PCR) and PCR processes.

Table 2-12: The reaction set up for a reverse transcription

Components	20µl Reaction	Final Concentration
RNA template	X	3µg
Oligo-dT/Random Primer/ Specific Primer	1	0.5µg/10-20pmol for specific primer
RNase-Free H ₂ O	to 12.5	
5X Reverse Transcription Buffer	4	
RNase Inhibitor	0.5	20U
dNTP Mix	1	0.5mM each dNTP
Reverse Transcriptase	1	

2.5.3 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed using PrecisionFAST 2X qPCR SYBR Master Mix (PrimerDesign) according to the manufacturer's protocol. Briefly, 6.9µl of cDNA product was mixed with 0.6µl of primer mix (primer sequences used for qRT-PCR is outlined in **Table 2-13**) and 7.5µl of 2X SYBR master mix to make up a 15µl reaction. The experiment was performed in triplicates for each sample. Next, the reaction tubes were placed in a 7500 real-time PCR system (Applied Biosystems) and subjected to one round of enzyme activation at 95°C for 2 minutes, followed by 40 cycles of denaturation (95°C, 5 seconds) and annealing/extension (60°C, 20 seconds) with fluorescent data collection at the end of each cycle. The results obtained were in the form of threshold cycles (C_T values). The relative abundance of mRNA in each sample was calculated using the $2^{-\Delta\Delta C_T}$ method, using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. The fold change of the knockout cells was normalized to its corresponding wild-type (WT) samples in time-course experiments. The equations used are as follows:

$$\Delta\Delta C_T = \Delta C_T (\text{KO sample}) - \Delta C_T (\text{WT sample})$$

$$\Delta C_T = C_T (\text{gene of interest}) - C_T (\text{housekeeping gene})$$

$$\text{Fold gene expression} = 2^{-\Delta\Delta C_T}$$

$$= 2^{-((C_T \text{ of KO gene} - C_T \text{ of KO } \beta\text{-actin}) - (C_T \text{ of WT gene} - C_T \text{ of WT } \beta\text{-actin}))}$$

Table 2-13: List of primers used for quantitative real-time PCR

Gene	Accession Number	Amplicon size (bp)	Forward Primer Sequence (5' → 3')	Reverse Primer Sequence (5' → 3')
GAPDH	NM_002046.4	102	CCACTCCTCCA CCTTTGAC	ACCCTGTTGCT GTAGCCA
IBV (+)gRNA	NC_001451.1	100	AGTAGCTTGGA AACGAACGGT	CGGCACTGGCA TCTTTAGAC
IBV(+)sgRNA2	NC_001451.1	106	CTATTATACTA GCCTTGCGCTA GA	GTGCCGTCATA GCTATAGA

2.6 Protein Expression and Analysis

2.6.1 DNA Plasmid Transfection in H1299 Cells

Plasmid transfection to H1299 cells was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Briefly, H1299 cells were seeded at 1×10^6 cells per well in a 6-well plate to achieve 70% confluency on the day of transfection. In a separate microfuge tube, 3.5 μ g of DNA plasmid diluted in 150 μ l of OPTI-MEM media was mixed with 6 μ l of Lipofectamine 2000 diluted in 150 μ l of OPTI-MEM media and incubated at 37°C. After 5 minutes of incubation, the DNA-lipid complex was added to adherent H1299 cells in a dropwise fashion in the 6-well plate. The plate was then incubated for 6 hours, after which the medium was replaced with standard RPMI culture medium to reduce the cytotoxicity of Lipofectamine 2000.

2.6.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

At indicated time points, the cells were washed twice with PBS and harvested using cell scrapers (TPP). After centrifugation at 13,000rpm for 1 minute, the supernatant was decanted and lysed in M-PER protein extraction reagent (Thermo Scientific). The cell lysate was mixed with Laemmli sample containing 100mM DTT following protein quantification using a spectrophotometer. The protein samples were boiled at 95°C for 10 minutes and short-spanned. Equal amounts of protein samples were loaded onto the lanes on the pre-casted 10% SDS-PAGE gels. Electrophoresis was performed using the Bio-Rad Mini-PROTEAN II system at a constant 20 microampere in the presence of 1X gel running buffer. The resolved proteins were transferred onto a 0.2 μ M nitrocellulose membranes using the Bio-Rad Trans-Blot system at 4°C in the presence of 1X Towbin wet transfer buffer.

2.6.3 Western Blot

Following the transfer of SDS-PAGE resolved proteins onto the nitrocellulose membrane, the membrane was treated with 5% bovine serum albumin (BSA) in Tris-buffered saline (20mM Tris-HCl pH7.4, 150mM NaCl and 0.1% Tween 20) to block the non-specific antibody binding sites. Next, the membrane was incubated with 1µg/ml primary antibodies at 4°C overnight. After washing the membrane five times for 5 minutes each, the membrane was incubated with 1:2000 diluted anti-mouse, anti-goat or anti-rabbit IgG antibodies conjugated with horseradish peroxidase (HRP) at room temperature for 1 hour. Next, the membrane was washed for 25 minutes, and the proteins were detected using a chemiluminescence detection kit (homemade) and medical X-ray film (FujiFilm) according to the manufacturer's protocol. For chemiluminescence, 1ml of solution A and 3µl of solution B were used. To re-probe the same membrane with another antibody, the membrane was stripped using a western blot stripping buffer (ThermoFisher Scientific) according to the manufacturer's protocol. After stripping, the membrane was washed with Tris-buffered saline and re-blocked with BSA before incubation with another antibody.

2.6.4 Densitometry and Statistical Analysis

X-ray films from western blots were saved as 8-bit TIFF images, and semi-quantitative measurements of band intensities were performed using ImageJ developed by National Institute of Health (NIH). The band intensities were normalized with the corresponding loading controls, such as β -actin and GAPDH. Quantitative or semi-quantitative data were expressed as the mean and standard error of the mean (SEM) from at least three biological replicates. Paired t-tests were performed on qPCR and plaque assay data, and p-values were determined using GraphPad PRISM statistical software to indicate the significance levels. Asterisks were indicated to show significant differences between the indicated samples and the control sample (*, $p \leq 0.05$; **, $p \leq 0.01$).

2.7 IMMUNOFLUORESCENCE (IF)

1.5 x 10⁴ cells were seeded to each well of a 4-chamber slide and incubated at 37°C overnight before IBV infection to perform immunofluorescence. At indicated time points post-infection, the cell culture medium was removed and rinsed three times using 1X PBS. Next, the cells were fixed with 400µl of 4% PFA at 37°C for 10 minutes. Following the removal and washing of the cells with PBS, the cells were permeabilized by adding 400µl of permeabilization solution (0.1% Triton X-100 in 1X PBS) and incubated at room temperature for 15 minutes. After cell permeabilization, the cells were blocked by adding 500µl of 2% BSA in 1X PBS and incubated at room temperature for 1 hour. Subsequently, the cells were incubated with 1:5000 diluted primary antibodies in 0.1% BSA at 4°C overnight. After removing the primary antibody solution and washing the cells thrice in 1X PBS, the cells were incubated with 1:1000 diluted anti-rabbit IgG antibodies conjugated with Alexa Fluor 594 fluorescent dye along with DAPI as a counterstain at room temperature protected from light for 45 minutes. Fluorescent images were then captured using a Zeiss LSM510 confocal microscope according to the manufacturer's protocol.

2.8 LUCIFERASE ASSAY

Luciferase assay was performed using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's protocol. H1299 cells plated on 6-well plates were transfected with 0.1µg firefly luciferase reporter constructs and 2µg of IBV RNA by electroporation for 16 hours. At the indicated time point, the cells were lysed with 200µl passive lysis buffer. Next, 20µl of cell lysate was mixed with 50µl of the substrate, and its luminescent signal was determined using an Infinite 200 PRO plate reader. 50µl of Stop & Glo reagent was added to quench the firefly luciferase reaction, and the luminescent signal specific for Renilla luciferase was determined. The reporter activity was calculated by normalizing the firefly luciferase readout to that of the Renilla luciferase. Each transfection and luciferase assay were performed in three biological replicates.

2.9 CRISPR/CAS9 GENOME EDITING

2.9.1 Gene Targeting Strategy

According to the RNMT (CCDS77156.1) and CLP1 (CCDS7964.1) sequences obtained from the NCBI database, single guide RNA sequences targeting RNMT and CLP1 were designed using the single guide RNA designer tool from ATUM (<https://www.atum.bio/eCommerce/cas9/input>). The selection of single guide RNA for molecular cloning is subjected to the following conditions: 1) On-target score is above 70/100, 2) single guide RNA targets all splice variants, 3) sequence is unique and is only found in indicated genes' mRNA and 4) sequence is closer towards the 5' of the genes' mRNA. The single guide RNA sequences selected for RNMT and CLP1 are shown in **Table 2-14** and synthesized by Integrated DNA Technologies (IDT).

Table 2-14: Single guide RNA sequences of RNMT and CLP1

Target Gene single guide RNA	Sequence (5' → 3')
CLP1 Oligo1	CACCGAGGACTTCCGGCGGGAATGT
CLP1 Oligo2	AAACACATTCCCGCCGGAAGTCCTC
RNMT Oligo1	CACCGACAGTGTCAGCAGCGGTATG
RNMT Oligo2	AAACCATACCGCTGCTGACACTGTC

Next, the annealed oligos were ligated to *BsmBI*-digested plentiCRISPR_V2 (Addgene #52961), which contained a U6 promoter-driven single guide RNA vector and an EF-1 α promoter-driven Cas9. The ligated plasmids were then transformed to *E. coli* competent cells and incubated overnight. Positive clones were confirmed by colony PCR and confirmed by sequence analysis.

2.9.2 Plasmid Transfection and Drug Selection

H1299 cells were seeded in 6-well plates for transfection with *plentiCRISPRv2-CLP1* and *plentiCRISPRv2-RNMT* using Lipofectamine 2000 (Invitrogen). In each well, 2 μ g of the CRISPR/Cas9 vectors and 6 μ l of the transfection reagent were used, with *pPuro-GFP* transfected to a separate well as a positive control for transfection

efficiency. The transfection procedure was performed according to the manufacturer's protocol. After a 48-hour recovery period, the culture medium was replaced with culture medium supplemented with 2.5µg/ml of puromycin (Invivogen) until a successful selection. Finally, the cells were isolated by limiting dilution as outlined by Addgene to achieve single clones from a pooled population.

2.9.3 Knockout Cell Line Validation

A portion of cells which have been expanded were then lysed to extract the RNA for DNA sequence analysis. RT-PCR of gene region containing RNMT and CLP1 target sequences was performed to validate the generation of the knockout cell line according to manufacturer's protocol. The primers listed in **Table 2-15** were used for PCR first to generate the DNA fragments containing the target sites. Next, the RT-PCR reaction was set up, and the amplicons were then resolved using agarose gel electrophoresis and sequenced.

Table 2-15: Primers used in sequence analysis for the detection of indels

Target Gene	Sequence (5' → 3')
CLP1 Forward Primer	ACTGTGCTGCTCCCTAAATC
CLP1 Reverse Primer	CACCACAATGAAGCCAGCTAC
RNMT Forward Primer	ATCTGGAAGAAGGACACAGC
RNMT Reverse Primer	GAACATCCACAACACCTTCC

Following preliminary confirmation of RNMT and CLP1 knockouts, the protein products of RNMT and CLP1 were confirmed by sequencing and western blot using RNMT- and CLP1-specific antibodies.

2.10 SEQUENCE ANALYSIS OF WILD-TYPE AND MUTANT IBV

Viral RNA was extracted from purified IBV viruses using TRIzol LS reagent (Ambion) according to the manufacturer's protocol. RT-PCR was performed using the M-MLV reverse transcriptase (Promega) and Phusion HiFi *Taq* polymerase (Thermo Scientific). More than 60 primers were specifically designed and used for amplification and sequencing of the WT and mutant viruses. **Table 2-16** outlines the list of primers used and their annealing temperatures. Automated DNA sequencing was carried out using PCR products and specific primers by 1st BASE sequencing service.

2.11 (3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE) (MTT) ASSAY

An MTT assay was performed to determine the cell viability of knockout cells. To perform the MTT assay 10µl of MTT solution (5mg/ml in PBS) was added to each well and incubated at 37°C for 4 hours. Next, the culture medium was aspirated and replaced with 100µl DMSO to dissolve the MTT formazan. The plate was then wrapped in foil to protect from light. The absorbance was read at OD₅₇₀ and OD₆₀₀ using the Infinite 200 Pro microplate reader.

2.12 CO-LOCALIZATION STUDIES

Full-length RNMT and CLP1 proteins were fused to enhanced Green Fluorescent Protein (eGFP), and IBV proteins were fused to mCherry fluorescent protein by molecular cloning methods. Both constructs were transfected to H1299 cells, and after 36 hours post-transfection, the cells were visualized using a fluorescent microscope equipped with a camera. The images were analyzed using ImageJ software.

Table 2-16: List of primers and annealing temperatures used in IBV sequencing

Primer Code	Nucleotide Sequence (5' → 3')	Nucleotide position (5')	Amplicon (bp)	Tm (°C)
IBVLeader	CTATTATACTAGCCTTGCGCTAGA	26	1, 509	60
IBV1535R	CCATCCAAAATTGCAGACC	1535		
IBV18294F	GTTGCGCAAGACCTTGTGTCT	18294	1324	65.6
IBV19618R	CCGCTAGGCAACGTTATTCCA	19618		
IBV15644F	AACCAGGCCGCCATTGAATA	15644	425	62.4
IBV16069R	GCTGCATGAGAACATGCAG	16069		
IBV13894F	GTCTAGAGGAACAGGACCAACT	13894	902	62.6
IBV14796R	CCACTAGCATTGTGTGTTGTG	14796		
IBV11148F	GCGTGTAACAGATAGACG	11148	475	57.9
IBV11623R	ACTCTACGCTACAATGTGC	11623		
IBV12500F	ACTACGCGATACTGAAGATGG	12500	539	62.4
IBV13039R	AAAACAGGAACACCAGCACC	13039		
IBV9288F	AGCCTGTGGTTCAGTTGGTT	9288	1029	63.7
IBV10317R	CAGTAGTGTGCACCAACTCG	10317		
IBV8115F	TAAACCCGGTGTTTTCTGTGG	8115	679	62
IBV8794R	GGTCCAAAGCATAAGCTAACC	8794		
IBV5338F	GGGTGGAAGGTAATGCTA	5338	678	59
IBV6016R	CCTGCTCTACACTATAAGCGTG	6016		
IBV3910F	GTGCCAGTTCTTTCATCAGG	3910	543	61.9
IBV4453R	AGTCTGTAGGATCTCCACCT	4453		
IBV2548F	GGTAAGACTGTCACCTTTGG	2543	491	61
IBV3039R	ATCTTCATCAAGAGGCAGAGGG	3039		
IBV1113F	GGCACTGGCTATTTTTGAG	1113	59.7	519
IBV1632R	TACACGCTCACAGCAATG	1632		
IBV8569F	CTCGTAAGCTGTATGATGGC	8569	1530	60.7
IBV10099R	CTACTGGGTCATACCATTGAC	10099		

Table 2-16: List of primers and annealing temperatures used in IBV sequencing (Continued)

Primer Code	Nucleotide Sequence (5' → 3')	Nucleotide position (5')	Amplicon (bp)	Tm (°C)
IBV14521F	GCAGGTCTACAGGCGAGTTAAT	14521	1504	60.9
IBV16025R	GTATACTGCAAGGCCTATAGC	16025		
IBV15956F	AAGCGTACTACAGTACAAGGTCC	15956	1479	60.9
IBV17435R	CATCTGCACTATCCTTGGTC	17435		
IBV7151F	TCCGCCGGTAGTATGGAAGTT	7151	1505	61.6
IBV8656R	CAAATTCAGAGCCGCGAATA	8656		
IBV2873F	TGATGACATCGAGGAGGAAG	2873	1477	61.8
IBV4351R	AACAATTTCCATCGCGCCAC	4351		
IBV12808F	GGAGAATAAGGATTGGTACGACCC	12808	782	61.8
IBV13590R	CCTTGTTAAAGTGACCTGGC	13590		
IBV10017F	GTTTGTGCTGAAGTGCCTTTC	10017	1472	63.4
IBV11489R	GGCCATGCTATATTAGCACCAC	11489		
IBV1425F	GCCAAAGGCACACAGGTAGTT	1425	1519	64.4
IBV2944R	TAGTGTCACACTCCTCAGCTTC	2944		
IBV4269F	TATTTGCAAACGCTTGCGCA	4269	1500	64.5
IBV5769R	CACGGTCTTATAGCTAGCGACA	5769		
IBV11414F	TGGCACAGAGTTACACCCAA	11414	1481	64.6
IBV12895R	AAAGCACGTCGTACAATAGGTCCC	12895		
IBVNSP16F	CGGGATCCATGTCAGCATGGACGTGTGGT	19509	908	71.2
IBVNSP16R	CCGCTCGAGCTACATAGTGACACACAAA	20417		
IBV5678F	TGTCTCCACAATTCCTAAAGACGC	5678	2000	65.7
IBV7678R	CCCAGGACACAAAACCAGGAA	7678		
IBV25624F	GCGTGCTTTTAGTTCAAT	25624	1991	52.6
IBV27614R	TGCTCTAACTCTATACTAG	27614		
IBV23835F	CAGACCCAAAAAGTCTGTT	23835	2009	56.8
IBV25843R	AGTTTTCCCTTGAATACT	25843		

Table 2-16: List of primers and annealing temperatures used in IBV sequencing (Continued)

Primer Code	Nucleotide Sequence (5' → 3')	Nucleotide position (5')	Amplicon (bp)	Tm (°C)
IBV22037F	AACCTGATGGCTCAATTGCC	22037	1991	62.2
IBV24027R	AGTCTAGACTGTGCCAAAGG	24027		
IBV20148F	TTGGGTGCAAGTGAAAAGG	20148	2039	61.7
IBV22186R	GGACCTTATCCATACGCGTT	22186		
IBV13483F	GGAGATCCTGCTTTGTTAGT	13483	7002	60.2
IBV20485R	AACCACTAGGTGGTCTGA	20485		
IBV6530F	CGCCATGTTAAACCTACAGC	13579	7049	61.8
IBV13579R	TGACCTGGCTTTACCGTT	6530		

CHAPTER 3

FUNCTIONAL CHARACTERIZATION OF RNA (GUANINE-7-) METHYLTRANSFERASE (RNMT) WITH CORONAVIRUS NSP3 PAPAIN-LIKE PROTEASE

3 RNA (Guanine-7-) Methyltransferase (RNMT)

In eukaryotic cells, the capping of mRNA at the 5' ends are an essential post-transcriptional event which functions to allow efficient splicing, stability and translation of mRNA. The presence of the cap marks these mRNA transcripts for a series of processing events required for protein translation, such as the recruitment of Eukaryotic Initiation Factor 4F (eIF4F) and the cap-binding complex.

Generally, the mRNA transcript is modified at the first nucleotide by the addition of the 5' capped structure; a 7-methylguanosine linked to the first transcribed nucleotide by a 5'-5' triphosphate bridge. The formation of the cap structure is performed in three sequential enzymatic steps. First, the 5' triphosphate of the nascent mRNA (pppN) is hydrolyzed by an RNA 5'-triphosphatase (TPase) to generate the diphosphate 5'-ppN, to which GMP will be added by the RNA guanylyltransferase to produce a cap intermediate (GpppN). Finally, an RNA guanine-7 methyltransferase (RNMT) then methylates the attached GMP (cap) at the N7 position by utilizing the methyl donor AdoMet to produce the final cap-0 structure (m7GpppN) and a by-product, AdoHcy (S-adenosyl homocysteine).

Regardless of where the virus is residing in the eukaryotic host cell upon entry, it is imperative to solve the capping problem for efficient viral replication. Eukaryotic viruses have evolved a variety of ways to cope with mRNA capping to safeguard the stability of viral genomic RNA. For DNA viruses, the straightforward solution is by exploiting the host cellular capping machinery. In cases where utilization of the host capping machinery is not possible, ssRNA viruses, such as the influenza A viruses, have been known to steal short 5'-capped transcripts, produced by the cellular DNA dependent RNA polymerase, and use them to prime the transcription of viral mRNA, in a process known as cap-snatching.

Because both genomic and subgenomic CoV mRNAs have a 5' cap structure, CoVs are known to encode its capping apparatus such as m7G-MTase in NSP 14 and 2'-O-MTase in NSP16, which are reported to be host-derived (435). However, it remains

unclear whether CoVs also concurrently use host RNMT to boost its viral replication, or if RNMT interacts with other viral proteins to advance their infection.

In our previous siRNA screening data, we identified RNA (Guanine-7-) Methyltransferase (RNMT) as a potential target exploited for IBV infection. Here, we functionally characterized RNMT and demonstrated their importance in IBV infection through RNMT KO cells, termed RNMT^{-/-}. Based on these findings, we identified NSP3 papain-like protease (PL_{pro}) as a potential viral interacting partner of RNMT.

3.1 Complete construction of *plentiCRISPR-RNMT*

Two-complementary *RNMT*-targeting single guide RNAs were designed to induce efficient knockout of the human *RNMT* gene. The *RNMT* gene consists of fifteen exons alternatively spliced to produce five RNMT isoforms. The single guide RNA designer tool from ATUM (<https://www.atum.bio/eCommerce/cas9/input>) was used to assess off- and on-target efficiency. Based on optimal off-target scores combined with the highest possible on-target scores, exon 6 of the RNMT gene was chosen for targeting (**Figure 3-1A**).

Cloned lentiviral construct, termed *plentiCRISPR-RNMT*, was generated by ligating the annealed single guide RNAs into the RNA scaffold induced by the U6 promoter in the lentiCRISPRv2 vector. The lentiviral construct also encodes for SpCas9 under the control of the EF-1 α promoter. The lentiCRISPRv2 vector also contains a FLAG tag fused to the SpCas9 cDNA sequence followed by DNA sequences encoding a P2A self-cleaving peptide and the Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE) for enhanced gene expression (**Figure 3-1B**). Following *BsmBI* digestion of lentiCRISPRv2 and ligation of annealed inserts bearing the target site of RNMT, the cloned plasmids were sent for DNA sequencing (**Figure 3-1C**). The resultant construct was used in all following experiments.

3.2 Generation of RNMT double allele knockout in H1299 cells

IBV adapted human non-small lung carcinoma cells H1299 expressing RNMT were utilized in the generation of RNMT KO cells. H1299 cells were transfected with 2µg of *plentiCRISPR-RNMT*. As the lentiviral construct does not contain an enhanced Green Fluorescent Protein (eGFP) transcript, an eGFP-C2 plasmid was transfected to another batch of H1299 cells in parallel as an indication of transfection efficiency. At day 2, the fluorescence analysis of eGFP expression showed high transfection efficiency (**Figure 3-2A**). At 24-hours post-transfection, the culture medium was replaced with fresh DMEM medium containing 2.5µg/ml of puromycin for one week to remove the non-transfected cells. Subsequently, a monoclonal cell line was generated from a polyclonal pool of stable cells by limiting dilution into 96-well plates to isolate single clones for genotypic and phenotypic characterization. Cell growth was monitored daily, and stable colonies were transferred into larger vessels. Once the cell numbers from each clone were sufficient, the cells were harvested for total RNA and analysed by RT-PCR (**Figure 3-2B**) and DNA sequencing.

DNA sequencing chromatograms obtained from H1299 cells transfected with *plentiCRISPR-RNMT* showed an insertion of one thymidine nucleotide from the cut site (3-bp upstream of PAM) as a result of double-stranded break formation by SpCas9 and subsequent non-homologous end joining (**Figure 3-2C**). The indel formation induced a frameshift mutation, leading to the translation of a premature stop codon (**Figure 3-2D**). Western blot analysis of gene knockout using anti-RNMT antibody showed a double allele knockout of RNMT (**Figure 3-2E**), indicating the successful generation of RNMT^{-/-} cells.

A **B**

Figure 3-2 *plentiCRISPR-RNMT* transfection efficiency in H1299 cells and RT-PCR amplification of RNMT exon 6 region.

- (A)** Immunofluorescence analysis of eGFP expression two days post-transfection. H1299 cells were transfected with either 2 μ g of eGFP-C2 (positive control) or *plentiCRISPR-RNMT*. Immunofluorescence analysis of eGFP at day 2 post-transfection indicates high transfection efficiency. An untransfected control was included as a negative control.
- (B)** PCR amplicons of RNMT exon 6 from various *plentiCRISPR-RNMT* transfected clones. Following the generation of a monoclonal cell line, total RNA was isolated from *plentiCRISPR-RNMT* transfected H1299 cells and used as a template for RT-PCR. Amplification of RNMT using sequence-specific primers resulted in a 700bp PCR product, which was sequenced using the forward PCR primer.

3.3 RNMT gene knockout is not a limiting factor to cell growth

The generation of a homozygous knockout cell line is sometimes lethal to the cells. In order to validate whether RNMT knockout was lethal, an MTT assay was performed over three days. To monitor the cell viability of RNMT^{-/-} cells, both WT H1299 and RNMT^{-/-} cells were seeded at 8×10^3 , 4×10^3 and 2×10^3 cells per well for days 0, 1 and 2 respectively based on H1299 doubling time. The absorbance readings of each well were taken at two wavelengths, 570nm for the formazan product and 650nm for the reference wavelength. The difference between the two wavelengths was generated by the microplate scanner and normalized to the WT H1299 cells assayed for the day.

Figure 3-3 Cell viability of RNMT^{-/-} cells were marginally affected by RNMT gene knockout.

The number of viable cells was measured by recording changes in absorbance at 570nm using a microplate spectrophotometer, with 650nm as a reference wavelength. Data are represented as mean percent cell viability $\pm$ SEM from three biological replicates.

By the third day of the MTT assay, RNMT^{-/-} showed only a slight decrease in cell viability, indicating that RNMT cell viability status could be discounted as a limiting factor affecting IBV infection in the subsequent time-course experiments.

3.4 RNMT^{-/-} suppresses IBV infection from the early replication stages of the viral life cycle

To examine changes which may occur during a coronaviral infection in both WT H1299 and RNMT^{-/-} cells, an IBV infection time course was performed. Since one IBV infection cycle usually lasts 16-18 hours, a 24-hour time course was deemed ideal for detecting changes in viral transcription, translation and egress in both WT and RNMT^{-/-} cells. During the 24 hours, total RNA, protein lysate and virus supernatant were collected at blocks of 4-hour intervals starting from 8-hour post-infection (h.p.i). Total RNA isolated from cells were reverse-transcribed and applied in qPCR to detect IBV gRNA and sgRNA expression levels, which correspond to the replication stages of the CoV life cycle. On the other hand, protein lysates were applied in western blot to detect changes in viral protein translation, while virus supernatant was used in the study of virus egress through quantification of viruses via plaque assays.

At the end of the 24 hours, both IBV gRNA (**Figure 3-4A**) and sgRNA (**Figure 3-4B**) of RNMT^{-/-} cells exhibited a reduction at all indicated time points compared to the WT cells, except for an increase in IBV gRNA in RNMT^{-/-} cells at 24 h.p.i. This is attributed to a secondary viral infection, where progeny virions emerging from the primary infection begins to infect the neighbouring cells. In WT cells, viral replication is not affected by any gene knockouts and hence, viral replication is going as per normal and cells die at 20 hours at the end of a primary infection (one IBV life cycle: 16-18 hours). However, in RNMT^{-/-} cells, the absence of RNMT may have hampered viral replication and as such, fewer cell deaths were observed since the number of progeny viruses produced were lower compared to WT cells. This then allows the progeny viruses emerging from the primary infection to infect the neighbouring surviving cells. The fold change in IBV sgRNA was statistically significant ($p=0.0082$), indicating that RNMT knockout affects IBV RNA replication.

A **B**

Figure 3-4 RNMT knockout reduces IBV genomic (gRNA) and subgenomic RNA (sgRNA) replication in RNMT^{-/-} cells.

- (A)** Bar diagram showing the fold change of IBV genomic RNA (gRNA) expression in WT H1299 and RNMT^{-/-} cells. Both WT H1299 and RNMT^{-/-} cells were infected at MOI-1 and cells were harvested at indicated hour post-infection (h.p.i). Total RNA lysate was isolated and subjected to RT-qPCR. Data represent the mean $\Delta\Delta CT \pm$ SEM of three biological replicates.
- (B)** Bar diagram showing the fold change of IBV subgenomic RNA (sgRNA) expression in WT H1299 and RNMT^{-/-} cells. Both WT H1299 and RNMT^{-/-} cells were infected at MOI-1 and cells were harvested at indicated hour post-infection (h.p.i). Total RNA lysate was isolated and subjected to RT-qPCR. Data represent the mean $\Delta\Delta CT \pm$ SEM of three biological replicates. The difference in fold change between WT H1299 RNMT^{-/-} sgRNA was statistically significant ($p=0.0082$).

Since a decrease in viral RNA replication would often lead to a decrease in viral protein translation, a western blot was performed to compare the IBV structural proteins produced in the WT H1299 and RNMT^{-/-} cells. At each time point, RNMT^{-/-} cells showed a reduction in IBV structural protein production compared to the WT cells, with more than 50% decrease in IBV M production from 12 h.p.i through 24 h.p.i (**Figure 3-4C**), indicating that the decreased RNA replication perpetuates into IBV protein translation.

C

Figure 3-4 RNMT knockout reduces IBV structural protein translation in RNMT^{-/-} cells.

(C) Western blot analysis of WT H1299 (+) and RNMT knockout (-) cells. 50µg of total protein lysate was loaded in each lane, incubated with anti-IBV S and anti-IBV M and detected indirectly via a secondary antibody with chemiluminescence. A β-actin antibody was used to detect the 43 kDa beta-actin protein, which was used as an internal loading control. The relative fold changes in each protein are indicated in the table for each lane.

Finally, to investigate if low viral RNA and protein translation levels equate to fewer progeny viruses, a plaque assay was performed to quantify the viruses produced in both WT H1299 and RNMT^{-/-} cells at each time point.

Consistent to the previous outcome observed in the qPCR and western blots, the virus titration of RNMT^{-/-} cells also showed a statistically significant decrease in comparison to the WT H1299 cells in the plaque assay ($p=0.0036$) (**Figure 3-4D**). In addition, immunofluorescence staining of IBV infection also revealed a decrease in IBV infection; the infection was restricted to the cytoplasm of the cell (**Figure 3-4E**). Collectively, these results suggest that RNMT may function as an enhancer in IBV infection; deletion of RNMT results in a decrease in IBV infection.

3.5 Overexpression of wild type RNMT rescued RNMT function to near wild-type levels in RNMT^{-/-} cells

To further validate the function of RNMT as a viral enhancer, a knockout recovery of RNMT^{-/-} cells were performed by overexpressing the WT RNMT gene. To this end, the full-length WT RNMT gene was ligated into a PXJ40-FLAG vector using *Bam*HI and *Bgl*II restriction enzymes. 10µg of RNMT overexpression plasmid, termed PXJ40-FLAG-RNMT, was then transfected to RNMT^{-/-} cells. In parallel, WT H1299 cells were also transfected with eGFP-C2 as an indicator of transfection efficiency before being subjected to a 24-hour IBV infection time-course experiment.

At each indicated time point post-infection, RNMT^{-/-} cells overexpressed with a WT RNMT gene had been able to recover and increase IBV gRNA (**Figure 3-5A**) and sgRNA (**Figure 3-5B**) to near WT H1299's IBV gRNA and sgRNA expression levels at 12 h.p.i and 20 h.p.i, indicating that WT RNMT overexpression can recover IBV replication in RNMT^{-/-} cells. In addition to an increase in IBV RNA replication, protein translation of IBV M and N protein had also marginally increased with RNMT overexpression compared to the WT cells (**Figure 3-5C**). On the other hand, virus titer of RNMT overexpressed RNMT^{-/-} cells had increased by at least 1.5 fold at all indicated h.p.i except at 24 h.p.i (**Figure 3-5D**), as all cells have lysed by 20 h.p.i and have not proceeded into a secondary infection.

A **B**

Figure 3-5 Overexpression of wild type RNMT rescues IBV genomic (gRNA) and subgenomic RNA (sgRNA) replication to near wild type levels in RNMT^{-/-} cells.

(A) Bar diagram showing fold change of IBV genomic RNA (gRNA) expression in WT H1299 and RNMT^{-/-} cells transfected with a control plasmid (eGFP-C2) and a RNMT overexpression plasmid (PXJ40-FLAG-RNMT), respectively. The transfected cells were infected at MOI-1 and harvested at indicated hour post-infection (h.p.i). Total RNA lysate was isolated and subjected to RT-qPCR. Data represent mean $\Delta\Delta CT \pm SEM$ of three biological replicates.

(B) Bar diagram showing fold change of IBV subgenomic RNA (sgRNA) expression in WT H1299 and RNMT^{-/-} cells transfected with a control plasmid (eGFP-C2) and a RNMT overexpression plasmid (PXJ40-FLAG-RNMT), respectively. The transfected cells were infected at MOI-1 and harvested at indicated hour post-infection (h.p.i). Total RNA lysate was isolated and subjected to RT-qPCR. Data represent mean $\Delta\Delta CT \pm SEM$ of three biological replicates.

C

D

Figure 3-5 Overexpression of wild type RNMT rescues IBV structural protein translation and viral titer in RNMT^{-/-} cells.

(C) Western blot analysis of WT H1299 and RNMT^{-/-} cells transfected with a control plasmid (eGFP-C2) and a RNMT overexpression plasmid (PXJ40-FLAG-RNMT), respectively. 50µg of total protein lysate was loaded in each lane, incubated with anti-IBV N, anti-IBV M, and anti-Flag Tag and detected indirectly via a secondary antibody with chemiluminescence. A GAPDH antibody was used to detect the 37 kDa GAPDH protein, which was used as an internal loading control. The relative fold changes in each protein are indicated in the table for each lane.

(D) Bar diagram showing the fold change in viral titer in WT H1299 and RNMT^{-/-} cells transfected with a control plasmid (eGFP-C2) and a RNMT overexpression plasmid (PXJ40-FLAG-RNMT), respectively. Both WT H1299 and RNMT^{-/-} cells were infected at MOI-1 and harvested at indicated hour post-infection (h.p.i). Data represent the mean fold change ± SEM of the virus titer recovered from plaque assays of three biological replicates.

3.6 Overexpression of wild type RNMT promotes IBV replication in H1299 cells

Since RNMT overexpression had led to a recovery of IBV replication in RNMT^{-/-} cells, RNMT overexpression in WT H1299 cells should result in an enhanced IBV infection compared to its WT counterparts with regular RNMT expression. To confirm our hypothesis, WT H1299 cells were transfected with a RNMT overexpression plasmid (PXJ40-FLAG-RNMT). In parallel, another batch of WT H1299 cells were also transfected with eGFP-C2 as an indicator of transfection efficiency before being subjected to a 24-hour IBV infection time-course experiment. As shown in **Figure 3-6A** and **Figure 3-6B**, RNMT overexpression had further promoted IBV replication in WT H1299 cells, with a statistically significant increase in IBV gRNA expression ($p=0.007$).

At the same time, IBV structural protein translation and viral titer have also increased in cells transfected to overexpress RNMT (**Figure 3-6C** and **Figure 3-6D**). These results further supported RNMT as a host factor exploited by IBV to promote infection.

3.7 RNMT^{-/-} cell-adapted IBV formed smaller plaques in H1299 cells

To elucidate the potential interacting viral partner(s) of RNMT, serial passage of IBV in RNMT^{-/-} cells were performed to allow the WT IBV to adapt to RNMT^{-/-} cells. The strategy of continuously passaging IBV in the KO cells increase the susceptibility of its viral interacting partner to gain mutations to compensate for the loss of RNMT. Mutations identified in the viral genome will then be mapped to the corresponding viral protein for further analysis. To this end, WT IBV was continuously passaged in two cell types: (1) H1299 cells and (2) RNMT^{-/-} cells at MOI-1 for 24 hours. After which, cell lysates and their supernatants were harvested and subjected to 3 freeze-thaw cycles to release the additional virus in the cell, and the virus titer at passage 1, 10 and 20 were quantified by western blot and plaque assay. At the end of 20 passages, WT IBV has shown a 1 log increase in viral titer (**Figure 3-7A**) and 30% and 77% increase in the translation of IBV N and M protein products respectively (**Figure 3-7B**), implying that WT IBV had adapted well to RNMT^{-/-} cells.

Since well-adapted viruses tend to cause aggressive infections in the cell line that was used for the adaptation, H1299-adapted IBV and RNMT^{-/-} cell-adapted IBV at its 1st, 10th and 20th passages were also observed for changes in plaque size as a marker for IBV infectivity. Before serial passaging, WT IBV was known to form larger plaques on H1299 cells in plaque assays. However, plaques created by RNMT^{-/-} cell-adapted IBV became 74% smaller with increased passages in plaque assays using WT H1299 cells (**Figure 3-7C**). Likewise, plaques were formed in plaque assays using RNMT^{-/-} cells become 76.5% larger with increased passages. This indicates that RNMT^{-/-} cell-adapted IBV was successfully generated, and RNMT^{-/-} cell-adapted IBV was no longer able to infect WT H1299 cells as efficiently like before.

A **B**

Figure 3-7 Serially passaged IBV displayed adaptation to RNMT^{-/-} cells by passage 20.

(A) Bar diagram showing the viral titer of RNMT^{-/-} cell-adapted IBV at passages 1, 10 and 20. RNMT^{-/-} cells were infected with IBV at MOI-1 for 24 hours for a total of 20 days. Culture supernatants from passages 1, 10, and 20 were harvested at 24 h.p.i and subjected to plaque assay. Virus titers were expressed as the logarithm of plaque-forming units (PFU) per ml of supernatant.

(B) Western blot analysis of WT IBV adaptation to RNMT^{-/-} cells at passages 1, 10 and 20. RNMT^{-/-} cells were infected with IBV at MOI-1 for 24 hours for a total of 20 days. 50µg of total protein lysate was loaded in each lane, incubated with anti-IBV M and anti-IBV N and detected indirectly via a secondary antibody with chemiluminescence. A GAPDH antibody was used to detect the 37 kDa GAPDH protein, which was used as an internal loading control. The relative fold changes in each protein are indicated in the table for each lane, calculated as the band intensities of each passage divided by passage 1 of the corresponding protein.

C

Cell	Passage	Plaque Size (mm ²)	
		Mean	Standard Deviation
H1299	1	4.66	1.606
	10	2.51	0.596
	20	1.21	0.757
RNMT ^{-/-}	1	2.97	1.218
	10	12.66	4.43
	20	12.62	1.779

Figure 3-7 RNMT^{-/-} cell-adapted IBV produced larger plaques on RNMT^{-/-} cells and smaller plaques on H1299 cells in plaque assays.

(C) Culture supernatants obtained from passage 1, 10 and 20 were serially diluted in a series of 10, 100, 1000, 10,000, 100,000, 1,000,000 times (-1 to -6) and subjected to plaque assays in H1299 cells and RNMT^{-/-} cells. The mean plaque size for each adapted IBV is indicated in the table.

3.8 RT-PCR sequencing of RNMT^{-/-} cell-adapted IBV genomic RNA revealed multiple mutations in NSP3 Papain-like protease

To screen for mutations which may be found in the gRNA of RNMT^{-/-} adapted IBV, plaques formed in passage 20 plaque assay plates in **Figure 3-7C** and its passage-matched H1299 adapted IBV were isolated and agar meshed in the culture medium. In this instance, only larger plaques from the 10⁻⁵ and 10⁻⁶ dilution were selected to ensure that IBV isolated from these plaque assays consist of only the well-adapted viruses. The first supernatant obtained was then used to amplify the adapted IBVs through infection with either RNMT^{-/-} or H1299 cells. Once sufficient RNMT^{-/-} and H1299 adapted IBV was obtained, both adapted viruses' RNA was isolated, subjected to RT-PCR procedures and sent for sequencing. To ensure quality sequencing reads, PCR primers were designed in a staggering manner to cover the entire IBV gRNA (refer to **Table 2-14** for primer sequences used for this study).

NSP	IBV Nucleotide Position	Mutation	Outcome
3	6143	T → G	L1199C
	6144	G → C	
	6152	C → G	T1202S
	6922	T → G	L1459V

Table 3-1 RNMT^{-/-} cell-adapted IBV showed multiple mutations in non-structural protein 3 (NSP3) papain-like protease (PL_{pro}). IBV agar plaques obtained from WT H1299 and RNMT^{-/-} plaque assays were meshed to release viruses into the culture supernatant. The supernatant was subsequently used to infect H1299 or RNMT^{-/-} cells for virus amplification. IBV gRNA was isolated using TRIzol LS according to manufacturer's protocol. RT-PCR sequencing was performed using primers staggered across the entire IBV genome. IBV nucleotide position refers to IBV Beaudette strain.

Based on sequencing results and comparison of common mutations found in H1299- and RNMT^{-/-} cell-adapted IBV, RNMT^{-/-} cell-adapted IBV bears 4 unique nucleotide changes in NSP3 PL_{pro}, resulting in 3 protein mutations (L1199C, T1202S and L1459V) (**Table 3-1**). Reference to the functional domains in NSP3 revealed that L1199C and T1202S were located on the ubiquitin-like domain of PL_{pro}, while L1459V were located on the C-terminal of PL_{pro}.

3.9 Papain-like protease and RNMT co-localization in H1299 cells

We next investigated the co-localization of NSP3 PL_{pro} and RNMT when both proteins were co-transfected. It is known that NSP3 PL_{pro} localizes to the cytoplasm and RNMT to the nucleoplasm and cytoplasm. However, NSP3 PL_{pro} can also be found in the nucleoplasm. H1299 cells were transiently transfected with a vector expressing eGFP-tagged RNMT at the N-terminus (eGFP-RNMT) together with NSP3 PL_{pro} tagged with mCherry at the C-terminus (PL_{pro}-mCherry). The cells were analysed by confocal microscopy 24 hours after transfection and showed that both RNMT and NSP3 PL_{pro} co-localized in the nucleoplasm and cytoplasm (**Figure 3-8**).

3.10 RNMT is involved in the replication stages of the virus life cycle

The inherent function of RNMT and the co-localization of NSP3 PL_{pro} and RNMT in the cytoplasm and nucleoplasm of the cell suggested that RNMT was unlikely to be involved in the IBV attachment and entry stages in IBV infection. To prove that RNMT was not involved in IBV attachment and entry, 2.5 μg of IBV gRNA was electroporated into H1299 and RNMT^{-/-} cells to bypass the attachment and entry stages of the IBV life cycle. At 16 h.p.i, whole-cell lysate (WCL) and culture supernatants were harvested to quantify virus particles from infected cells and to determine IBV RNA expression levels. As shown in **Figure 3-9A**, IBV gRNA and sgRNA levels in RNMT^{-/-} cells decreased compared to H1299 cells, despite introducing an equal amount of IBV gRNA into each cell type. Moreover, virus titer levels in RNMT^{-/-} cells are 1.2 log lower than H1299 cells (p=0.0069) (**Figure 3-9B**). These results confirm that RNMT is

indeed not involved in the entry and attachment stages of the IBV life cycle, as the same amount of IBV RNA electroporated still resulted in lower levels of IBV RNA expression levels and viral titer produced.

Since RNMT was not involved in the attachment/entry stages of the IBV life cycle, we next investigated if RNMT was involved in IBV release. To this end, culture supernatants and total lysates were harvested 12 h.p.i to detect viral proteins and enumerate virion particles. As shown in **Figure 3-9C**, while IBV M and N detected in the WCL of RNMT^{-/-} was only marginally lower than those detected in H1299 cells, the ratio between the amount of viral proteins detected in the WCL and supernatant were not significantly different between the WT and RNMT^{-/-} cells, implying that RNMT did not affect the release of virions to the supernatant. Consistent with western blot data presented in **Figure 3-4C** at the same h.p.i, this points RNMT to be involved in the replication steps of the IBV life cycle after virus entry.

3.11 Discussion

Capping is a common modification made to the 5' end of eukaryotic mRNA transcripts. In addition to providing mRNA stability, the 5' cap is also an essential component for the recruitment of the eukaryotic translation machinery. Most RNA viruses require the use of mRNA-cap dependent cellular translation machinery to translate their viral genome into proteins (436). In the case of CoVs, 5' cap structure found in its genomic RNA permits the translation of the replicase gene in its first step upon virus entry. Moreover, the cap protects the viral RNA from degradation by cellular nucleases (437) and circumvent viral RNA recognition by the cellular innate immune response (310). While CoVs acquire their cap structure using virus-encoded capping enzymes, the potential exploitation of cellular capping machinery in tandem or other processes of IBV infection remains unexplored, given the similarities in the capping process shared by cellular and viral RNA transcripts (438).

Based on previous siRNA screening study, the cellular RNA methyltransferase, RNMT, had been identified as a potential target in IBV infection (429). In this study, we followed up on this lead by further dissecting the functional role of RNMT in CoV infection through the generation of RNMT double allele KO stable cell lines using CRISPR/Cas9 genome editing technique and IBV as the model CoV. As the purpose of our genome editing efforts was to generate loss-of-function of RNMT in H1299 cells, the type of indels created in RNMT^{-/-} (i.e., addition/deletion of nucleotides) were beyond the user's control. The resultant RNMT KO cell line, termed RNMT^{-/-}, has been verified through sequencing and western blot analysis. RNMT^{-/-} cells' RNA were routinely sequenced to ensure the genetic integrity of the RNMT^{-/-} cell line to ensure that RNMT remains knocked out in subsequent passages.

With the generation of RNMT^{-/-}, we next examined the viability of these KO cells using MTT assay and revealed that RNMT KO did not significantly affect cell viability. The observation was not surprising owing to the presence of other mRNA methyltransferases in the cell, such as cap methyltransferase 1 and 2 (CMTR1/2) (439). Recently, the discovery of a novel cap methyltransferase, Adenosine N6-methyltransferase (CAPAM), was found to catalyse N6-methylation of the first

transcribed nucleotide adenosine to create the cap structure m7G(5')ppp(5')m6Am (440-443). Structural comparisons between RNMT and other methyltransferases (CAPAM, CMTR1 and CMTR2) showed homology in their catalytic regions (440, 444). This suggests that the presence of other cellular methyltransferases may help to support mRNA capping even in the absence of RNMT.

However, the presence of CAPAM and CMTR1 did not help to compensate for the function of RNMT, as RNMT^{-/-} showed a reduction in IBV RNA replication, structural protein production and virus titer in comparison to the WT H1299 cells. This result is consistent to results generated by previous siRNA screening and suggested that RNMT likely functions as an enhancer in IBV infection, as RNMT KO diminished viral production. Furthermore, RNMT overexpression studies performed through the transfection of WT RNMT gene into H1299 and RNMT^{-/-} cells confirmed our hypothesis. Overexpression of WT RNMT in WT cells had further boosted IBV production, while an RNMT overexpression in RNMT^{-/-} cells helped partially rescue the cells and enhance IBV production levels to near WT infection levels. These results imply that IBV showed a preference for RNMT over CAPAM and CMTR1, as RNMT methylate on the N7 position of the added guanosine identical to the IBV RNMT counterpart NSP14 to form the cap structure m7G(5')ppp(5')A. Conversely, CAPAM methylate on the N6 position of the added adenosine to form the cap structure m7G(5')ppp(5')m6Am, while CMTR1 forms m7G(5')ppp(5')Am through methylation on the O2 position of the first transcribed ribose.

Next, to identify the viral protein responsible for the changes in IBV infection between WT and RNMT^{-/-} cells, serial IBV passage in both WT and RNMT^{-/-} cells were performed to understand how IBV adapted to compensate for RNMT loss by comparing viral gene mutations. At the end of passage 20, we observed that IBV which had been serially passaged in RNMT^{-/-} cells displayed lower syncytium formation and formed smaller plaques on plaque assays. In addition, through sequencing of RNMT-adapted IBV, unique mutations such as L1199C, T1202S and L1459V were identified in the NSP3 PL_{pro}, suggesting PL_{pro} is a potential viral partner of RNMT, closely working with PL_{pro} in polyprotein processing. Given the low infectivity of RNMT-

adapted IBV in WT cells, these results also hint that PL_{pro} may become a promising target in the generation of coronaviral vaccines.

Finally, we also demonstrated that this RNMT-PL_{pro} interaction occurred in the replication stages of the CoV life cycle. This observation is congruent to the intrinsic function of NSP14. Like other CoVs, IBV replicates in the cytoplasm of infected cells and adopt the eukaryotic capping pathway which involves other NSPs: 1) NSP13 hydrolyzes the γ -phosphate from the 5'-triphosphate RNA, 2) transfer of GMP to the 5'-triphosphate by a GTase to form GpppN, 3) guanosine methylation by NSP14 in the presence of a methyl donor S-adenosyl-L-methionine (SAM) to form a cap-0 structure (7mGpppN) and 4) NSP10/NSP16 complex methylate the ribose group of the first transcribed nucleotide to convert the cap-0 into a cap-1 structure (438). Moving forward, the viral NSP14 may also be a potential antiviral target, as RNA methylation provides a molecular signature for discrimination between self and non-self RNA. Double-stranded RNA, 5'-triphosphate RNA and incorrectly capped RNA can be detected by intracellular PRRs, such as RIG-I and Mda5 (312, 326). On the other hand, miscapped RNA can be recognized by IFN-induced protein with tetratricopeptide repeats 1 (IFIT1) and inhibits its translation (445). Thus, compounds blocking NSP14 could help to limit viral replication and promoting viral clearance. Together, this study characterized the role of RNMT in IBV replication and presented PL_{pro} as a potential viral partner to RNMT, which will be useful as a target in the development of antivirals and vaccines. However, the use of knockout models without RNMT expression may not be physiological, and hence it would be interesting to study RNMT-NSP3 interaction *in vivo* using a mouse model.

CHAPTER 4

FUNCTIONAL CHARACTERIZATION OF CLEAVAGE AND POLYADENYLATION FACTOR I SUBUNIT 1 (CLP1) WITH NSP3, 5, 15 AND 16

4 CLEAVAGE AND POLYADENYLATION FACTOR I SUBUNIT 1 (CLP1)

mRNA polyadenylation is a critical post-transcriptional modification in eukaryotes. The 3' end cleavage of nascent mRNAs, followed by the addition of a poly(A) tail plays an essential role in many aspects of cellular metabolism of mRNA, including nuclear export of mature mRNAs, mRNA stability and increasing mRNA translation efficiency.

The cellular process of mRNA polyadenylation involves two distinct phases. In the first phase, short poly(A) tail of about 10 nts is synthesized based on interactions between the polyadenylation-related proteins and the polyadenylation signal (PAS) hexamer AAUAAA or its variant (AGUAAA, AUUAAA or UAUAAA) located some 10-30 nts upstream of the poly(A) cleavage site. On recognition of the poly(A) cleavage site, the second phase begins with the rapid addition of 200 nts.

As opposed to eukaryotic mRNAs which are used only for translation, polyadenylation of viral RNA in RNA viruses may be involved in both replication and translation. In CoV, how long the poly(A) tract is generated from a short poly(U) tract has not been described in literature so far, although it has been postulated to result from a stuttering mechanism by viral RNA-dependent RNA polymerase during the synthesis of gRNA or sgRNA, or by a cellular cytoplasmic poly(A) polymerase.

In our previous siRNA screening data, we identified Cleavage and Polyadenylation Factor I Subunit 1 (CLP1) as a potential target exploited for IBV infection. Here, we functionally characterized CLP1 and demonstrated their importance in IBV infection through CLP1 KO cells, termed CLP1^{-/-}. Based on these findings, we identified NSP3, NSP5, NSP15, and NSP16 as potential viral interacting partners of CLP1. The results presented here provide the first evidence that will help in elucidating coronaviral polyadenylation.

4.1 Complete construction of *plentiCRISPR-CLP1*

Two-complementary *CLP1*-targeting single guide RNAs were designed to induce efficient knockout of the human *CLP1* gene. The *CLP1* gene consists of three exons alternatively spliced to produce two CLP1 isoforms. The single guide RNA designer tool from ATUM (<https://www.atum.bio/eCommerce/cas9/input>) was used to assess off- and on-target efficiency. Based on optimal off-target scores combined with the highest possible on-target scores, exon 3 of the CLP1, which can be found on both CLP1 isoforms, was chosen for targeting (**Figure 4-1A**).

Cloned lentiviral construct, termed *plentiCRISPR-CLP1*, was generated by ligating the annealed single guide RNAs into the RNA scaffold induced by the U6 promoter in the lentiCRISPRv2 vector. The lentiviral construct also encodes for SpCas9 under the control of the EF-1 α promoter. The lentiCRISPRv2 vector also contains a FLAG tag fused to the SpCas9 cDNA sequence followed by DNA sequences encoding a P2A self-cleaving peptide and the Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE) for enhanced gene expression (**Figure 4-1B**). Following *BsmBI* digestion of lentiCRISPRv2 and annealed inserts bearing the target site of CLP1, the cloned plasmids were sent for DNA sequencing (**Figure 4-1C**). The resultant construct was used in all following experiments.

Figure 4-1 The molecular cloning strategy of *plentiCRISPR-CLP1*.

- (A)** Human CLP1 target sequence used for single guide RNA design, indicated with the number of exons (blue rectangles). The sequence selected was based on the on- and off-target efficiency assessed by ATUM. The supposed cut-site (red triangle) and subsequent PAM sequence (in red letters) are as indicated.
- (B)** Schematics of the lentiCRISPRv2 vector. The lentiviral vector contains a U6 promoter which drives the expression of the single guide gRNA scaffold. *BsmBI* restriction sites were used for the cloning of the single guide RNA sequence. The EF-1α promoter drives the expression of SpCas9 fused to a FLAG tag followed by P2A self-cleaving sequence and WPRE.
- (C)** DNA sequencing chromatogram indicating the correct insertion of CLP1 single guide RNA (boxed in grey) into lentiCRISPRv2.

4.2 Generation of CLP1 double allele knockout in H1299 cells

IBV adapted human non-small lung carcinoma cells H1299 expressing CLP1 were utilized in the generation of CLP1 KO cells. H1299 cells were transfected with 2µg of *plentiCRISPR-CLP1*. As the lentiviral construct does not contain an eGFP transcript, an eGFP-C2 plasmid was transfected to another batch of H1299 cells in parallel as an indication of transfection efficiency. At day 2, fluorescence analysis of eGFP expression showed high transfection efficiency (**Figure 4-2A**). At 24-hours post-transfection, the culture medium was replaced with fresh DMEM medium containing 2.5µg/ml of puromycin for 1 week to remove the non-transfected cells. Subsequently, a monoclonal cell line was generated from a polyclonal pool of stable cells by limiting dilution into 96-well plates to isolate single clones for genotypic and phenotypic characterization. Cell growth was monitored daily, and stable colonies were transferred into larger vessels. Once the cell numbers from each clone were sufficient, the cells were harvested for total RNA and analysed by RT-PCR (**Figure 4-2B**) and DNA sequencing.

DNA sequencing chromatograms obtained from H1299 cells transfected with *plentiCRISPR-CLP1* showed a deletion of one adenine nucleotide from the cut site (3-bp upstream of PAM) as a result of double-stranded break formation by SpCas9 and subsequent non-homologous end joining (**Figure 4-2C**). The indel formation induced a frameshift mutation, leading to the translation of a premature stop codon (**Figure 4-2D**). Western blot analysis of gene knockout using anti-CLP1 antibody showed a double allele knockout of CLP1 (**Figure 4-2E**), indicating the successful generation of CLP1^{-/-} cells.

A **B**

Figure 4-2 *plentiCRISPR-CLP1* transfection efficiency in H1299 cells and RT-PCR amplification of CLP1 exon 3 region.

(A) Immunofluorescence analysis of eGFP expression two days post-transfection. H1299 cells were transfected with either 2 μ g of eGFP-C2 (positive control) or plentiCRISPR-CLP1. Immunofluorescence analysis of eGFP at day 2 post-transfection indicates transfection efficiency. An untransfected control was included as negative control.

(B) PCR amplicons of CLP1 exon 3 from various plentiCRISPR-CLP1 transfected clones. Following the generation of monoclonal cell line, total RNA was isolated from plentiCRISPR-CLP1 transfected H1299 cells and used as a template for RT-PCR. Amplification of CLP1 using sequence-specific primers resulted in a 240bp PCR product, which was sequenced using the forward PCR primer.

C

D

E

Figure 4-2 Sequencing data and western blot analysis of CLP1 clone #2 evidenced a double allele KO of CLP1 in *plentiCRISPR-CLP1* transfected H1299 cells.

- (C) Sequencing chromatogram of CLP1 clone #2 evidenced a 1-bp adenine deletion, with the wild-type (WT) sequence presented as a comparison.
- (D) The deletion of an adenine nucleotide resulted in a frameshift mutation, leading to the nonsense translation of CLP1 followed by the premature stop codon at amino acid position 232.
- (E) Western blot analysis of CLP1 expression in WT H1299 and CLP1^{-/-} (KO) cells. 50µg of total protein lysate was loaded in each lane, incubated with anti-CLP1 and detected indirectly via a secondary antibody with chemiluminescence. A GAPDH antibody was used to detect the 37 kDa GAPDH protein, which was used as an internal loading control.

4.3 CLP1 gene knockout is not a limiting factor to cell growth

The generation of a homozygous knockout cell line is sometimes lethal to the cells. In order to validate whether CLP1 knockout was lethal, an MTT assay was performed over three days. To monitor the cell viability of CLP1^{-/-} cells, both WT H1299 and CLP1^{-/-} cells were seeded at 8×10^3 , 4×10^3 and 2×10^3 cells per well for days 0, 1 and 2 respectively based on H1299 doubling time. The absorbance readings of each well were taken at two wavelengths, 570nm for the formazan product and 650nm for the reference wavelength. The difference between the two wavelengths was generated by the microplate scanner and normalized to the WT H1299 cells assayed for the day.

Figure 4-3 Cell viability of CLP1^{-/-} cells were marginally affected by CLP1 gene knockout.

The number of viable cells was measured by recording changes in absorbance at 570nm using a microplate spectrophotometer, with 650nm as a reference wavelength. Data are represented as mean percent cell viability $\pm$ SEM from three biological replicates.

By the third day of the MTT assay, CLP1^{-/-} showed a slight decrease in cell viability, indicating that CLP1 cell viability status could be discounted as a limiting factor affecting IBV infection in the subsequent time-course experiments.

4.4 CLP1^{-/-} suppresses IBV infection from the early replication stages of the viral life cycle

To examine changes which may occur during a coronaviral infection in both WT H1299 and CLP1^{-/-} cells, an IBV infection time course was performed. Since one IBV infection cycle usually lasts 16-18 hours, a 24-hour time course was deemed ideal for detecting changes in viral transcription, translation, and egress in both WT and CLP1^{-/-} cells. During the 24 hours, total RNA, protein lysate and virus supernatant were collected at blocks of 4-hour intervals starting from 8 h.p.i. Total RNA isolated from cells were reverse-transcribed and applied in qPCR to detect IBV gRNA and sgRNA expression levels, which correspond to the replication stages of the CoV life cycle. On the other hand, protein lysates were applied in western blot to detect changes in viral protein translation, while virus supernatant was used in the study of virus egress through quantification of viruses via plaque assays.

At the end of the 24 hours, both IBV gRNA (**Figure 4-4A**) and sgRNA (**Figure 4-4B**) of CLP1^{-/-} cells exhibited at least 60% decrease at all indicated time points compared to the WT cells, except for an increase in IBV gRNA and sgRNA in CLP1^{-/-} cells at 24 h.p.i (IBV gRNA, $p=0.2536$; IBV sgRNA: $p=0.1159$). This is attributed to a secondary viral infection, where progeny virions emerging from the primary infection begins to infect the neighbouring cells. In WT cells, viral replication is not affected by any gene knockouts and hence, viral replication is going as per normal and cells die at 20 hours at the end of a primary infection (one IBV life cycle: 16-18 hours). However, in CLP1^{-/-} cells, the absence of CLP1 may have hampered viral replication and as such, fewer cell deaths were observed since the number of progeny viruses produced were lower compared to WT cells. This then allows the progeny viruses emerging from the primary infection to infect the neighbouring surviving cells. Notably, IBV gRNA replication was the most significantly affected, with only a maximum of 30% expression in CLP1^{-/-} cells (compared with wild-type cells) across 8 to 20 h.p.i.

A **B**

Figure 4-4 CLP1 knockout reduces IBV genomic (gRNA) and subgenomic RNA (sgRNA) replication in CLP1^{-/-} cells.

(A) Bar diagram showing the fold change of IBV genomic RNA (gRNA) expression in WT H1299 and CLP1^{-/-} cells. Both WT H1299 and CLP1^{-/-} cells were infected at MOI-1 and cells were harvested at indicated hour post-infection (h.p.i). Total RNA lysate was isolated and subjected to RT-qPCR. Data represent mean $\Delta\Delta CT \pm$ SEM of three biological replicates.

(B) Bar diagram showing the fold change of IBV subgenomic RNA (sgRNA) expression in WT H1299 and CLP1^{-/-} cells. Both WT H1299 and CLP1^{-/-} cells were infected at MOI-1 and cells were harvested at indicated hour post-infection (h.p.i). Total RNA lysate was isolated and subjected to RT-qPCR. Data represent mean $\Delta\Delta CT \pm$ SEM of three biological replicates.

Since a decrease in viral RNA replication would often lead to a decrease in viral protein translation, a western blot was performed to compare the IBV structural proteins produced in the WT H1299 and CLP1^{-/-} cells. At each time point, CLP1^{-/-} cells showed a reduction in IBV structural protein production compared to the WT cells, with more than 50% decrease in IBV M production from 12 h.p.i through 24 h.p.i. (**Figure 4-4C**), indicating that the decreased RNA replication perpetuates into IBV protein translation.

Finally, to investigate if low viral RNA and protein translation levels equate to fewer progeny viruses, a plaque assay was performed to quantify the viruses produced in both WT H1299 and CLP1^{-/-} cells at each time point.

Consistent to the previous outcome observed in the qPCR and western blots, the virus titration of CLP1^{-/-} cells also showed a statistically significant decrease in comparison to the WT H1299 cells in the plaque assay ($p=0.0174$) (**Figure 4-4D**). In addition, immunofluorescence staining of IBV infection also revealed a decrease in IBV infection; the infection was restricted to the cytoplasm of the cell (**Figure 4-4E**). Taken as a whole, these results suggest that CLP1 may function as an enhancer in IBV infection; deletion of CLP1 results in a decrease in IBV infection.

D **E**

Figure 4-4 CLP1 knockout reduces IBV viral titer and the number of infected cells on immunofluorescence (IF).

(D) Bar diagram showing the fold change in viral titer in WT H1299 and CLP1^{-/-} cells. Both WT H1299 and CLP1^{-/-} cells were infected at MOI-1 and harvested at indicated hour post-infection (h.p.i). Data represent the mean fold change $\pm$ SEM of the virus titer recovered from plaque assays of three biological replicates. The fold change in the viral titer between WT H1299 and CLP1^{-/-} was statistically significant. *, $p=0.0174$.

(E) IF images of WT H1299 and CLP1^{-/-} cells at 6-hour post infection (h.p.i). WT H1299 and CLP1^{-/-} cells were infected at MOI-1 and subjected to IF using anti-IBV and Alexa-594 conjugated anti-rabbit antibodies.

4.5 Overexpression of wild type CLP1 rescued CLP1 function to near wild-type levels in CLP1^{-/-} cells

To further validate the function of CLP1 as a viral enhancer, a knockout recovery of CLP1^{-/-} cells were performed by overexpressing the WT CLP1 gene. To this end, the full-length WT CLP1 gene was ligated into a PXJ40-FLAG vector using *BamHI* and *BglII* restriction enzymes. 10µg of CLP1 overexpression plasmid, termed PXJ40-FLAG-CLP1, was then transfected to CLP1^{-/-} cells. In parallel, WT H1299 cells were also transfected with eGFP-C2 as an indicator of transfection efficiency before being subjected to a 24-hour IBV infection time-course experiment.

At each indicated time point post-infection, CLP1^{-/-} cells overexpressed with a WT CLP1 gene had been able to recover and increase IBV gRNA (**Figure 4-5A**) and sgRNA (**Figure 4-5B**) to near WT H1299's IBV gRNA and sgRNA expression levels from 12 h.p.i to 24 h.p.i. This indicates that WT CLP1 overexpression can recover IBV replication in CLP1^{-/-} cells. In addition to an increase in IBV RNA replication, protein translation of IBV N protein had also marginally increased with CLP1 overexpression compared to the WT cells (**Figure 4-5C**). On the other hand, virus titre in CLP1 overexpressed CLP1^{-/-} cells had increased compared to CLP1^{-/-} cells from 16 h.p.i to 24 h.p.i (**Figure 4-5D**).

A **B**

Figure 4-5 Overexpression of wild type CLP1 rescues IBV genomic (gRNA) and subgenomic RNA (sgRNA) replication to near wild type levels in CLP1^{-/-} cells.

(A) Bar diagram showing fold change of IBV genomic RNA (gRNA) expression in WT H1299 and CLP1^{-/-} cells transfected with a control plasmid (eGFP-C2) and a CLP1 overexpression plasmid (PXJ40-FLAG-CLP1), respectively. The transfected cells were infected at MOI-1 and harvested at indicated hour post-infection (h.p.i). Total RNA lysate was isolated and subjected to RT-qPCR. Data represent mean $\Delta\Delta CT \pm SEM$ of three biological replicates.

(B) Bar diagram showing fold change of IBV subgenomic RNA (sgRNA) expression in WT H1299 and CLP1^{-/-} cells transfected with a control plasmid (eGFP-C2) and a CLP1 overexpression plasmid (PXJ40-FLAG-CLP1), respectively. The transfected cells were infected at MOI-1 and harvested at indicated hour post-infection (h.p.i). Total RNA lysate was isolated and subjected to RT-qPCR. Data represent mean $\Delta\Delta CT \pm SEM$ of three biological replicates.

C

D

Figure 4-5 Overexpression of wild type CLP1 rescues IBV structural protein translation and viral titer in CLP1^{-/-} cells.

(C) Western blot analysis of WT H1299 and CLP1^{-/-} cells transfected with a control plasmid (eGFP-C2) and a CLP1 overexpression plasmid (PXJ40-FLAG-CLP1), respectively. 50µg of total protein lysate was loaded in each lane, incubated with anti-IBV N, anti-IBV M and anti-CLP1 and detected indirectly via a secondary antibody with chemiluminescence. A GAPDH antibody was used to detect the 37 kDa GAPDH protein, which was used as an internal loading control. The relative fold changes in each protein is indicated in the table for each lane.

(D) Bar diagram showing the fold change in viral titer in WT H1299 and CLP1^{-/-} cells transfected with a control plasmid (eGFP-C2) and a CLP1 overexpression plasmid (PXJ40-FLAG-CLP1) respectively. Both WT H1299 and CLP1^{-/-} cells were infected with IBV at MOI-1 and harvested at indicated hour post-infection (h.p.i). Data represent the mean fold change ± SEM of the virus titer recovered from plaque assays of three biological replicates.

4.6 Overexpression of wild type CLP1 promotes IBV replication in H1299 cells

Since CLP1 overexpression had led to a recovery of IBV replication in CLP1^{-/-} cells, CLP1 overexpression in WT H1299 cells should result in an enhanced IBV infection compared to its WT cells with regular CLP1 expression. To confirm our hypothesis, WT H1299 cells were transfected with a CLP1 overexpression plasmid (PXJ40-FLAG-CLP1). In parallel, another batch of WT H1299 cells were also transfected with eGFP-C2 as an indicator of transfection efficiency before being subjected to a 24-hour IBV infection time-course experiment. As shown in **Figure 4-6A** and **Figure 4-6B**, CLP1 overexpression had further promoted IBV replication in WT H1299 cells, with a statistically significant increase in IBV gRNA expression ($p=0.0027$) and sgRNA expression ($p=0.014$).

Figure 4-6 Overexpression of wild type CLP1 promotes IBV genomic (gRNA) and subgenomic RNA (sgRNA) replication in wild type H1299 cells.

(A) Bar diagram showing fold change of IBV genomic RNA (gRNA) expression in WT H1299 cells transfected with either a control plasmid (eGFP-C2) or a CLP1 overexpression plasmid (PXJ40-FLAG-CLP1). The transfected cells were infected at MOI-0.5 and harvested at indicated hour post-infection (h.p.i). Total RNA lysate was isolated and subjected to RT-qPCR. Data represent mean $\Delta\Delta CT \pm$ SEM of three biological replicates. The difference in fold change between WT H1299 transfected with eGFP-C2 and PXJ40-FLAG-CLP1 gRNA was statistically significant. **, $p=0.0027$.

(B) Bar diagram showing fold change of IBV subgenomic RNA (sgRNA) expression in WT H1299 cells transfected with either a control plasmid (eGFP-C2) or a CLP1 overexpression plasmid (PXJ40-FLAG-CLP1). The transfected cells were infected at MOI-0.5 and harvested at indicated hour post-infection (h.p.i). Total RNA lysate was isolated and subjected to RT-qPCR. Data represent mean $\Delta\Delta CT \pm$ SEM of three biological replicates. The difference in fold change between WT H1299 transfected with eGFP-C2 and PXJ40-FLAG-CLP1 sgRNA was statistically significant. *, $p=0.014$.

At the same time, IBV M had shown a moderate increase in protein expression, while IBV N did not show any noticeable difference in its protein expression (**Figure 4-6C**). On the other hand, virus titre had also significantly increased in cells transfected to overexpress CLP1 at all time-points ($p=0.005$) (**Figure 4-6D**). These results further supported CLP1 as a host factor exploited by IBV to promote infection.

C

D

Figure 4-6 Overexpression of wild type CLP1 promotes IBV structural protein translation and viral titer in wild type H1299 cells.

(C) Western blot analysis of H1299 cells transfected with either a control plasmid (eGFP-C2) or a CLP1 overexpression plasmid (PXJ40-FLAG-CLP1). 50µg of total protein lysate was loaded in each lane, incubated with anti-IBV M, anti-IBV N and anti-CLP1 and detected indirectly via a secondary antibody with chemiluminescence. A β-actin antibody was used to detect the 43 kDa β-actin protein, which was used as an internal loading control. The relative fold changes in each protein are indicated in the table for each lane.

(D) Overexpression of CLP1 in H1299 cells improves IBV replication. Bar diagram showing the fold change in viral titer in WT H1299 cells transfected with either a control plasmid (eGFP-C2) or a CLP1 overexpression plasmid (PXJ40-FLAG-CLP1). Both H1299 cells transfected with either eGFP-C2 or PXJ40-FLAG-CLP1 were infected at MOI-0.5 and harvested at indicated hour post-infection (h.p.i). Data represent the mean fold change ± SEM of the virus titer recovered from plaque assays of three biological replicates. **, 0.005.

4.7 CLP1^{-/-} cell-adapted IBV formed smaller plaques in H1299 cells

To elucidate the potential interacting viral partner(s) of CLP1, serial passage of IBV in CLP1^{-/-} cells were performed to allow the WT IBV to adapt to CLP1^{-/-} cells. The strategy of continuously passaging IBV in the KO cells increase the susceptibility of its viral interacting partner to gain mutations to compensate for the loss of CLP1. Mutations identified in the viral genome will then be mapped to the corresponding viral protein for further analysis. To this end, WT IBV was continuously passaged in two cell types: (1) H1299 cells and (2) CLP1^{-/-} cells at MOI-1 for 24 hours. After which, cell lysates and their supernatants were harvested and subjected to 3 freeze-thaw cycles to release the additional virus in the cell, and the virus titer at passage 1, 10 and 20 were quantified by western blot and plaque assay. At the end of 20 passages, WT IBV has shown a 2-log increase in viral titer (**Figure 4-7A**) and 70% and 80% increase in the translation of IBV N and M protein products respectively (**Figure 4-7B**). This implies that WT IBV had adapted well to CLP1^{-/-} cells.

Since well-adapted viruses tend to cause aggressive infections in cell line that was used for the adaptation, H1299-adapted IBV and CLP1^{-/-} cell-adapted IBV at its 1st, 10th and 20th passages were also observed for changes in plaque size as a marker for IBV infectivity. Before serial passaging, WT IBV was known to form larger plaques on H1299 cells in plaque assays. However, plaques created by CLP1^{-/-} cell-adapted IBV became 18% smaller with increased passages in plaque assays using WT H1299 cells (**Figure 4-7C**). Likewise, plaques were formed in plaque assays using CLP1^{-/-} cells became 36.2% larger with increased passages. This indicates that CLP1^{-/-} cell-adapted IBV was successfully generated, and CLP1^{-/-} cell-adapted IBV was no longer able to infect WT H1299 cells as efficiently like before.

A **B**

Figure 4-7 Serially passaged IBV displayed adaptation to CLP1^{-/-} cells by passage 20.

- (A)** Bar diagram showing the viral titer of CLP1^{-/-} cell-adapted IBV at passages 1, 10 and 20. CLP1^{-/-} cells were infected with IBV at MOI-1 for 24 hours for a total of 20 days. Culture supernatants from passages 1, 10, and 20 were harvested at 24 h.p.i and subjected to plaque assay. Virus titers were expressed as the logarithm of plaque-forming units (PFU) per ml of supernatant.
- (B)** Western blot analysis of WT IBV adaptation to CLP1^{-/-} cells at passages 1, 10 and 20. CLP1^{-/-} cells were infected with IBV at MOI-1 for 24 hours for a total of 20 days. 50µg of total protein lysate was loaded in each lane, incubated with anti-IBV M and anti-IBV N and detected indirectly via a secondary antibody with chemiluminescence. A GAPDH antibody was used to detect the 37 kDa GAPDH protein, which were used as an internal loading control. The relative fold changes in each protein are indicated in the table for each lane, calculated as the band intensities of each passage divided by passage 1 of the corresponding protein.

C

Cell	Passage	Plaque Size (mm ²)	
		Mean	Standard Deviation
H1299	1	1.57	0.47
	10	1.382	0.562
	20	1.303	0.641
CLP1 ^{-/-}	1	2.2864	0.859
	10	3.487	0.639
	20	3.581	1.203

Figure 4-7 CLP1^{-/-} cell-adapted IBV produced larger plaques on CLP1^{-/-} cells, and smaller plaques on H1299 cells in plaque assays.

(C) Culture supernatants obtained from passage 1, 10 and 20 were serially diluted in a series of 10, 100, 1000, 10 000, 100 000, 1 000 000 times (-1 to -6) and subjected to plaque assays in H1299 and CLP1^{-/-} cells. The mean plaque size for each adapted IBV is indicated in the table.

4.8 RT-PCR sequencing of CLP1^{-/-} cell-adapted IBV genomic RNA revealed multiple mutations in NSP3, NSP5, NSP15 and NSP16

To screen for mutations which may be found in the gRNA of CLP1^{-/-} adapted IBV, plaques formed in passage 20 plaque assay plates in **Figure 4-7C** and its passage-matched H1299 adapted IBV were isolated and agar meshed in the culture medium. In this instance, only larger plaques from the 10⁻⁵ and 10⁻⁶ dilution were selected to ensure that IBV isolated from these plaque assays consist of only the well-adapted viruses. The first supernatant obtained was then used to amplify the adapted IBVs through infection with either CLP1^{-/-} or H1299 cells. Once sufficient CLP1^{-/-} and H1299 adapted IBV was obtained, both adapted viruses' RNA was isolated, subjected to RT-PCR procedures and sent for sequencing. To ensure quality sequencing reads, PCR primers were designed in a staggering manner to cover the entire IBV gRNA (refer to **Table 2-14** for primer sequences used for this study).

NSP	IBV Nucleotide Position	Mutation	Outcome
3	4690	T → C	S715P
5	9697	GTG deletion	G379 deletion
15	18945	T → G	S1A
16	19512	G → C	A2P

Table 4-1 CLP1^{-/-} cell-adapted IBV showed multiple mutations in non-structural protein 3 (NSP3), NSP5, NSP15 and NSP16. IBV agar plaques obtained from WT H1299 and CLP1^{-/-} plaque assays were meshed to release viruses into the culture supernatant. The supernatant was subsequently used to infect H1299 or CLP1^{-/-} cells for virus amplification. IBV gRNA was isolated using TRIzol LS according to manufacturer's protocol. RT-PCR sequencing was performed using primers staggered across the entire IBV genome. IBV nucleotide position refers to IBV Beaudette strain.

Based on sequencing results and comparison of common mutations found in H1299- and CLP1^{-/-} cell-adapted IBV, CLP1^{-/-} cell-adapted IBV bears 4 unique nucleotide changes in the NSP3, NSP5, NSP15 and NSP16 (**Table 4-1**). Reference to mutation location found in NSP3 showed that S715P is located on the transmembrane domain of PL_{pro}. On the other hand, G379 deletion is located on Domain III of NSP5 M_{pro}. Lastly, S1A and A2P are both located on the N-termini of NSP15 and NSP16,

respectively. These results suggest that NSP3, NSP5, NSP15 and NSP16 are the putative interacting partners of CLP1.

4.9 NSP5 and NSP15 co-localizes individually with CLP1 in H1299 cells

We next investigated the co-localization of NSP3, NSP5, NSP15, NSP16 and CLP1 when both proteins were co-transfected. It is known that NSP3, NSP5, NSP15 and NSP16 localize to the cytoplasm, and CLP1 to the nucleoplasm and cytoplasm. To this end, H1299 cells were transiently transfected with a vector expressing eGFP-tagged CLP1 at the N-terminus (eGFP-CLP1) together with either NSP3, NSP5, NSP15 or NSP16 tagged with mCherry at the C-terminus (NSP3/5/15/16-mCherry). Due to the large size of NSP3, only the c-terminal half of NSP3 was cloned into the vector. The cells were analysed by confocal microscopy 24 hours after transfection and showed that both NSP5 and NSP15 individually co-localized with CLP1 in the nucleoplasm and cytoplasm (**Figure 4-8**).

However, we were unable to obtain co-localization images for NSP3 and NSP16. In the case of NSP3, this might be attributed to the unstable expression of the C-terminal half of NSP3, as NSP3 is a multi-domain protein which requires interactions with the N-terminal half of NSP3 to express correctly. Conversely, NSP16-mCherry plasmid may be poorly transfected into H1299 cells due to the large size of the plasmid at 10kb.

4.10 CLP1 is involved in the replication stages of the virus life cycle

The inherent function of CLP1 and the co-localization of CLP1 separately with NSP5 and NSP15 in the cytoplasm and nucleoplasm of the cell suggested that CLP1 was unlikely to be involved in the IBV attachment and entry stages in IBV infection. To prove that CLP1 was not involved in IBV attachment and entry, 2.5 µg of IBV gRNA was electroporated into H1299 and CLP1^{-/-} cells to bypass the attachment and entry stages of the IBV life cycle. At 16 h.p.i, WCL and culture supernatants were harvested to quantify virus particles from infected cells and to determine IBV RNA expression levels. As shown in **Figure 4-9A**, IBV gRNA and sgRNA levels in CLP1^{-/-} cells decreased compared to H1299 cells, despite introducing an equal amount of IBV RNA into each cell type. Moreover, virus titer levels in CLP1^{-/-} cells were 2 logs lower than H1299 cells ($p=0.0028$) (**Figure 4-9B**). These results confirm that CLP1 is indeed not involved in the entry and attachment stages of the IBV life cycle, as the same amount of IBV RNA electroporated still resulted in lower levels of IBV RNA expression levels and viral titer produced.

Figure 4-9 CLP1 is not involved in the IBV attachment and entry stages of the viral life cycle.

(A) Bar diagram showing fold change of IBV genomic RNA (gRNA) and subgenomic RNA (sgRNA) expression in WT H1299 and CLP1^{-/-} cells. H1299 and CLP1^{-/-} cells are electroporated with 2.5µg of IBV gRNA and harvested at 16-hour post infection (h.p.i). Total RNA lysate was isolated and subjected to RT-qPCR. Data represent the mean $\Delta\Delta CT \pm SEM$ of three biological replicates.

(B) IBV titer in H1299 cells remained higher than CLP1^{-/-} cells, even though equal amounts of IBV gRNA was electroporated to both cell types. Culture supernatants obtained from H1299 and CLP1^{-/-} cells at 16-hour post infection (h.p.i) were serially diluted in a series of 10, 100, 1000, 10 000, 100 000, 1 000 000 times (-1 to -6) and subjected to plaque assays in Vero cells. Virus titers were expressed as the logarithm of plaque-forming units (PFU) per ml of supernatant. **, $p = 0.0028$.

Since CLP1 was not involved in the attachment/entry stages of the IBV life cycle, we next investigated if CLP1 is involved in IBV release. To this end, culture supernatants and total lysates were harvested 12 h.p.i to detect viral proteins and enumerate virion particles. As shown in **Figure 4-9C**, while IBV M and N detected in the WCL of CLP1^{-/-} is only marginally lower than those detected in H1299 cells, the ratio between the amount of viral proteins detected in the WCL and supernatant were not significantly different between the WT and CLP1^{-/-} cells, implying that CLP1 does not affect the release of virions to the supernatant. Consistent with western blot data presented in **Figure 4-4C** at the same h.p.i, this points CLP1 to be involved in the replication steps of the IBV life cycle after virus entry.

4.11 Discussion

As small infectious entities reliant on the host cell for survival, viruses often evolve to adapt to different cellular environments to exploit the translation machinery. As opposed to eukaryotic mRNAs which are used only for translation, polyadenylation of viral RNA in RNA viruses are usually involved in multiple processes of the virus life cycle, including translation and replication. RNA viruses have developed several mechanisms for synthesizing poly(A) tail based on genetic features to ensure the stability of their gRNA. For instance, influenza viruses utilize short oligo(U) stretches located at the 5' end of negative-strand gRNA as a PAS by the viral RNA polymerase possessing a stuttering mechanism during positive-strand synthesis (446). Conversely, polioviruses utilize a homopolymeric stretch on the negative-strand as a template for poly(A) tail addition during positive-strand synthesis (447).

Although the mechanism for CoV polyadenylation remains elusive, it is postulated that a stuttering mechanism based on the short poly(U) stretch found in the negative-strand genome is utilized (448). Notably, the poly(A) tail and hexamer AGUAAA are essential elements responsible for CoV polyadenylation. Other studies on CoV poly(A) tail length regulation have been reported, where the CoV poly(A) tail length increased in the early stages of infection, and gradually decreased after hitting peak tail length of approximately 65 nts in the advanced stages of viral infection in both cell culture and animals (431). The change in poly(A) tail length appears to function in viral translation regulation, as a longer CoV poly(A) tail is associated with better translation efficiency. However, the mechanism by which the CoV poly(A) tail is regulated remains unclear.

In the previous study (429), we identified the eukaryotic cleavage and polyadenylation factor I subunit 1, CLP1, as a potential cellular target in IBV infection. In this study, we followed up on this lead by further dissecting the functional role of CLP1 in CoV infection through the generation of CLP double allele KO stable cell lines using CRISPR-CAS9 genome editing technique and IBV as the model CoV. The resultant CLP1 KO cell line, termed CLP1^{-/-}, has been verified through sequencing and western blot analysis. CLP1^{-/-} cells were routinely sequenced to ensure the genetic integrity of CLP1^{-/-} cells to ensure that CLP1 knockout was permanent.

With the successful generation of CLP1^{-/-}, we next evaluated the effect of CLP1 KO in H1299 cells by using MTT assay to measure cell viability. We reported that CLP1 KO does not significantly affect cell viability, although it was noted that CLP1^{-/-} doubling time was longer compared to its WT counterpart. While knowledge of CLP1 on polyadenylation is limited, studies on other polyadenylation factors, such as the polyadenylation/cleavage stimulatory factor (CstF), have shown that the increase in 64-kDa subunit of polyadenylation/cleavage stimulatory factor (CstF-64) is associated with G0 to S phase transition and concomitant proliferation of cells (449). Thus, the increased doubling time of CLP1 KO may be due to aberrations in mRNA maturation, as CLP1 is involved in transcription termination and disassembly of the elongation complex (Figure 4-10).

Figure 4-10 Schematic of the eukaryotic polyadenylation complex. Polyadenylation requires a host of factors, including polymerase II, cleavage and polyadenylation specific factor (CPSF) complex, Cleavage Stimulatory Factor (CstF), CFI, and CFII. The subunit structures and molecular weights of CPSF and CstF are indicated. CFII is composed of CLP1 and PCF11. The sequence elements that comprise the poly(A) signals are represented in black rectangles, and the site of cleavage and subsequent polyadenylation site is shown by a red arrow bolt. The figure is taken from (450).

However, despite the minute role it plays in the polyadenylation process, CLP1^{-/-} showed a reduction in IBV RNA replication, structural protein translation, and viral titer in comparison to the WT cells. This result is consistent with our previous screening data that CLP1 likely functions as an enhancer in IBV infection, as CLP1 KO diminished viral production. To confirm our hypothesis, we overexpressed CLP1 by

transfecting WT CLP1 gene in both WT H1299 and CLP1^{-/-} cells and showed that CLP1 overexpression in WT H1299 cells boosted IBV infection, while CLP1 overexpression in CLP1^{-/-} cells partially rescued the KO cells to near WT IBV infection levels.

Next, to identify the viral proteins responsible for the changes in IBV infection between WT and CLP1^{-/-} cells, serial IBV passage in both WT and CLP1^{-/-} cells were performed to understand how IBV adapted to compensate for CLP1 loss. To prevent including random mutations that may arise as a result of virus passages, we compare genomic RNA sequences between WT- and CLP1^{-/-}-adapted IBV. At the end of 20 passages, we observed that IBV which have been serially passaged in CLP1^{-/-} cells displayed less aggressive syncytium formation and smaller plaque diameter on plaque assays. Furthermore, through sequencing data obtained from WT and CLP1^{-/-}-adapted IBV, we identified 4 NSPs – NSP3, NSP5, NSP15 and NSP16 – to be the likely viral interacting partners of CLP1.

The identification of these NSPs with CLP1 is congruent to their roles in RNA processing and protein translation. Firstly, NSP3 and NSP5 are involved in polyprotein processing of IBV, with NSP3 processing pp1a and pp1ab to produce NSP2 and NSP3, while NSP5 proteolytically processes the remaining polyprotein to generate the rest 14 NSPs. As demonstrated in previous studies, the length of the poly(A) tail contributes to the translation and replication efficiency of CoV defective interfering RNAs (451, 452). Therefore, lapses in polyadenylation would subsequently affect the CoV translation and replication. Under normal circumstances, RNA transcripts with a proper poly(A) tail length permits translation initiation and further proteolytic processing. In CLP1 knockout cells, however, polyadenylation might terminate prematurely, resulting in inefficient initiation of translation and downstream proteolytic processing by NSP3 and NSP5.

Conversely, NSP15 is known to preferentially cleave dsRNAs immediately 3' of uridylates (317). It is a crucial component of the replication/transcription complex of CoV RNA synthesis machinery, as a site-directed mutagenesis study of HCoV-229E NSP15 showed that substitution of pp1ab residue Asp-6408 with Ala led to a complete

blockage of viral RNA synthesis (317). Under normal circumstances following CoV RNA replication, CLP1 adds a poly(A) tail to the 3'-ends of the transcripts, while a poly(U) tract is generated on the minus-strand of the genomic RNA, allowing NSP15 to recognize the ends of an RNA transcript and cleave at the 3' of uridylates as part of RNA processing. With CLP1 knockout, a poly(A) tail may terminate prematurely, causing NSP15 to erroneously cleave the anticoding sequence at the 3' end of its mRNA if the short sequence GU(U) is found at the 3'-end of the transcript. Interestingly, NendoU may be regulated by NSP16, as 2'-O-ribose methyl groups rendered RNA substrates insensitive to NendoU-mediated cleavage (317). Therefore, the detection of NSP16 mutation along with NSP15 in this study suggest that there may be a functional cooperation between NSP15 and NSP16, and this may provide a rationale for the coordinated release of the adjacent NSP15 and NSP16 subunits from pp1ab by NSP3 (453).

Lastly, NSP16 may be indirectly involved with CLP1 in CoV replication, since mRNA capping always begin shortly after transcription initiation and precedes other mRNA processing events such as polyadenylation. In one study, it has been shown that deletion or ablation of NSP16 expression leads to abolishment of RNA synthesis in SARS-CoV (329), implying that NSP16 is critical in coronavirus replication. In the case of CLP1-NSP16 interaction, NSP16 may contribute to the stability of IBV genomic and subgenomic transcripts and allows the transcription of its RNA to proceed as per normal, while CLP1 and its polyadenylation complex is recruited to terminate transcription. In the absence of CLP1, replication and transcription of these RNA transcripts may terminate prematurely, thus preventing the RNA transcripts from proceeding to translation. Altogether, NSP3, NSP5, NSP15 and NSP16 may be working collectively with CLP1 in CoV RNA maturation.

Together, this study has characterized the role of CLP1 in IBV replication and presented NSP3, NSP5, NSP15, and NSP16 as the potential interacting viral partners to CLP1. While the phenotypes of the resulting virus mutants have not been confirmed by reverse genetics, published studies on CoVs encoding mutant forms of NSP15 and NSP16 have shed some light on the phenotypes of two of these virus mutants. In one study, mutant MHV-A59 viruses encoding mutant forms of NSP15 have been found to

impair viral replication and induce rapid cell death during infection of bone marrow-derived macrophages (BMDMs) (454). These NSP15 mutant viruses also stimulate early induction of Type I IFN response in macrophages, mediating the evasion of dsRNA sensors and limiting apoptosis in macrophages (454). Likewise, disruption of a KDKE motif in MERS-CoV NSP16 showed that while marginal impact on virus propagation was observed in the absence of 2' O-MTase activity, significant attenuation was observed *in vivo* and in human airway cell cultures (455). Further studies on identifying the interaction mode of CLP1 with these NSPs would reveal mechanistic details of these mutations in CoV replication and aid in the development of antivirals and vaccines.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The work presented in this dissertation focuses on the functional characterization of coronavirus-host interactions between RNMT and NSP3, and between CLP1 and NSP3, NSP5, NSP15, and NSP16, using IBV as a model coronavirus. This chapter lists the main conclusions from the work done thus far and suggests the future direction for investigation.

5 FUNCTIONAL CHARACTERIZATION OF RNA (GUANINE-7-) METHYLTRANSFERASE (RNMT) WITH CORONAVIRUS NSP3 PAPAIN-LIKE PROTEASE

5.1.1 Main Conclusions

The main conclusions from the experiments presented in chapter 3 are listed as follows:

- RNMT knockout stable cell lines were achieved via CRISPR/Cas9 genome editing
- Cell viability of RNMT^{-/-} was discounted as a limiting factor in IBV infection
- qPCR, western blot and plaque assay analysis suggest RNMT is an enhancer in IBV infection
- Overexpression of wild-type RNMT in RNMT^{-/-} cells can enhance IBV infection and partially recovers infectivity observed in H1299 cells
- Serial IBV passage in knockout cells renders IBV to adapt to RNMT^{-/-} and loses infectivity to WT H1299 cells
- RT-PCR sequencing of IBV gRNA suggests RNMT may interact with IBV PL_{pro} domain of NSP3
- Co-localization of RNMT and PL_{pro} showed that both proteins co-localize in the nucleus and cytoplasm of H1299 cells
- Direct electroporation of IBV gRNA in H1299 and RNMT^{-/-} cells implies that RNMT is not involved in virus attachment and entry
- Comparison of IBV collected from culture supernatant, and cell lysate indicates RNMT is not involved in virus assembly and egress

5.1.2 Unanswered questions and future directions

- The direct physical interaction between RNMT and PL_{pro} has not been verified by pull-down studies. As such, a pull-down study could be performed to determine if there is direct interaction between these proteins. If a direct interaction between RNMT and PL_{pro} is found, structural biology techniques such as NMR, crystallography, or X-ray diffraction may be employed to determine the critical residues involved in the interaction.
- RNA viruses are known to adopt both conventional and unconventional mechanisms for capping viral mRNA, such as cap snatching (influenza viruses) or recruiting the 43S pre-initiation complex (picornaviruses) (310). Therefore, it would be pertinent to study whether RNMT plays a role in cap formation, and whether CoV uses both viral and eukaryotic mRNA capping mechanisms during infection.
- The formation of correct mRNA caps can prevent viral RNA recognition by the cellular innate immune response. As such, it would be intuitive to investigate the immune pathways which may be up/downregulated in RNMT^{-/-} cells. So far, RNMT has been known to be regulated by IFN type I from microarray experiments (456), hence it would be useful to begin with a study of the IFN pathway in RNMT^{-/-} cells, and how changes in IFN type I can affect IBV replication.
- Recently, it was reported that Thr-77 phosphorylation of RNMT by CDK-cyclin-B1 could regulate mRNA cap methyltransferase activity during the early G1 phase of the cell cycle, and preventing RNMT Thr77-phosphorylation can inhibit cell proliferation (457). This suggests that RNMT may play a role in the cell cycle, and the RNMT KO may have led to lower IBV replication. Thus, it would be interesting to evaluate if IBV replication is indeed affected by the changes in the cell cycle of RNMT^{-/-} cells by performing G0/G1, G1/S, and G2/M cell synchronization experiments.

5.2 FUNCTIONAL CHARACTERIZATION OF CLEAVAGE AND POLYADENYLATION FACTOR I SUBUNIT 1 (CLP1) WITH NSP3, NSP5, NSP15 AND NSP16

5.2.1 Main Conclusions

The main conclusions from the experiments presented in chapter 4 are listed as follows:

- CLP1 knockout stable cell line was achieved via CRISPR/Cas9 genome editing
- Cell viability of CLP1^{-/-} was discounted as a limiting factor in IBV infection
- qPCR, western blot and plaque assay analysis suggest CLP1 is an enhancer in IBV infection
- Overexpression of wild-type CLP1 in CLP1^{-/-} cells can enhance IBV infection and partially recovers infectivity observed in H1299 cells
- Serial IBV passage in knockout cells renders IBV to adapt to CLP1^{-/-} and loses infectivity to H1299 cells
- RT-PCR sequencing of IBV gRNA suggests CLP1 likely interacts with IBV NSP3, NSP5, NSP15 and NSP16
- Co-localization of CLP1 and NSP5, NSP15, showed that these proteins co-localize in the nucleus and cytoplasm of H1299 cells
- Direct electroporation of IBV gRNA in H1299 and CLP1^{-/-} cells implies that CLP1 is not involved in virus attachment and entry
- Comparison of IBV collected from culture supernatant, and cell lysate indicates CLP1 is not involved in virus assembly and egress

5.2.2 Unanswered questions and future directions

In this study, the functional role of CLP1 and its involvement in the coronavirus replication cycle was dissected in a series of studies based on wild-type and CLP1^{-/-} cells using IBV. While our experimental results point to NSP3, NSP5, NSP15, and NSP16 as the likely interacting partners, several questions remain on the interaction between these proteins. Further experiments are proposed as follows to answer these questions:

- The direct physical interactions between CLP1 and NSP3, NSP5, NSP15 and NSP16 have not been verified by pull-down studies. As such, a pull-down study could be performed to determine if there is direct interaction between these proteins. If a direct interaction between CLP1 and its NSP is found, structural biology techniques such as NMR, crystallography, or X-ray diffraction may be employed to determine the critical residues involved in the interaction.
- In previous studies (451, 452), it was reported that a longer CoV poly(A) tail is associated with higher replication and translation efficiency. Hence, it would be interesting to explore if CLP1 has been utilized in CoV polyadenylation by generating mutant IBV with impaired viral CLP1 counterpart.

5.3 FINAL REMARKS

CoVs are respiratory pathogens that continuously circulate and evolve in several hosts, including poultry, bats, domestic pets and humans. The emergence of novel CoV strains and re-emergence of existing CoVs that can cause human epidemics and pandemics remains a significant public health threat. Since viral spread and pathogenesis depends on virus-host interactions and inhibition of host pathways involved in viral restriction, identification of novel virus-host interactions may result in novel antiviral strategies. As such, it is hoped that the findings reported in this dissertation may enthruse further investigation on host factors regulating CoV replication and pathogenesis to facilitate the development of CoV antiviral drugs and vaccines.

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APPENDIX

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