Generating Biologic and Genetic Research Tools to Investigate Serotype I Feline Coronaviruses

Robert Christian Mettelman

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For Libby and the tremendous amount of support, patience, and good humor you sustained to complete this journey with me.
I now have additional questions…

- Sentiment expressed in the Baker Lab
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<tr>
<td>(-)</td>
<td>negative-sense</td>
</tr>
<tr>
<td>(+)</td>
<td>positive-sense</td>
</tr>
<tr>
<td>2.2</td>
<td>second target, second exon of the feline <em>ifnar2</em></td>
</tr>
<tr>
<td>2.2 Poly</td>
<td>Fcwf-4 CU IFNαR 2.2 polyclonal population</td>
</tr>
<tr>
<td>2' 5'-OAS</td>
<td>2'5'-oligoadenylate synthase</td>
</tr>
<tr>
<td>3CLpro</td>
<td>3C-like protease</td>
</tr>
<tr>
<td>Ac</td>
<td>acidic domain</td>
</tr>
<tr>
<td>ACE2</td>
<td>angiotensin converting enzyme 2</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>ADE</td>
<td>antibody-dependent enhancement</td>
</tr>
<tr>
<td>AmpR⁺</td>
<td>ampicillin resistance cassette positive</td>
</tr>
<tr>
<td>APN</td>
<td>aminopeptidase N</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell activating factor</td>
</tr>
<tr>
<td>BMDM</td>
<td>bone marrow-derived macrophages</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>C</td>
<td>capacitance</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase activation and recruitment domains</td>
</tr>
<tr>
<td>Cardif</td>
<td>CARD adapter-inducing interferon beta</td>
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</table>
E envelope
EAV equine arteritis virus
eIF eukaryotic initiation factor
EMEM minimal essential medium Eagle
EndoU endoribonuclease
ER endoplasmic reticulum
ERGIC reticulum-Golgi intermediate compartment
EV empty vector
ExoN exoribonuclease
f feline
FADD fas-associated death domain
FBS / FCS fetal bovine serum / fetal calf serum
FCoV feline coronavirus
FcR Fc receptor
Fcwf-4 felis catus whole fetus 4
FECV feline enteric coronavirus
FeLV feline leukemia virus
FF firefly luciferase
FIP feline infectious peritonitis
FIPV feline infectious peritonitis virus
g g-force (m/s²)
G2M coronavirus group 2 marker
GM-CSF granulocyte monocyte cell stimulating factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>gRNA</td>
<td>genomic RNA</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HCoV</td>
<td>human coronavirus</td>
</tr>
<tr>
<td>HE</td>
<td>hemagglutinin-esterase</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HEK 293T/17</td>
<td>human embryonic kidney 293T clone 17</td>
</tr>
<tr>
<td>HII</td>
<td>hyper interferon-inducing</td>
</tr>
<tr>
<td>HIS</td>
<td>hyper interferon-sensitive</td>
</tr>
<tr>
<td>HP</td>
<td>high-passage</td>
</tr>
<tr>
<td>HPE</td>
<td>hours post-electroporation</td>
</tr>
<tr>
<td>HPI</td>
<td>hours post-infection</td>
</tr>
<tr>
<td>HR</td>
<td>heptad repeat</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IBV</td>
<td>Infectious bronchitis coronavirus</td>
</tr>
<tr>
<td>ic</td>
<td>infectious clone</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>fifty-percent inhibitory concentration</td>
</tr>
<tr>
<td>ICTV</td>
<td>International Committee on Taxonomy of Viruses</td>
</tr>
<tr>
<td>IFIT</td>
<td>IFN-induced proteins with tetratricopeptide repeats</td>
</tr>
<tr>
<td>IFITM</td>
<td>IFN-induced transmembrane proteins</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IFNαR</td>
<td>interferon alpha receptor</td>
</tr>
<tr>
<td>IFNαR2</td>
<td>interferon alpha receptor domain 2</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
</tbody>
</table>
IL interleukin
IPS-1 interferon beta promoter stimulator protein 1
IRAK interleukin 1 receptor associated kinase
IRF interferon regulatory factor
IRN IFNα receptor null
ISG interferon-stimulated gene
ISRE interferon-stimulated response elements
Jak Janus kinase
KanR+ kanamycin resistance cassette positive
kb kilobase
kDa kilo Dalton
L leader
LGP2 laboratory of genetics and physiology 2
LP low-passage
LTR long terminal repeat
Luc luciferase
M membrane protein; molar
M-CSF monocyte cell stimulating factor
MAVS mitochondrial antiviral signaling protein
mCEACAM murine carcinoembryonic antigen-related cell adhesion molecules
MDA5 melanoma differentiation-associated gene 5
MEM minimal essential medium
MERS-CoV Middle East respiratory syndrome coronavirus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Term</th>
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<tbody>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MHV</td>
<td>mouse/murine hepatitis virus</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>Mpro</td>
<td>main protease</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MTase</td>
<td>methyltransferase</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation factor 88</td>
</tr>
<tr>
<td>N</td>
<td>nucleocapsid; amino-terminal</td>
</tr>
<tr>
<td>NAB</td>
<td>nucleic-acid binding domain</td>
</tr>
<tr>
<td>Neu 5,9 Ac2</td>
<td>5-N-acetyl-9-O-acetylNeuraminic acid</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>ng</td>
<td>nanograms</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NLR</td>
<td>nucleotide-binding oligomerization domain like receptors</td>
</tr>
<tr>
<td>NPC1</td>
<td>Niemann-Pick C1</td>
</tr>
<tr>
<td>nsp</td>
<td>nonstructural protein</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>NTD</td>
<td>amino-terminal domain</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethylenimine</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque-forming units</td>
</tr>
<tr>
<td>PKR</td>
<td>protein kinase R</td>
</tr>
<tr>
<td>PLP1</td>
<td>papain-like protease 1</td>
</tr>
<tr>
<td>PLP2</td>
<td>papain-like protease 2</td>
</tr>
<tr>
<td>pmol</td>
<td>picomole</td>
</tr>
<tr>
<td>poly (A)</td>
<td>poly adenosine</td>
</tr>
<tr>
<td>poly (U)</td>
<td>poly uridylate</td>
</tr>
<tr>
<td>pp</td>
<td>polyprotein</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>R</td>
<td>Resistance domain</td>
</tr>
<tr>
<td>RBD</td>
<td>receptor binding domain</td>
</tr>
<tr>
<td>RD</td>
<td>repressor domain</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>Rev Gen</td>
<td>reverse genetics</td>
</tr>
<tr>
<td>RFS</td>
<td>ribosomal frameshift</td>
</tr>
<tr>
<td>RIG-I</td>
<td>retinoic acid-inducible gene 1</td>
</tr>
<tr>
<td>RIP1</td>
<td>receptor interacting protein 1</td>
</tr>
</tbody>
</table>
RL  renilla luciferase
RLR  retinoic acid-inducible gene I-like receptors
RNP  ribonucleoprotein
RT-PCR  reverse-transcription PCR
RTC  replicase-transcriptase complex
S  spike
s  second
SARS-CoV  severe acute respiratory syndrome coronavirus
sgRNA  subgenomic RNA; short-guide RNA
SPF  specific pathogen-free
ss  single-stranded
STAT  signal transducer activator of transcription
STING  stimulator of interferon genes
SUD  SARS-CoV unique domain
TC  tissue culture
TCID$_{50}$  fifty-percent tissue culture infectious dose
TGEV  transmissible gastroenteritis virus
TIL  toll/interleukin-1 receptor
TLR  toll-like receptors
TMPRSS2  transmembrane serine protease 2
TNF-α  tumor necrosis factor alpha
TP  transducing particle
TRAF  tumor necrosis factor receptor-associated factor
TRIF  TIR domain-containing adaptor inducing IFN beta
TRS   transcription-regulating sequence
TRS-B transcription-regulating sequence (body)
TRS-L transcription-regulating sequence (leader)
ts   temperature-sensitive
Ubl  ubiquitin-like domain
UCD  University of California-Davis
UTR  untranslated region
V    volt
VISA virus-induced signaling adapter
VLP  virus-like particle
VSV  vesicular stomatitis virus
w/v  weight to volume
WT   wild type; wild-type
Y    coronavirus highly conserved domain
α    alpha; anti-
β    beta
γ    gamma
λ    lambda
μF   microfarad
μg   microgram
μL   microliter
μM   micromolar
ABSTRACT

Serotype I feline infectious peritonitis virus (FIPV) is an alphacoronavirus of high veterinary importance due to the 99% mortality rate observed in cats. Since the 1980s, robust experimentation on these viruses has been limited by availability of culturable pathogenic strains, few permissive cell lines, and a lack of standardized methods to study their basic virology. These complications have resulted in variable and conflicting literature reports, have slowed study of clinical strains and hindered effective vaccine design. The goal of this dissertation was to develop a laboratory “toolbox” containing standardized methods, permissive cell lines and genetic techniques to alleviate some of the technical barriers to investigating type I feline coronavirus (FCoV). To this end, our group characterized the replication kinetics of serotype I FIPV Black in three feline cell lines. From this work, colleagues and I described a new macrophage-like cell line, termed Fcwf-4 Cornell University (CU), which rapidly grew FCoV to high titer. Using these cells, we established a standardized plaque assay to accurately determine type I FCoV titer. To facilitate study of live-attenuated and clinical isolate FCoVs, I used Crispr/Cas to disrupt IFNαR, the major antiviral signaling component, in two feline cell lines: AK-D and Fcwf-4 CU. Further, I isolated a clone, termed Fcwf-4 CU IRN, and demonstrated that expression of a feline protease enhanced FIPV infection in these cells. Finally, using a deep-sequenced laboratory strain of FIPV Black, we constructed an infectious clone (ic) reverse genetics system and successfully produced icFIPV.
CHAPTER ONE
INTRODUCTION

The Biology of Coronaviruses

Overview and Classification.

Coronaviridae are a family of enveloped, positive-sense RNA viruses that, along with the Arteriviridae, Mesoniviridae, and Roniviridae, make up the taxonomic order Nidovirales (Figure 1). Contained within the Coronaviridae are two subfamilies: the newly defined Letovirinae housing the Alphaeltovirus genus, and the much larger Orthocoronavirinae subfamily composed of four monophyletic genus clusters: Alpha-, Beta-, Delta- and Gammacoronavirus. The Betacoronavirus genus is further divided into four lineages, A-D. Together, the Orthocoronavirinae represent the majority of known and studied coronavirus species and include important animal, human and zoonotic pathogens (International Committee on Taxonomy of Viruses (ICTV), 2018).

Viruses in the order Nidovirales, including coronaviruses are organized into the above taxonomic groups based on phylogenetic similarity of the positive-sense RNA genomes. All Nidoviruses have large RNA genomes, with the Coronaviridae family boasting the largest known, containing between 27 and 32 kilobases (kb). A unique feature of Nidovirus genomes, and the one from which the name was derived, is the expression of genes via the synthesis of a 3’ nested set of 7-9 subgenomic RNAs (sgRNAs) (Masters, 2006) – nido meaning “nest” in Latin. Other features common to Nidoviruses include a highly conserved genome organization with a large 5’ replicase,
arising from ribosomal frameshift translation, which precedes a set of structural and group-specific accessory genes, as well as a set of viral nonstructural proteins processed from the replicase polyprotein by replicase-encoded viral proteases. The major differences between members of the Nidovirus order are morphologic – wide variations in structural protein type, number and size results in unique virion and nucleocapsid constructions (Fehr and Perlman, 2015).

Figure 1: Phylogenetic Classification of Orthocoronavirinae Subfamily. The taxonomic tree depicted here is based on the 2018 report from the International Committee on Taxonomy of Viruses (ICTV).

Genome Organization.

Coronaviruses (CoVs) carry single copy, non-segmented, single-stranded, positive-sense RNA genomes, which range in size between 27-32 kb (Figure 2). The 5’ end of the genome is capped with a guanine residue methylated at the N7 position while the 3’ end terminates with repeating adenosine monophosphate residues, termed the
poly (A) tail. These terminal features mimic host messenger (m) RNA thus the CoV genomic RNA can be directly translated on host ribosomes following membrane fusion.

The 5’ terminal region of the CoV genome is highly structured and contains a large (210-530 bp) untranslated region (5’ UTR) comprising seven stem-loops (Brown et al., 2007; Guan et al., 2011; Liu et al., 2009; Yang and Leibowitz, 2015). These stem-loops directly associate with host ribosomes as well as the virus-encoded RNA-dependent RNA polymerase (RdRp) and are critical to virus replication. The 5’ UTR also includes a 70-100 nt leader (L) sequence (Lai et al., 1984, 1983), the 3’ end of which houses the 6-8 nucleotide transcription-regulating sequence (TRS-L [Leader]). Identical TRS segments are also present on the 5’ end of each of the 7-9 subgenomic mRNAs (TRS-B [Body]) acting as cis-regulatory elements (Budzilowicz et al., 1985; Masters, 2006) critical for the hallmark discontinuous transcription (Sawicki et al., 2007) observed during Nidovirus replication (discussed in detail below).
Figure 2: Feline Coronavirus Genome Organization, Replicase Processing and Subgenomic mRNAs. The typical coronavirus genome organization is depicted using feline infectious peritonitis virus (FIPV) as a model. The replicase, comprising ORF1a and ORF1b separated by a ribosomal frameshift site (RFS), contain nonstructural proteins (nsps) 1-16. The two replicase polyproteins (pp1a and pp1ab) are depicted. Arrows indicate PLP1/2 or 3CLpro cleavage sites; numbers below indicate nsp number. Specific nsp components: nsp3, papain-like protease 1/2 (PLP1/2); nsp5, 3C-like protease (3CL); nsp12, RNA-dependent RNA polymerase (RdRp); nsp13, helicase (HEL); nsp14, exoribonuclease (ExN); nsp15, endoribonuclease (EnU); nsp16, 2-O-methyltransferase (MT). The structural proteins spike (S), envelope (E), membrane (M), nucleocapsid (N) and accessory protein regions ORF3 (3a, 3bc) and ORF7 (7a, 7b) are shown. The 5' leader (L) and body (B) transcription-regulating sequences (TRS-) are indicated on genomic and subgenomic mRNAs 1-7. Bottom arrow indicates direction of translation.
The first two thirds (~20 kb) of the CoV genome downstream of the 5’ UTR is dominated by the replicase polyprotein coding region. Translation of two open reading frames, ORF1a and ORF1b produce two replicase polyproteins (pp) 1a and 1ab (Lai et al., 1994). While pp1a is translated directly from ORF1a, the larger pp1ab is produced due to a -1 nt ribosomal frameshift (RFS) that occurs at a tandem pseudoknot-slippery sequence in ORF1 allowing continual translation through ORF1b. Together, the replicase polyproteins encode 16 nonstructural proteins (nsp1-16) that have wide-ranging activities including formation of the replicase-transcriptase complex (RTC) and modulation of innate immune signaling. Downstream of the replicase, the final third (~10 kb) of the CoV genome encodes the structural and accessory genes. CoV structural proteins include spike (S), envelope (E), membrane (M), and nucleocapsid (N) (de Haan and Rottier, 2005; Masters, 2006). Several members of the Betacoronavirus genus, including murine hepatitis virus and bovine coronavirus, also encode a fifth structural gene, hemagglutinin-esterase (HE) (Cornelissen et al., 1997; Kazi et al., 2005; Klausegger et al., 1999). Interspersed within the structural proteins are the accessory genes, which are unique to different CoV subfamilies. Accessory proteins are largely dispensable for replication but often provide a selective pathogenic advantage (Zhong et al., 2016) such as antagonism of innate immunity. The genome of the feline alphacoronavirus FIPV, the focus of this dissertation, contains five such accessory proteins encoded on two polycistronic gene regions, ORF3 (3a, 3b, 3c) and ORF7 (7a, 7b) (Kipar and Meli, 2014). FIPV 7a, and ORF3, have been implicated in antagonism of host innate response during infection, however the mechanisms of this activity are not fully understood (Dedeurwaerder, 2014; Dedeurwaerder et al., 2014, 2013).
The 3’ terminal end of the CoV genome contains a 270-500 bp untranslated region (3’ UTR) that directly precedes the poly (A) tail. Like the 5’ end, the 3’ UTR is also highly structured (Goebel et al., 2004; Yang and Leibowitz, 2015) and contains several cis-acting elements essential for coronavirus replication [reviewed in (Yang and Leibowitz, 2015)] as well as a putative molecular switch predicted to be involved in a transition event during RNA synthesis (Goebel et al., 2004).

**Virion Structure.**

The **coronavirus virion.** Coronaviruses are such named due to the solar halo or corona-like, Latin for crown, pattern of the virion when observed using EM microscopy. This crown-like appearance is due to the large, trimeric, club-like spike (S) structures, which jut out from the host membrane-derived lipidic envelope that forms the outside of the roughly spherical virion (Benjamin W Neuman et al., 2006). Two to three other viral proteins are also anchored in the envelope: the abundant membrane (M) and the more sparse envelope (E) proteins are present on all coronaviruses, while the hemagglutinin-esterase (HE) protein is found on only a subset of **Betacoronviruses** (de Haan and Rottier, 2005; Masters, 2006). Coronavirus virions are approximately 100-125 nm in diameter as determined by studies using cryo-electron microscopy (Benjamin W Neuman et al., 2006) and cryo-electron tomography (Bárcena et al., 2009). Contained within the virion is the ribonucleoprotein (RNP) core composed of the ~ 30 kb genomic RNA encased in an oligomer of nucleocapsid (N) protein oriented with helical symmetry. A typical coronavirus virion is shown in **Figure 3.**
Spike protein. The spike (S) protein is the main determinant of coronavirus host-range and tissue tropism as it functions to both engage host receptors as well as mediate the membrane fusion events that deliver the viral RNP to the cell cytoplasm. For example, small sequence variations of the feline coronavirus spike protein can lead to drastic changes in cell tropism and virulence (Chang et al., 2012; Licitra et al., 2013; Rottier et al., 2005). The mature spike is a trimeric type I glycoprotein of approximately 150 kDa. During protein synthesis, the S homotrimer is heavily glycosylated on 21-35 N-glycosylation sites and tethered to the virus envelope outer leaflet via a membrane-anchoring sequence and a short hydrophilic CTD tail (de Haan and Rottier, 2005). The
ectodomain makes up the majority of the S molecule and, for most CoVs, is cleaved into two polypeptide regions, S1 and S2 by host proteases (Abraham et al., 1990; Luytjes et al., 1987). The amino-terminal S1 “head” region contains the receptor-binding domain (RBD), while the carboxy-terminal S2 “stalk” houses the components of a class I fusion protein (Bosch et al., 2003; Collins et al., 1982; de Haan and Rottier, 2005; Walls et al., 2019). Many coronaviruses engage host peptidases as entry receptors, although this is not universally true. A partial list of entry receptors used by alpha- and betacoronaviruses is provided in Table 1.

Envelope protein. The envelope (E) is a small, 8-12 kDa integral membrane protein and a minor component of the coronaviral envelope (Raamsman et al., 2000; Siddell, 1995). The protein has a short hydrophilic amino-terminal end followed by a hydrophobic membrane domain and a hydrophilic carboxy-terminal domain. The E protein functions in assembly and release of the virus through potential interaction with, or modulation of the M protein, or by influencing the curvature of the virion envelope (Boscarino et al., 2008; Corse and Machamer, 2000; Fischer et al., 1998; Ye and Hogue, 2007). A portion of the CTD has also been shown to have ion-channel activity, which has importance in determining SARS-CoV virulence (Nieto-Torres et al., 2014; Verdiá-Báguena et al., 2012).
Membrane protein. The membrane (M) protein is a small (25-30 kDa), highly abundant component of the coronaviral envelope and directs the complete assembly of mature virion (de Haan and Rottier, 2005; Fehr and Perlman, 2015; Masters, 2006). Owing to its description as the virion “building block”, the M protein has been shown to interact with all other structural components via its five, largely hydrophobic domains [reviewed in (de Haan and Rottier, 2005)]. The amino-terminal ectodomain is glycosylated and is followed by three hydrophobic transmembrane domains and a large
carboxy-terminal endodomain (Armstrong et al., 1984; Nal et al., 2005). On the envelope, the M protein dimer has been shown by cryo-EM and tomography to adopt two conformations, which influence membrane curvature and interactions with other virion components including the nucleocapsid (N) protein (Neuman et al., 2011).

**Nucleocapsid protein.** The nucleocapsid (N) phosphoprotein is the most abundant viral protein expressed during infection and, together with the CoV RNA genome, forms the protective and stable ribonucleoprotein (RNP) core (Laude and Masters, 1995; Narayanan et al., 2003). The N protein ranges in size between 43 and 50 kDa and amino acid identity varies between coronavirus subfamilies. The highly-basic amino-terminal and carboxy-terminal domains of the nucleocapsid associate with RNA by different mechanisms, possibly utilizing both ends for optimal binding (Chang et al., 2006; Hurst et al., 2009) and the N protein has been shown to bind specifically to the TRS and packaging signal of the CoV genome (Molenkamp and Spaan, 1997; Stohlman et al., 1988). Additionally, phosphorylation of the N protein has been suggested to impose structural changes that increase affinity for viral, rather than host RNAs (Stohlman and Lai, 1979). When in complex, the N protein and RNA form a compact structure with helical symmetry referred to as the ribonucleoprotein (RNP) core or complex. Other interacting partners include nsp3 (Hurst et al., 2013, 2009), a major component of the replicase-transcriptase complex (RTC), and the M protein (Sturman et al., 1980) highlighting potential roles for the N protein in promoting transcription and packaging newly-synthesized genomic RNAs into virions.

**Hemagglutinin-esterase.** Several members of the Betacoronavirus genus cluster encode a fifth structural protein: the hemagglutinin-esterase (HE) (Brian et al.,
1995). The HE protein is a 60-65 kDa type I transmembrane protein of 424-439 amino acids that contains an amino-terminal signal peptide ectodomain, a transmembrane domain and a small, cytoplasmic, carboxy-terminal tail (de Haan and Rottier, 2005; Hogue et al., 1989). The mature protein is a glycosylated homodimer linked by disulfide bonds that juts out from the viral envelope, resembling the spike protein (Kienzle et al., 1990). Much like the hemagglutinin of influenza C virus, the CoV HE protein dimer binds and cleaves sialic acid residues found on surface glycoproteins (Cornelissen et al., 1997). HE proteins from specific coronavirus species have also been shown to have acetyl-esterase activity, a function not present in the HE of influenza C or other CoVs (Klausegger et al., 1999). Although the exact role of HE during CoV infection and replication is not fully understood, it has been suggested that HE may enhance S-mediated virus entry and/or spread through mucosal tissue, thereby contributing to pathogenesis (Cornelissen et al., 1997).
Figure 4: The Coronavirus Replication Cycle. A typical coronavirus replication cycle is shown in 6 steps: 1) CoV spike engages the host receptor at the cell surface and membrane fusion, mediated by host proteases at the surface or within endosomes, allows uncoating and insertion of the RNP into the cytoplasm. 2) gRNA associates with host ribosomes and translation of the replicase pp1a and 1ab occurs. 3) Pp1a/1ab are processed by viral proteases (PLP1/2 and 3CLpro) releasing 16 nsps, which form the RTC on host-derived DMVs. 4) The RTC produces subgenomic RNAs by discontinuous transcription that are translated into structural and accessory proteins, and replicates gRNA, which becomes engulfed by N protein to form RNP cores. The structural proteins traffic from the ER to the ERGIC. 5) RNP cores bud into the ERGIC and are surrounded by the viral envelope containing the structural proteins to form the mature virion. 6) Mature virions are released due to vesicle fusion at the cell surface.
Replication Cycle.

Overview. Replication of CoVs (Figure 4) is initiated through engagement of the CoV spike protein with the corresponding target cell receptor. The virus is then taken into intracellular vesicles through receptor-mediated endocytosis (Gallagher and Buchmeier, 2001), and following a membrane fusion event (Bosch et al., 2003), the single-stranded RNA genome is released into the cell cytoplasm (Fehr and Perlman, 2015). In some cases, CoV virions fuse directly at the cell plasma membrane and immediately release genomic RNP into the cytoplasm (Millet and Whittaker, 2015). Fusion at the cell surface or within vesicles may be linked to the overall stability of the virus spike protein, the density or presence of host proteases and/or specific entry cofactors such as tetraspanins involved in promoting membrane fusion (Belouzard et al., 2012; Earnest et al., 2017; Millet and Whittaker, 2015). In either case, the positive-sense genomic RNA enters the cytoplasm and is directly translated on host ribosomes (Lai et al., 1994), which initiate production of the CoV replicase polyproteins pp1a and pp1ab (Lai et al., 1994). The large replicase polyproteins are processed through catalytic cleavage by viral proteases encoded on the replicase (Baker et al., 1993; Kanjanahaluethai et al., 2003; Masters, 2006) into 16 nonstructural proteins (nsp1-16) (de Haan and Rottier, 2005). Each nsp has a distinct function that is conserved among CoV orthologues (Gorbalenya et al., 1989; Su et al., 2014). Of note here are nsp12, encoding the RNA-dependent RNA polymerase (RdRp), and nsps 3, 4 and 6, which modify host membranes to form the double-membrane vesicles (DMV) that house the replicase-transcriptase complex (RTC). The RTC has two primary functions: copy the full-length RNA genome (replication) and transcribe the structural and accessory genes
(discontinuous transcription) generating a nested set of subgenomic mRNAs. The structural gene products S, E and M (and HE, if present) are inserted into the endoplasmic reticulum (ER) and traffic to the endoplasmic reticulum-Golgi intermediate compartment (ERGIC). In the cytoplasm, the nucleocapsid protein surrounds newly-synthesized genomic RNA then buds into the ERGIC where it becomes enveloped by the other structural proteins thus forming complete virions. Finally, virions are transported in smooth-walled vesicles to the cell surface where they fuse with the plasma membrane and release infectious CoV particles.

**Attachment and entry.** The coronavirus spike protein is the main determinant of virus attachment and entry as it possesses both a receptor binding domain (S1) and is a type I fusion protein via the S2 domain. The interaction between the coronavirus spike protein and cognate host cell receptor is the first step in initiating the replication cycle. CoV host range, tissue tropism and zoonotic emergence are largely dependent on the specificity of the S to a particular receptor, however, may also be determined by the presence and density of host proteases involved in promoting membrane fusion. These interactions are highly species specific, but are also mutable as has been shown both experimentally (Saif, 2004) and naturally with the zoonotic emergence of both SARS- and MERS-CoV. The specific location of the receptor binding domain (RBD) varies along the S1 domain and different CoV species utilize one or both for this function. The galectin-like amino-terminal domain of S1 binds sialic-acid moieties as observed in mouse hepatitis virus (MHV) (mCEACAM) (Nedellec et al., 1994) and HCoV-OC43 (Qian et al., 2015). The carboxy-terminal domain of S1 is more commonly utilized and engages peptidases as identified for HKU1 (Qian et al., 2015), feline CoV (APN)
(Tresnan et al., 1996; Tresnan and Holmes, 1998), SARS-CoV (ACE2) (Li et al., 2003) and MERS-CoV (Raj et al., 2013). Interestingly, a neurotropic strain of murine coronavirus (MHV-JHM) has also been shown to enter cells without specific receptor engagement (Gallagher et al., 1992) possibly owing to a highly fusogenic, metastable spike protein.

In all cases, following engagement of the host receptor, the S2 region of S catalyzes a membrane fusion event, which allows transit of the genomic RNA from virion to the cytoplasm. This fusion process requires significant structural changes to spike and has been recently visualized by cryo-EM (Walls et al., 2017). The S conformational changes are triggered following cleavage by host proteases (in some cases pH-dependent) and drives the rapid release and insertion of the fusion peptide, which is located in the center of the pre-fusion spike trimer, into the host membrane. For most CoV spike proteins, cleavage of S occurs at a defined junction between S1 and S2; however, certain S proteins have been shown to have alternative or additional cleavage sites along S1, which can impact cell tropism and pathogenesis (Belouzard et al., 2012, 2009; Licitra et al., 2013; Millet and Whittaker, 2015). Proteolytic processing of the spike protein occurs in several locations depending on the CoV, including at the cell surface (Earnest et al., 2017; Glowacka et al., 2011; Park et al., 2016), within early endosomes (Millet and Whittaker, 2014) and in complex with lysosomes (Bosch et al., 2008). This process allows spatiotemporal control of viral entry (Rottier and Bosch, 2008) and has implications in cell tropism, entry efficiency and pathogenicity (Matsuyama et al., 2005; Park et al., 2016). The membrane fusion event mediated by the S2 region of the spike is typical of other type I fusion proteins (Harrison, 2015) and
includes a three step process: i) insertion of fusion peptide into the outer leaflet of the host membrane; ii) a fold-back event bringing the outer leaflets of the host and viral membranes into close proximity; iii) a hemi-fusion event in which the lipid bilayers of the host and viral membrane fuse. The end result of this fusion event is the release of the genomic RNP core into the cytoplasm.

**Replicase protein translation, processing and formation of the replicase-transcription complex (RTC).** Once within the cytoplasm, translation of the CoV genomic RNA occurs on host ribosomes (Lai et al., 1994). The 5’ end of the genome is capped with a methylated guanine residue that precedes a highly-structured untranslated region containing seven stem-loops (Yang and Leibowitz, 2015). The 5’ structure of the RNA engages host translation machinery components eIF4 and the ribosomal 40 S subunit to form the pre-initiation complex. The 60 S ribosomal subunit then associates with this complex and begins translation of the first set of open reading frames encoding the coronavirus replicase [reviewed in (Nakagawa et al., 2016)].

The replicase region comprises the first two-thirds of the genome and houses all the components necessary to drive formation of the replicase-transcriptase complex (RTC), a structure that functions to replicate the full-length genome and transcribe the downstream structural and accessory genes. The replicase is translated from two open reading frames, ORF1a and ORF1b that produce two co-terminal polyproteins (pp) 1a and 1ab (Lai et al., 1994). A large pseudoknot, preceded by a 5’-UUUAAC-3’ “slippery” sequence, is present at the junction between ORF1a and ORF1b and directs production of either pp1a (75%) or pp1b (25%). For production of pp1a, the ribosome translates the entire ORF1a by unwinding and continuing through the pseudoknot before terminating
at a downstream stop codon. To produce pp1ab, the pseudoknot causes the ribosome to temporarily pause at the “slippery” sequence leading to a -1 nt ribosomal frameshift and subsequent continuation of translation through ORF1b.

The functional components of the ~20 kb replicase are a set of 16 nonstructural proteins, which are released from the co-terminal polyproteins 1a and 1ab through catalytic cleavage by a set of 2-3 replicase-encoded viral proteases. Contained on nsp3 are one or two papain-like proteases (PLP1; PLP2) (Baker et al., 1993; Kanjanahaluethai et al., 2003) that process the nsp1/2, 2/3, and 3/4 junctions in trans through recognition of a conserved LXGG motif. Nsp5 encodes the 3C-like protease (3CLpro; also termed Main protease or Mpro), a serine protease, which is released by autocatalytic cleavage and recognizes a conserved LQS motif to release nsps 5-16. Each nonstructural protein has a distinct function that is conserved among CoV orthologues (Gorbalenya et al., 1989; Su et al., 2014) and summarized Table 2. Together, these components mediate the assembly of the cytoplasmic replicase-transcriptase complex (RTC), the site of de novo RNA synthesis and transcription of subgenomic RNAs. Within hours of infection, nsp3, 4 and 6 (Angelini et al., 2013) induce large alterations of the cellular reticulovesicular membrane network forming perinuclear convoluted membranes from which the double-membrane vesicles (DMVs), the scaffolds of the RTC, are derived (Gosert et al., 2002; Knoops et al., 2008; Prentice et al., 2004; Shi et al., 1999; van der Meer et al., 1999). Other notable components of the RTC include nsp12 and 13 that encode the RdRp and helicase, respectively.
<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>FUNCTION(S)</th>
<th>REFERENCE</th>
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| nsp1    | Cellular mRNA degradation  
Blocks host translation  
Innate immune antagonism | (Tanaka et al., 2012)  
(Huang et al., 2011)  
(Kamitani et al., 2009)  
(Kamitani et al., 2006) |
| nsp2    | Not required for replication  
Binds prohibitin proteins | (Cornillez-Ty et al., 2009)  
(Graham et al., 2005) |
| nsp3    | Multi-domain transmembrane protein; DMV formation  
(Ub1 / Ac) Associate with N protein  
(Macromod) IFN antagonism, PLP2 interaction  
(PLP1/2) LXGG-specific protease (nsp1/2, 2/3, 3/4 junctions)  
Deubiquitinase-mediated IFN antagonism  
DeISGylase: deconjugates ISG15  
(DPUP, Ub12, NAB, G2M, SUD, Y) unknown functions | (Chen et al., 2015)  
(Chatterjee et al., 2009)  
(Frieman et al., 2009)  
(Serrano et al., 2009)  
(Eriksson et al., 2008)  
(Varma et al., 2008)  
(Serrano et al., 2007)  
(Egloff et al., 2006)  
(Ziebuhr et al., 2001)  
(Deng et al., 2019a) |
| nsp4    | DMV formation | (Gadlage et al., 2010)  
(Clementz et al., 2008) |
| nsp5    | (3CL pro / M pro) RLG-specific protease (nsp4-16 junctions) | (Lu et al., 1995) |
| nsp6    | DMV formation | (Oostra et al., 2008) |
| nsp7    | Forms hexadecameric complex with nsp8  
Potential processivity clamp for RNA polymerase (nsp12) | (Zhai et al., 2005) |
| nsp8    | Forms hexadecameric complex with nsp7  
Potential processivity clamp for RNA polymerase (nsp12)  
Potential primase | (Imbert et al., 2006)  
(Zhai et al., 2005) |
| nsp9    | RNA binding protein | (Egloff et al., 2004) |
| nsp10   | Cofactor for nsp14 and nsp16  
Promotes 2-O-MT and ExoN activities of nsp14 and nsp16, rsp. | (Decroly et al., 2011)  
(Bouvet et al., 2010) |
| nsp11   | Small peptide formed at end of pp1a | (de Haan and Rottier, 2005) |
| nsp12   | (RdRp) RNA-dependent RNA polymerase | (Xu et al., 2003) |
| nsp13   | DNA/RNA helicase; unwinds with 5’ to 3’ polarity  
5’ triphosphatase | (Ivanov et al., 2004b)  
(Ivanov and Ziebuhr, 2004) |
| nsp14   | (N7 MTase) Addition of 5’ cap to viral mRNAs  
(ExoN) exoribonuclease; RNA proofreading activity | (Eckerle et al., 2010)  
(Chen et al., 2009)  
(Eckerle et al., 2007)  
(Minskaia et al., 2006) |
| nsp15   | (EndoU) Endoribonuclease of ss/dsRNAs at uridylate residues  
Prevents dsRNA accumulation; strong IFN antagonist | (Deng et al., 2019b)  
(Deng et al., 2017)  
(Kindler et al., 2017)  
(Bhandiwaj et al., 2006)  
(Bhandiwaj et al., 2004)  
(Ivanov et al., 2004a) |
| nsp16   | (2’-O-Methyltrasferase); Prevents RNA detection by MDA5 | (Züst et al., 2011)  
(Decroly et al., 2008) |
Interestingly, several of the nonstructural proteins, including the papain-like protease (Clementz et al., 2010; Devaraj et al., 2007; Frieman et al., 2009; Li et al., 2011; Mielech et al., 2014; Rose and Weiss, 2009; Sun et al., 2012), and the EndoU encoded on nsp15 (Deng et al., 2019b, 2017; Deng and Baker, 2018; Kindler et al., 2017) have been demonstrated to have additional functions and antagonize innate immunity.

**RNA replication and transcription.** Within the RTC, replication of the full-length gRNA occurs and is used as a template to generate a set of subgenomic (sg) messenger (m) RNAs that encode the structural proteins, spike (S), envelope (E), membrane (M), nucleocapsid (N) and hemagglutinin-esterase (HE), if present (de Haan and Rottier, 2005; Masters, 2006), as well as the group-specific accessory proteins. Together, the structural and accessory genes comprise the final third of the genome, downstream of the replicase. The number of sgRNAs differs between coronaviruses but is typically between 7 and 9. The positive sense (+) sg mRNAs are 5’ capped (Nakagawa et al., 2016) and contain a 70-100 nt leader (L) sequence downstream of the cap, which includes a 6-9 nt section called the transcription-regulating sequence (TRS-B [Body]). The TRS-B is identical to the TRS at the 5’ end of the gRNA (termed TRS-L [Leader]) and the exact composition of the TRS is highly conserved within each
CoV subfamily. Additionally, the sgRNAs are 3' co-terminal with genomic RNA and thus form the nested-set of subgenomic mRNAs that partly define the Nidovirus order.

The “discontinuous transcription” model (Figure 5) proposed by Sawicki et al. (Sawicki and Sawicki, 1995) is widely accepted as the mechanism through which sg mRNAs are produced. In this model, the full-length genomic RNA acts as a template for the generation of negative-sense (-) sgRNAs, which in turn become templates for transcription of the positive-sense sg mRNAs. To produce the (-) sgRNAs, the viral RdRp binds the gRNA at the 3’ poly (A) tail and extension occurs 3’ to 5’ until a TRS-B is reached. A looping event brings the TRS-B in proximity to the TRS-L located at the 5’ end of the gRNA and here the polymerase strand-switches to produce a complimentary replicate of the 5’ gRNA leader. When extension terminates, the negative-sense RNAs possess an anti-sense leader sequence and a poly (U) tail (Sethna et al., 1991). From the limited population of negative-sense sgRNA templates, the viral RdRp produces one-hundred times more positive-sense sgRNAs, which are 5’-capped and exit the RTC to be translated on host ribosomes into S, E, M, N and accessory proteins (de Haan and Rottier, 2005; Fehr and Perlman, 2015; Masters, 2006; Sawicki et al., 2007).

Owing to the co-terminal nature of the 3’ ends of the sgRNA, the identical TRS compositions and the strand-jumping tendency of the viral RdRp, CoV genomic recombination events can occur. Recombination helps drive viral adaptation, evolution, zoonotic emergence and the generation of quasispecies during an infection. The strain variation and difficulty in investigating feline coronaviruses, as will be discussed below, is likely due in large part to recombination.
Figure 5: Model for Discontinuous Negative-strand Transcription. The viral RNA-dependent RNA polymerase binds the 3’ end of the positive-strand (solid black line) RNA and initiates elongation of the negative-strand (red dotted line) RNAs until a body transcription-regulating sequence (TRS-B) is encountered. Strand-switching occurs, which pairs the TRS-B with the leader-containing TRS (TRS-L) on the positive strand. Transcription of the negative strand re-initiates creating a complement to the TRS-L on the 3’ end and forming a dsRNA intermediate. The complete negative-strand is used as a template to produce 100-1000 times more positive-sense subgenomic mRNAs. Adapted from (Masters, 2006).
As a final note, the formation of double-stranded RNA intermediates in the RTC during replication presents a unique challenge to coronaviruses as dsRNAs are highly potent pathogen-associated molecular patterns (PAMP). As mentioned above and will be discussed below, the activity of at least one nonstructural protein, the EndoU encoded on nsp15, has been shown to maintain association of dsRNA with RTC thereby preventing detection of this viral PAMP by cytoplasmic pathogen recognition receptors.

**Virion assembly and release.** In addition to the nonstructural proteins, the structural nucleocapsid (N) protein has also been shown to localize to the replicase-transcriptase complex (Bost et al., 2000; van der Meer et al., 1999). Here, the N protein binds newly-synthesized, full-length coronavirus genomic RNA through associations with the 5’ leader, TRS and 3’ UTR to form a ribonucleoprotein (RNP) core (Molenkamp and Spaan, 1997; Shi and Lai, 2005; Stohlman et al., 1988). The stable RNP core is maintained by the large number of N-N interactions, which together form a structure with helical symmetry (de Haan and Rottier, 2005).

Following translation on host ribosomes, the other structural proteins S, E, M and HE localize to the endoplasmic reticulum (ER) via encoded signal peptides (Fehr and Perlman, 2015). The proteins are inserted in the ER and oriented such that the protein ectodomains extend into the ER lumen, then transported via the host secretory pathway to the ER-Golgi intermediate compartment (ERGIC), the site of mature virion assembly (Krijnse-Locker et al., 1994; Tooze et al., 1984). Cytoplasmic RNP cores bud into the ERGIC and become surrounded by host membranes with embedded structural proteins, thus giving rise to the mature coronavirus virion. The M protein, often referred to as the
virion “building block” directs the majority of the protein-protein interactions that necessitate the formation of the mature virion including the accumulation/incorporation of S into the envelope and directing the RNP core to bud into the ERGIC (de Haan and Rottier, 2005). However, several groups have shown that M protein alone is not sufficient to form virions and requires the E protein to form basic virus-like particles (VLPs), a process that is made more efficient by the N protein (Bos et al., 1996; Siu et al., 2008). Although the role of E is not fully defined, it may play a role in inducing proper membrane curvature (Corse and Machamer, 2000; Fischer et al., 1998; Raamsman et al., 2000), preventing M protein aggregation (Boscarino et al., 2008), altering the host secretory pathway (Ye and Hogue, 2007), or as is observed with other coronavirus proteins, may have pleiotropic effects in virion assembly and release. Assembled virions are transported in smooth-walled exocytic vesicles to the cell plasma membrane where the vesicles fuse and release the mature, infectious coronavirus particle (de Haan and Rottier, 2005; Masters, 2006).

Innate Immune Responses to RNA Virus Infection

Overview.

During virus infection, host cells detect extracellular and intracellular products of virus replication and mount an antiviral response. The so called “antiviral state” limits virus replication through production of directly-acting antiviral factors, blocking new infections, stimulation of cellular apoptosis and recruitment of innate and adaptive immune cells. Interferons (IFNs), host antiviral signaling molecules, are the master regulators of this antiviral state and are produced in response to detection of viral pathogen-associated molecular patterns (PAMPs), mainly viral RNAs, by host pattern
recognition receptors (PRRs). Autocrine and paracrine IFN signaling propagates through IFN receptors and stimulates expression of hundreds of IFN-stimulated genes (ISGs) that establish the rapid, pro-inflammatory antiviral state. In addition to interferon, other PRRs direct the synthesis and release of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), numerous interleukins (IL’s) and chemotactic cytokines resulting in an influx of neutrophils, macrophages, natural killer cells, lymphocytes and plasmacytoid dendritic cells (pDCs) to the sites of infection. Immune-activated macrophages and pDCs secrete high amounts of type I IFN that significantly enhance ISG production in surrounding tissue as well as collect viral antigens necessary to develop long-term immune memory. Together, the pro-inflammatory antiviral state is intended to limit the spread of the virus and prevent new infections during an intense yet brief window of time, while recruiting the cells necessary to generate long-lasting immunologic memory. As prolonged inflammation can lead to pathology, cells also encode a set of negative regulators to return tissues to homeostasis.

**Detection of RNA Viruses by Host Sensors.**

Cells distinguish self from non-self by detecting microbial- and viral-specific signatures collectively known as pathogen-associated molecular patterns (PAMPs), which are detected by a set of pattern-recognition receptors (PRRs). Host cells encode three major classes of PRRs: the cytoplasmic-resident retinoic acid-inducible gene I-like receptors (RLRs), the surface- and endosomal-resident toll-like receptors (TLRs) and the cytoplasmic-resident nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). Together, these PRRs detect virus components and trigger a signal
cascade to induce production type I and type III interferons and pro-inflammatory cytokines, which act in concert to orchestrate the “antiviral state” [reviewed in (Koyama et al., 2008; Loo and Gale, 2011; Takeuchi and Akira, 2009)].

**Retinoic-acid inducible gene-like receptors (RLRs).** The RLR class of pattern recognition receptors is the most well-studied and includes three members: retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). RIG-I and MDA5 are highly expressed in conventional dendritic cells (cDCs), macrophages and fibroblasts, but are also present in most tissues at a low basal level (Yoneyama et al., 2004). RLR expression is also tied to IFN signaling as cytoplasmic levels rapidly increase with IFN stimulation (Kang et al., 2004; Yoneyama et al., 2004; Yount et al., 2007). These two RLRs are individually or dually responsible for response to numerous RNA viruses [reviewed in (Loo and Gale, 2011)]. While RIG-I directs responses to Influenza, Japanese-Encephalitis virus and VSV, MDA5 is required for detection of Picornaviruses and, importantly, Coronaviruses (Deng et al., 2017; Kato et al., 2006; Kindler et al., 2017; Zalinger et al., 2015). A combined RIG-I and MDA5 response occurs following West Nile virus, Dengue and Reovirus infections (Fredericksen et al., 2008; Loo et al., 2008).

RIG-I and MDA5 proteins comprise two N-terminal caspase activation and recruitment domains (CARDs) followed by a DExD/H RNA helicase domain and a C-terminal repressor domain (RD) (Yoneyama et al., 2004). LGP2 does not contain a CARD domain and is thought to act as a negative regulator of RIG-I and MDA5 through associations with the RD (Saito et al., 2008). Both RIG-I and MDA5 bind viral RNAs via the RNA helicase and RD domains, which form a set of RNA-binding loops (Takahasi et
RIG-I binds short (19-1000 nt), cytoplasmic single-stranded (ss) and double-stranded (ds) RNA species, which are distinguished from host RNAs due to the lack of a 5’ CAP exposing a 5’ triphosphate group (Hornung et al., 2006; Kato et al., 2008; Pichlmair et al., 2006; Takahasi et al., 2008). MDA5 also binds cytoplasmic RNAs, however associations are only made with dsRNA species longer than 2 kilobases (Kato et al., 2008). Additionally, both RIG-I and MDA5 bind the products of the 2’5’-oligoadenylate synthase (OAS)-RNaseL system (Malathi et al., 2007), a set of interferon-stimulated genes that sense cytoplasmic dsRNAs and cleave host and viral RNA indiscriminately, thus creating a positive-feedback loop to potentiate RLR signaling. The N-terminal CARDs are held in an inactive “closed” conformation by the RD and released into an active, “open” conformation following RNA binding allowing signal propagation (Cui et al., 2008; Saito et al., 2008). Active RIG-I and MDA5 signal via homotypic CARD-CARD interactions with the mitochondrial antiviral signaling protein (MAVS) (Seth et al., 2005), also termed IPS-1 (Kawai et al., 2005), VISA (Xu et al., 2005) or Cardif (Meylan et al., 2005). MAVS is a membrane-associated, CARD-containing protein essential for RLR-dependent IFN production and resides on mitochondrial (Seth et al., 2005) and peroxisome (Dixit et al., 2010) membranes. Once associated, the RLR-MAVS complexes aggregate on host membranes to form the MAVS “signalome”, an intricate signaling platform modulated by a number of positive- and negative-regulators that functions to activate transcription of interferons and pro-inflammatory cytokines (Hiscott et al., 2006). Specifically, the MAVS signalome leads to downstream phosphorylation of interferon regulatory factor (IRF) 3 and IRF7, which form homodimers that translocate into the nucleus, bind target sequences and drive
expression of IFN. Interestingly, mitochondrial-associated and peroxisomal-associated MAVS signalome complexes are thought to differentially stimulate production of type I and type III interferons, respectively, seemingly by similar activation pathways (Coccia et al., 2004; Kotenko et al., 2003; Loo and Gale, 2011; Odendall et al., 2014; Onoguchi et al., 2007; Seth et al., 2005). In parallel, the MAVS signalome also results in the phosphorylation and subsequent ubiquitin-dependent proteasomal degradation of IκBα, releasing active NFκB into the nucleus leading to transcription of pro-inflammatory cytokines and interferons.

**Toll-like receptors (TLRs).** Mammalian cells express between 10 and 13 unique toll-like receptors (TLRs), which specifically associate with a broad range of viral, bacterial and fungal PAMPs. TLRs are single-span type I transmembrane proteins containing an N-terminal, leucine-rich ectodomain responsible for binding to PAMPs, a transmembrane domain, and a C-terminal, cytoplasmic toll/interleukin-1 receptor (TIL) domain, which allows homo- and heterodimer formation. The endosomal TLRs 3, 7, 8 and 9 are responsible for detection of phagocytosed viral materials or viruses that gain access to cells via the endosomal network. TLR7 and 9 are highly expressed in plasmacytoid dendritic cells (pDCs), a specialized innate-immune cell type responsible for the majority of type I interferon production in mammalian cells and the major modulator of cell-mediated immunity to coronavirus infection (Cervantes-Barragan et al., 2006). Within the endosome, TLR7 detects ssRNA species, while TLR9 binds non-methylated, CpG-containing DNA. TLR3 is more broadly expressed in mammalian tissues and detects endosomal dsRNA species [reviewed in (Iwasaki, 2012; Iwasaki and Medzhitov, 2004)]; it has also been shown to be important for appropriate innate
responses to coronaviruses including the human pathogen SARS-CoV (Totura et al., 2015).

Following binding to their respective ligands, TLR3 or TLR7 form homodimers through interactions between the cytosolic TIR domains. For TLR3, the TIR-TIR interactions form a complex with TIR domain-containing adaptor inducing IFN beta (TRIF), tumor necrosis factor receptor-associated factor (TRAF) 3, TRAF6, and receptor interacting protein 1 (RIP1) (Meylan et al., 2004; Oshiumi et al., 2003; Sato et al., 2003; Yamamoto et al., 2002). NFκB and AP-1 activation occurs via TRAF6/RIP1 resulting in pro-inflammatory cytokine production, while TRAF3 mediates IRF3 activation through the kinase activity of TBK1, resulting in type I IFN synthesis. The TLR7 TIR-TIR interaction forms a more traditional TLR signaling pathway mediated by interaction with myeloid differentiation factor 88 (MyD88). MyD88 proteins form a signaling complex with interleukin 1 receptor associated kinase (IRAK) 1, IRAK4, TRAFs 3 and 6 and Iκκα to promote NFκB-mediated cytokine production and IRF-7 mediated IFN synthesis (Häcker et al., 2006; Kawai et al., 2004; Uematsu et al., 2005).

**NOD-like receptors (NLRs).** NLRs are a third class of RLRs that detect both PAMPs and danger-associated molecular patterns (DAMPs), host-derived biomolecules that propagate pro-inflammatory signals following substantial tissue damage. The NLRs have a number of functions including the activation of the NLRP3 inflammasome and production of the pro-inflammatory cytokine IL-1β (Martinon et al., 2002), regulation of apoptosis, activation of NFκB and IFN synthesis, and as negative regulators of other RLRs (discussed below) [reviewed in (Carneiro et al., 2007; Lupfer and Kanneganti, 2013)]. Many of the antiviral NLRs, particularly the NLRP3 inflammasome components,
are interferon-stimulated geneses and thus are coordinated through the upstream activation of RLR and TLR signaling. Once formed, the NLRP3 inflammasome can be activated by a number of cell stresses to produce IL-1β, a highly pro-inflammatory cytokine with pleiotropic effects. This system is becoming increasingly appreciated in the antiviral response to RNA and DNA viruses including Orthomyxo-, Paramyxo-, Picrona-, Flavi-, Pox-, Herpes- and Adenoviridae [reviewed in (Lupfer and Kanneganti, 2013)]. The NLRP3 inflammasome had also been recently shown to be activated by SARS-CoV (Chen et al., 2019).

**Interferons and the Antiviral Effects of Interferon-stimulated Genes.**

Pro-inflammatory cytokines and interferons (IFNs) play an integral role in the innate immune system (de Weerd and Nguyen, 2012; Ivashkiv and Donlin, 2013; Pestka et al., 2004; Trinchieri, 2010) and are produced through the activity of the pattern recognition receptors discussed in detail above (Goubau et al., 2013; Iwasaki, 2012; Sen, 2002). These molecules have pleiotropic effects on various cell types and can direct both local and systemic responses. In most cases, the composition, intensity and duration of the IFN/cytokine response to viral infection determines if the virus is cleared, if immune memory is developed and if the host experiences immunopathologic side-effects. Thus, synthesis and signaling is tightly regulated.

**Interferons.** Interferons fall into three classes: type I comprising predominantly IFN-alpha (α) and -beta (β), type II or IFN-gamma (γ), and type III or IFN-lambda (λ). These different classes are structurally distinct, and as such, signal through different cell surface receptors, specific to each class. Type II (gamma) IFN is synthesized by specialized immune cells such as NK cells, CD4+ Th1 cells and CD8+ T cells and is
important for immune cell activation and adaptive immunity. Type I and III IFN, the focus of this discussion, play dominant roles as master regulators of the innate immune antiviral state. The effects of type I and type III IFN are largely cell-type specific and are determined both by the level and type of IFN transcription as well as the expression of the corresponding receptor. Type III IFNs are produced in greater amounts at barrier tissue sites (pulmonary and intestinal epithelia) and tend to stimulate a mild, localized response rather than the large, often systemic pro-inflammatory response observed with type I IFN. It should be noted that tissue-resident macrophages are largely insensitive to IFN-λ (Zanoni et al., 2017) and therefore, function in an IFN-stimulation paradigm that is not dependent directly on type III. As a result, IFN-λ is often triggered first in epithelial cells in an attempt to clear the infection. If the IFN-λ response is insufficient and the infection persists, then type I IFN, produced predominantly by recruited macrophages and dendritic cells, takes over. A great example is the relationship between type I and III interferons in response to influenza infection, which recapitulates the above model (Galani et al., 2017). Thus, it can be generally stated that IFN-λ is important for antiviral response at barrier sites, while type I IFN is broadly utilized in antiviral response [reviewed in (Donnelly and Kotenko, 2010; Lazear et al., 2015)].

The signaling pathways required to trigger synthesis of type I and III IFN discussed above, converge on the activation of a family of transcription factors termed interferon regulatory factors (IRFs) (Ito et al., 1999) with IRF3 and IRF7 being of particular importance to RLR-mediated synthesis of type I / III interferons (Marié et al., 1998). IRF3 is expressed in a broad range of cell types and low basal levels are present in the cytoplasm. Activation of IRF3 via the RLR signaling pathways leads to
homodimerization and translocation into the nucleus where it stimulates production of mainly IFN-β (or IFN-λ) and several IFN-α subtypes. Following downstream IFN signaling, IRF7, which is not constitutively expressed and acts as a positive feedback mechanism for IFN production, is activated by a similar pathway as IRF3 and promotes transcription of a different set of IFNs than IRF3 [reviewed in (Sen, 2002)]. Together, active IRF3 and IRF7 promote the synthesis of all type I / III IFNs, which are secreted from cells and stimulate autocrine (producer cell) and paracrine (surrounding cells) signal transduction pathways.

Type I and III IFNs signal through distinct receptors, IFNαR and IFNLR1, respectively, which activate a Janus Kinase / Signal Transducer Activator of Transcription (Jak/STAT) signal cascade resulting in the transcription of hundreds of interferon-stimulated genes (ISGs) (Randall and Goodbourn, 2008; Takeuchi and Akira, 2010; Wilkins et al., 2010; Yoneyama and Fujita, 2010). The type I IFN signal cascade is well defined. Association with type I IFNs causes dimerization of the two IFNαR component subunits, IFNαR1 and IFNαR2, leading to cross-phosphorylation of two JAKs associated with the cytoplasmic tail. The Janus Kinases allow association of STAT1 and STAT2, which are phosphorylated, dimerize and are released to bind cytoplasmic IRF9 (Meyer, 2009; Samuel, 2001; Yoneyama and Fujita, 2010). This complex, termed interferon-stimulated transcription factor 3 (ISGF3), enters the nucleus and binds consensus cis-acting DNA elements (interferon-stimulated response elements; ISRE) present on several hundred ISG promoters, which enhance transcription (Liu et al., 2012; Schoggins et al., 2014, 2011). The result is the production of hundreds of ISGs with a broad-range of antiviral activities.
**Antiviral activity of interferon-stimulated genes (ISGs).** The end result of the intricate interferon signaling cascade is the transcription of hundreds of interferon-stimulated genes (ISGs) in both infected and uninfected neighboring cells. These ISGs range in function from direct antiviral activity, immune cell regulation, negative and positive-IFN signaling feedback and many more detailed in Table 3. Together, these responses produce a host antiviral response against a multitude of RNA and DNA viruses and determine the outcome of infection, degree of associated sequelae and initiation of immune memory development. Several are discussed here, but please note that this is by no means an exhaustive list.
Multiple ISGs play roles in virus surveillance and inducing drastic cellular responses including shutdown of protein synthesis and/or programmed cell death (apoptosis). The importance of pattern recognition receptors cannot be understated as these allow host cells to determine if a virus is present in the tissue and ultimately

<table>
<thead>
<tr>
<th>ACTIVITY TYPE</th>
<th>SPECIFIC ISG(S)</th>
<th>FUNCTION(S)</th>
<th>LITERATURE REVIEW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Translation Inhibition / Apoptosis</td>
<td>PKR</td>
<td>(via eIF2a) Translation inhibition (via FADD and Caspase 8) Apoptosis</td>
<td>(Dauber and Wolff, 2009)</td>
</tr>
<tr>
<td></td>
<td>OAS / RNaseL</td>
<td>Binds dsRNA to induce total RNA degradation; Substrates for RLRs</td>
<td>(Chakrabarti et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>IFIT Family</td>
<td>Pleiotropic: Reduce cap-dependent translation; 5'ppp RNA binding; cell-intrinsic and cell-extrinsic immune responses</td>
<td>(Diamond and Farzan, 2013)</td>
</tr>
<tr>
<td>RNA Mutation</td>
<td>APOBEC</td>
<td>Cytidine deamination</td>
<td>(Stavrou and Ross, 2015)</td>
</tr>
<tr>
<td>Host or Viral Protein Modulation</td>
<td>ISG15</td>
<td>Pleiotropic regulation of cell processes: IFN signaling, translation, motility</td>
<td>(Perng and Lenschow, 2018)</td>
</tr>
<tr>
<td></td>
<td>Mx</td>
<td>Direct interaction with viral proteins (e.g. nucleocapsid and polymerase)</td>
<td>(Verhelst et al., 2013)</td>
</tr>
<tr>
<td>Virus entry and release</td>
<td>IFITM Family</td>
<td>Inhibit entry of enveloped viruses</td>
<td>(Diamond and Farzan, 2013)</td>
</tr>
<tr>
<td></td>
<td>Tethrin</td>
<td>Binds to virions and prevents virus release from membranes</td>
<td>(Sauter, 2014)</td>
</tr>
<tr>
<td></td>
<td>Viperin</td>
<td>Disruption of lipid rafts and lipid homeostasis</td>
<td>(Fitzgerald, 2010)</td>
</tr>
<tr>
<td>Inflammasome</td>
<td>RIG-I-mediated; NLRP3-mediated</td>
<td>Non-canonical Production of IL-1β; Modulation of NLRP3 inflammasome</td>
<td>(Kopitar-Jerala, 2017; Poeck et al., 2010)</td>
</tr>
<tr>
<td>T Cell and Cytolytic Cell Responses</td>
<td>IL-12</td>
<td>CD4+ Th1 differentiation; NK cell production of IFN-γ</td>
<td>(Biron et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>IL-21</td>
<td>CD8+ / NK cell cytolytic activity</td>
<td>(Strengell et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Perforin and Granzyme B</td>
<td>CD8+ / NK cell cytolytic activity</td>
<td>(Curtsinger et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>IL-15</td>
<td>Proliferation of memory T cells and NK cells</td>
<td>(Verbist and Klonowski, 2012)</td>
</tr>
<tr>
<td></td>
<td>IFN-γ</td>
<td>Activation of CD8+ T cells and Macrophages</td>
<td>(Samuel, 2001)</td>
</tr>
<tr>
<td></td>
<td>CCL3; CCL5; CXCL10</td>
<td>T cell chemoattractants</td>
<td>(Meager, 2006)</td>
</tr>
<tr>
<td>Antigen Processing / Presentation</td>
<td>Multiple</td>
<td>Upregulates MHC-I, antigen processing machinery and co-stimulatory molecules; dendritic cell expansion, others</td>
<td>(Gessani et al., 2014)</td>
</tr>
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</table>

Table 3: Type I Interferon Stimulated Genes and Antiviral Activities.
trigger the interferon signaling cascade. It is not surprising then, that these PRRs, including the RLRs, TLRs and NLRs, are significantly upregulated in response to interferon signaling and therefore provide enhanced virus surveillance in infected and uninfected cells. Additional cytoplasmic RNA binding proteins are also induced by interferon and include both protein kinase R (PKR) and 2′5′-oligoadenylate synthase (OAS). Following binding to cytoplasmic dsRNA, PKR has multiple roles in antiviral response including phosphorylation of eukaryotic initiation factor (eIF) 2α leading to host translation shutdown and promoting cellular apoptosis via the FADD/caspase8 pathway. 2′5′-OAS also binds cytoplasmic dsRNAs and produces the small signaling molecule 2′5′-oligoadenylate. This activates a second ISG, RNaseL, which non-specifically degrades host and viral RNAs resulting in a shutdown of protein synthesis and apoptosis. The products of RNaseL activity are also recognized by RIG-I and MDA5 and thus represent a positive IFN-signaling feedback loop. Other ISGs including the large family of cytoplasmic IFN-induced proteins with tetratricopeptide repeats (IFITs) and ISG15 have been shown to have pleiotropic effects on virus surveillance and fundamental cell processes. IFIT1 for example, is known to bind 5′-triphosphate-containing RNAs similar to RIG-I, sequestering viral mRNAs and preventing uncapped protein translation. IFIT1 has also been shown to inhibit cap-dependent translation through interaction with eIF3 thereby stalling the translation initiation complex or by directly preventing association of non-methylated, capped viral mRNAs. ISG15, which is one of the first and most abundant ISGs produced, is a ubiquitin-like polypeptide and is post-translationally conjugated to both host and viral proteins. As a result, ISG15 can caused proteasome-dependent protein degradation, impact the RLR/IFN signaling
cascades and cell processes such as translation, glycolysis and motility. The full spectrum of ISG15 activities and the mechanisms through which it acts are not fully understood, however it can be appreciated as a significant antiviral response mediator.

Another set of ISGs act to inhibit virion entry, maturation and release from cells. IFN-induced transmembrane proteins (IFITMs), are small host transmembrane proteins that broadly inhibit the entry of enveloped viruses such as coronaviruses, flaviviruses, influenza and others. While IFITMs are produced in cells at low basal levels, their expression increases significantly with IFN stimulation. These proteins appear to more potently inhibit viruses that fuse in late endosomes and although the exact antiviral mechanism is not known, they have been predicted to affect the curvature of endosomes to prevent fusion, alter the composition of lipids and host proteases and recently, disrupt the vesicular trafficking network completely. Another ISG, Viperin disrupts lipid homeostasis and lipid raft formation, critical sites for the budding and release of several host lipid membrane-derived enveloped viruses. Finally, the activity of an ISG called tetherin, physically binds to budding virions and significantly reduces the efficiency to release from the cell surface. The activity of these ISGs acts to slow virion production and help prevent entry of viruses into uninfected cells.

An early and robust interferon response is also important for modulating the “quality, quantity and balance” of host adaptive responses, a notion our group has utilized in the design of live-attenuated coronavirus vaccines. Type I interferon stimulation has been shown to increase surface levels of MHC-I, the machinery required to process and load antigens on MHC-I as well as and production of co-stimulatory molecules in, and specific expansion of, antigen presenting cells. Several ISGs have
also been shown to regulate T lymphocytes, which provide a critical adaptive response to viral infection and are involved in activating phagocytic cells, antigen-specific killing of virus-infected cells and cross-talk with B cells to establish humoral immunity. Among the T cell modulating ISGs are IFN-γ, IL’s -12, -15, -21 and several chemoattractant cytokines that recruit CD4+ and CD8+ T cells to sites of infection. B cell differentiation and isotype switching has also been observed, however the relationship between IFN and these processes is complex and not fully understood. Thus, type I and III interferons are critical modulators of innate antiviral immunity but are also important regulators in CMI, humoral and long-term adaptive immunity.

**Negative Regulators of Interferon Synthesis and Signaling.**

Prolonged inflammation resulting from sustained IFN signaling and ISG activity can lead to extensive tissue damage and sequelae long after viral clearance. These immune-mediated complications are collectively referred to as “immune pathologies” or “interferonopathies” and are observed following infection of multiple viruses including influenza, West Nile virus and SARS-CoV (Channappanavar et al., 2016; Garcia-Sastre and Biron, 2006; Le Goffic et al., 2006; Wang et al., 2004). As a result, cells encode a number of negative regulators that act to suppress IFN signaling at the transcription and post-transcriptional level [reviewed in (Arimoto et al., 2018)]. IRF2, for example, is a member of the interferon regulatory factor (IRF) protein family that has been shown to attenuate type I IFN gene synthesis by preventing transcription in the nucleus (Hida et al., 2000). Several other ISGs are known to suppress IFN through post-translational modification or direct interaction with components of RLR signaling pathways. NLRC5, a NOD-like receptor (NLR) family protein, disrupts RLR-induced antiviral responses...
through direct CARD-CARD sequestration of RIG-I and MDA5, preventing association with MAVS, while another NLR, NLXR1, disrupts the RLR signaling through competitive binding to MAVS (Moore et al., 2008). Other proteins affect IFN signaling through ubiquitin modifications that target RLRs. RNF125 is a K48 ubiquitin ligase that targets RIG-I and MDA5 causing degradation by the proteasome (Arimoto et al., 2007). CYLD, a deubiquitinase, removes poly-ubiquitin chains from activated RLRs, preventing downstream binding to MAVS (Zhang et al., 2008).

**Coronavirus-encoded Antagonists of Type I Interferon**

**Overview.**

Establishing successful viral infection is a complex interplay between host and viral factors. While the host attempts to detect and respond to unique virus signatures, viruses utilize encoded factors to antagonize these host pathways and establish an intracellular environment amenable to production of progeny virions [reviewed in (Katze et al., 2002)]. This competition is often referred to as the “Red Queen Hypothesis”, a literary allusion to continuous movement without progress, and is the driving force behind both virus and innate response evolution (Duggal and Emerman, 2012). The major obstacle to virus replication is the host master antiviral signaling molecule, interferon. Indeed, all successful viral pathogens, including the coronaviruses discussed in this dissertation, encode viral proteins that antagonize the synthesis, signaling and/or effector functions of the host interferon pathway. Importantly, virus strains encoding mutations in these interferon antagonists, creating so called hyper interferon-inducing (HII) or hyper interferon-sensitive (HIS) strains, have been shown to attenuate virus replication and are useful platforms for live-attenuated vaccine design (Deng et al.,
Coronaviruses carry a large, single-stranded RNA genome, which is replicated in the cell cytoplasm and forms double-stranded RNA intermediates during the process of discontinuous transcription. These single and double-stranded viral RNAs are known to be potent triggers of type I / III interferon synthesis and are detected by cytoplasmic and endosomal PRRs. Interestingly, coronavirus infection does not result in an early or robust type I IFN response and these viruses appear to replicate seemingly without innate immune detection by the host. In fact, this has become a hallmark of coronavirus infection kinetics and is observed in both human and animal cases (Channappanavar et al., 2016; Kint et al., 2014; Lau et al., 2013; Menachery et al., 2014). This subversion of the IFN signaling cascade is due to a complex and coordinated set of CoV-encoded interferon antagonists as well as several fundamental aspects of coronavirus replication biology. Indeed, over fifteen unique components of human and animal CoVs have been demonstrated to antagonize distinct elements of the IFN synthesis and signaling pathways or affect ISG function (Table 4). From these studies, several key conclusions can be drawn: First, all CoV have multiple antagonists to delay the IFN response, the majority of which are group-specific accessory genes, but several, such as the papain-like protease encoded on nsp3 and the EndoU encoded on nsp15, are highly conserved across all CoVs. Second, some antagonists are inherent aspects of CoV replication biology and may or may not have evolved specifically to prevent immune activation. Finally, the ability of CoV proteins to antagonize IFN may be cell-type specific as observed with MHV ns2 (Zhao et al., 2012). Together, these mechanisms allow productive replication of coronaviruses in various mammalian tissues without triggering an early innate immune response.
Synthesis. A number of coronavirus factors prevent the synthesis of type I interferons by preventing detection of cytoplasmic RNAs or by directly targeting components of the RLR or TLR signaling cascades. One aspect of coronavirus

<table>
<thead>
<tr>
<th>ANTAGONIST TARGET</th>
<th>VIRUS</th>
<th>ANTAGONIST</th>
<th>ACTIVITY / TARGET</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SARS-CoV</td>
<td>PLpro</td>
<td>Binds STING and blocks IRF3 translocation</td>
<td>(Sun et al., 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nsp14 and nsp16</td>
<td>RNA capping</td>
<td>(Totura and Baric, 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ORF3b</td>
<td>Binds and blocks IRF3 translocation</td>
<td>(Freundt et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ORF6</td>
<td>Blocks NF-κB signaling</td>
<td>(Hussain and Gallagher, 2010; Kopecy-Bromberg et al., 2007)</td>
</tr>
<tr>
<td>IFN Synthesis</td>
<td>MERS-CoV</td>
<td>ORF4a</td>
<td>Binds dsRNA; blocks PKR</td>
<td>(Niemeyer et al., 2013; Rabouw et al., 2016)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ORF4b</td>
<td>Prevents MAVS signaling by binding TBK1</td>
<td>(Yang et al., 2015)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PLpro</td>
<td>DUB activity prevents IRF3 signaling</td>
<td>(Mielech et al., 2014; Yang et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>MHV</td>
<td>Nsp15</td>
<td>Prevents MDA5, OAS and PKR detection of dsRNAs</td>
<td>(Deng et al., 2017; Kindler et al., 2017)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PLP2</td>
<td>DUB activity</td>
<td>(Mielech et al., 2015; Rose and Weiss, 2009)</td>
</tr>
<tr>
<td></td>
<td>HCoV 229E</td>
<td>Nsp15</td>
<td>Prevents MDA5, OAS and PKR detection of dsRNAs</td>
<td>(Deng et al., 2017; Kindler et al., 2017)</td>
</tr>
<tr>
<td>IFN Signaling</td>
<td>SARS-CoV</td>
<td>nsp1</td>
<td>Decreases STAT phosphorylation</td>
<td>(Wathelet et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PLpro</td>
<td>Interaction with several cell signaling components</td>
<td>(Clementz et al., 2010; Devaraj et al., 2007; Frieman et al., 2009; Li et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ORF3a</td>
<td>Degradation of IFNαR</td>
<td>(Minakshi et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ORF6</td>
<td>Blocks STAT1 translocation</td>
<td>(Frieman et al., 2007)</td>
</tr>
<tr>
<td>ISG Activity</td>
<td>MHV</td>
<td>ns2</td>
<td>Cleaves 2-5A and prevents RNase-L activation</td>
<td>(Zhao et al., 2012)</td>
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<tr>
<td></td>
<td></td>
<td>ORF5a</td>
<td>Uncharacterized phenotype</td>
<td>(Koetzner et al., 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nsp15</td>
<td>Binds dsRNAs and prevents OAS and PKR activation</td>
<td>(Deng et al., 2017; Kindler et al., 2017)</td>
</tr>
<tr>
<td></td>
<td>MERS-CoV</td>
<td>ORF4a</td>
<td>Binds dsRNA; blocks PKR</td>
<td>(Niemeyer et al., 2013; Rabouw et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>IBV</td>
<td>nsp2</td>
<td>Overexpress GADD34 to block PKR</td>
<td>(Wang et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>TGEV</td>
<td>ORF7</td>
<td>Activates PP1c-mediated inactivation of PKR and OAS</td>
<td>(Cruz et al., 2013, 2011)</td>
</tr>
<tr>
<td></td>
<td>FIPV</td>
<td>ORF7</td>
<td>Prevents phosphorylation of eIF2α</td>
<td>(Dedeuwaerder et al., 2014)</td>
</tr>
</tbody>
</table>

Table 4: Coronaviruses and Encoded Antagonists of the Type I IFN System.
replication biology, the formation of host-derived double-membrane vesicles (DMVs), is an important and well-conserved method to prevent RLR activation. These DMVs, which are the sites of genomic RNA replication and mRNA transcription, act to physically separate viral dsRNA replication intermediates from detection by MDA5 (Gosert et al., 2002; Knoops et al., 2008; Prentice et al., 2004; Shi et al., 1999; van der Meer et al., 1999). Once viral mRNAs are produced within the DMVs, they must enter the cytoplasm for translation. This is problematic as these viral RNAs contain a 5’ triphosphate, a potent activator of RIG-I. However, the conserved activity of nonstructural protein (nsp) 13 removes the 5’ triphosphate and further processing by nsps 10, 14 and 16 adds a methylated guanidine 5’ cap thus preventing the detection of this viral mRNA by RIG-I and allowing translation (Bouvet et al., 2010; Chen et al., 2009; Decroly et al., 2008; Ivanov and Ziebuhr, 2004; Züst et al., 2011). Our group and others have also demonstrated that other highly conserved components of the DMV-associated replicase-transcriptase complex (RTC), the papain-like protease and the EndoU, are important interferon antagonists. My contributions to determining the IFN antagonism functions of these proteins will discussed further below.

In many cases, however, DMV formation and viral mRNA capping are not sufficient to prevent detection of viral PAMPs, thus coronaviruses encode compensatory mechanisms to directly antagonize components of the RLR or TLR signaling cascades. The well-studied human coronaviruses, SARS- and MERS-CoV, for example, utilize several unique accessory proteins for this role. In SARS-CoV, ORF3b binds directly to IRF3 preventing nuclear translocation (Freundt et al., 2009), while ORF6 blocks NFκB and downstream STAT1 signaling by impeding protein import into the nucleus (Frieman
et al., 2007; Hussain and Gallagher, 2010; Kopecky-Bromberg et al., 2007). The MERS-CoV accessory protein ORF4a has been shown to bind cytoplasmic dsRNA species prevents RLR binding as well as PKR-mediated shutdown of host protein synthesis (Niemeyer et al., 2013; Rabouw et al., 2016). ORF4b, another MERS accessory protein prevents MAVS signaling by binding to TBK1 and preventing kinase activity (Yang et al., 2015). Together, these IFN synthesis antagonists represent a small number of studied viral factors and it is likely that other coronavirus species, including the large number of SARS-related bat CoVs, encode unique accessory proteins to prevent IFN synthesis.

**Signaling and activity of interferon-stimulated genes (ISGs).** Following synthesis, interferons are released and propagate the antiviral signal back to the producer cell (autocrine) and neighboring cells (paracrine) through engagement of the IFN alpha-receptor (IFNαR). This triggers the Jak/STAT signal cascade and results in ISG production. Not surprisingly, coronavirus IFN antagonists also target components of this signal pathway as well as specific activities of the ISGs produced. To prevent signaling, SARS-CoV reduces the cell-surface abundance of IFNαR, through the activity of the accessory protein, ORF3a (Minakshi et al., 2009). Further, SARS-CoV nsp1 and ORF6 antagonize the host protein STAT by decreasing the phosphorylation required for STAT activity and blocking STAT1 translocation, respectively (Frieman et al., 2007; Wathelet et al., 2007).

Further downstream, multiple coronaviruses encode ISG antagonists many of which target PKR and the 2’5’-OAS / RNaseL system. These two cytoplasmic systems bind dsRNAs and lead to shutdown of host protein synthesis and apoptosis by phosphorylation of eIF2α (PKR) or non-specific digestion of host and viral RNAs
(OAS/RNaseL), thus blocking the activity of these ISGs is critical for virus production. Several factors including the highly conserved EndoU encoded on nsp15 (Deng et al., 2019b, 2017; Kindler et al., 2017), and the MERS-CoV accessory protein ORF4a (Niemeyer et al., 2013; Rabouw et al., 2016) prevent ISG activation by binding and sequestering cytoplasmic dsRNAs. The MHV hepatocyte-specific accessory protein ns2 cleaves the small signaling molecule 2’5’-oligoadenylate preventing activation of RNaseL (Zhao et al., 2012). Others, such as MHV ORF5a, are known ISG antagonists however but their functions have not been fully described (Koetzner et al., 2010).

Generally speaking, coronaviruses that encode these downstream signaling and ISG antagonists are much more resistant to IFN treatment and are more likely to prevent de novo interferon activation of infected cells. FIPV, for example, remains highly resistant to serum IFN as is evident by the inability to treat feline peritonitis with intravenous IFN after infection is established (Weiss et al., 1990). Several studies investigating TGEV (Cruz et al., 2013, 2011) and FIPV (Dedeurwaerder et al., 2014, 2013) have determined that 7a, an accessory protein only encoded in several alphacoronaviruses, antagonizes the activity of ISGs and correspondingly causes insensitivity to IFN. In TGEV, 7a enhances the specificity of protein phosphatase 1c (PP1c) for eukaryotic initiation factor (eIF) 2α. This interaction causes increased dephosphorylation of eIF2α and results in prevention of host translation shutdown. In FIPV, the activity of 7a is associated with a second unique accessory region, ORF3. From clinical studies, it was determined that deletion of 7a or ORF3 from WT FIPV resulted in a significant reduction of FIP severity. Wild-type FIPV and FIPV-∆7a viruses stimulated similar, increased levels of type I IFN in feline macrophages and serum
during cat infection. Interestingly, when 7a is expressed alone it not sufficient to establish IFN resistance during VSV infection, suggesting that 7a requires additional FIPV components, presumably ORF3 to function. The mechanism of ORF3 activity and the characterization of other conserved or unique viral components responsible for IFN antagonism have yet to be evaluated.

**Experimental Examples of Highly Conserved Coronavirus Interferon Antagonists.**

The Baker Lab is interested in understanding how highly conserved nonstructural proteins contribute to antagonism of interferon. We predict that mutagenesis of these nsps, leading to a hyper-interferon-inducing (HII) virus phenotype, is a strategy that could be employed to attenuate most all coronavirus strains and provide a platform on which to construct live-attenuated vaccines. To test this hypothesis, colleagues and I studied two CoV factors, the PLP2 encoded on nsp3 and the EndoU encoded on nsp15 and determined if these conserved proteins antagonized interferon. This work laid the groundwork my dissertation research and formed the long-term goal of developing an effective vaccine for feline coronavirus.

**The feline coronavirus papain-like protease is an IFN antagonist.** CoVs encode one or two multifunctional papain-like protease(s) (PLP1 and/or PLP2), which catalyze three reactions within the host cell: processing of the nonstructural CoV replicase polyprotein (protease), cleavage of the host signaling molecule ubiquitin (DUB), and deconjugation of the antiviral signaling molecule interferon-stimulated gene (ISG) 15 (deISGylase). While protease activity is required for viral replication, it is unclear what role DUB or deISGylation play during infection. We and others hypothesize that PLP2 is an IFN antagonist acting through disruption of ub-dependent
signaling in the IFN synthesis pathway, and therefore DUB function is an interesting mutagenesis target for generating live-attenuated vaccine strains. The DUB activity of MERS-CoV (Bailey-Elkin et al., 2014) and Equine Arteritis Virus (EAV) (van Kasteren et al., 2013) PLPs have been shown to suppress innate immune responses by targeted disruption of critical ubiquitination marks of IFN synthesis pathways (Bibeau-Poirier and Servant, 2008). DUB activity, however, is not the only means by which PLP can antagonize innate immune responses. Several groups have experimentally determined that PLPs can indirectly antagonize IFN production through association with different cell signaling components (Clementz et al., 2010; Devaraj et al., 2007; Frieman et al., 2009; Li et al., 2011; Mielech et al., 2014; Rose and Weiss, 2009; Sun et al., 2012). SARS-CoV PLpro stabilizes the NF-κB inhibitor IκBα, preventing NF-κB nuclear translocation and subsequent IFN production (Frieman et al., 2009). By a similar mechanism, the PLPs of HCoV-NL63 and SARS-CoV were shown to associate with and destabilize stimulator of interferon genes (STING) dimers (Sun et al., 2012). The end result is disruption of IRF-3 nuclear translocation, a key element of the MAVS-mediated IFN synthesis pathway. Additionally, SARS-CoV PLpro has been shown to decrease STAT phosphorylation through proteasome-mediated degradation of ERK1 (Li et al., 2011), a component of the auto- and paracrine signaling pathways leading to ISG expression. These studies have largely been done on PLPs from the Betacoronavirus genus, and I was interested to understand how conserved these functions were in the alphacoronavirus feline infectious peritonitis virus (FIPV). Therefore, I characterized the activity of recombinant FIPV PLP2, and demonstrated that it functions as a robust protease (Figure 6A), delSGylase (Figure 6B) and DUB enzyme (Figure 6C) and is
able to antagonize MDA5-driven IFN transcription (Figure 6D). Thus, the PLP2 from the alphacoronavirus FIPV has the same conserved functions as other CoV PLPs, and therefore generating an FIPV PLP mutant virus may lead to increased IFN stimulation and virus attenuation.
Nonstructural protein 15 is a potent antagonist of type I interferon. Another effective method used by CoVs to prevent IFN synthesis and ISG activity is to avoid detection by PRRs entirely. Recently, our group and others demonstrated that the
endoribonuclease (EndoU) encoded on the highly conserved nonstructural protein 15 acts as a dominant interferon antagonist by preventing detection of dsRNA replication intermediates (Deng et al., 2019b, 2017; Kindler et al., 2017). In our 2017 study, mutant mouse hepatitis viruses (MHV) encoding a hexamer-destabilizing mutation (N15m1) or a catalytic site-inactivating mutation (N15m3) in EndoU stimulated high amounts of type I IFN during replication in bone marrow-derived macrophages (Figure 7A). The nsp15-mutant also promoted rapid macrophage apoptosis (Figure 7B-C) and prevented the activation of both PKR and the 2′5-OAS / RNaseL systems (data not shown). We determined that this virus was attenuated in tissue culture and did not cause pathology in mice (data not shown). Further, vaccination with the N15m1 virus was sufficient to protect mice from a lethal intracranial challenge of wild-type MHV (Figure 7D) demonstrating that nsp15 is a valuable target for generation of live-attenuated vaccine strains of other coronaviruses, such as FIPV.
A. MHV expressing mutations in EndoU (N15m1 and N15m3) induce early production of type I IFN in bone marrow-derived macrophages (BMDMs). BMDMs were infected with WT or mutant MHV at an MOI of 0.1. At indicated time points, total RNA was extracted and analyzed for mRNA levels of IFN-α11 by qPCR. The levels of mRNA relative to β-actin mRNA were expressed as $2^{-\Delta\Delta CT}$ [$\Delta\Delta CT = CT_{(\text{gene of interest})} - CT_{(\beta-\text{actin})}$]. Values were analyzed using a two-way ANOVA test by time. 

B. Representative electron micrograph images depicting induction of BMDM cell apoptosis following infection with mutant MHV or staurosporine positive control. No apoptosis is observed in mock or WT MHV-infected BMDMs. Arrows indicate nuclear chromatin condensation, a hallmark of apoptosis. 

C. B6 BMDMs were infected with WT or mutant MHV at a MOI of 0.1. At indicated time points, cell lysates were collected for the detection of cleaved–caspase-3 (active form), N protein, and β-actin by Western blotting. 

D. Primary challenge of C57/BL6 mice by N15m1 is protective against secondary challenge with WT virus. For the primary infection, mice were challenged intracranially (IC) with WT or N15m1 MHV. Primary WT infection was lethal by 10 days post-infection (DPI), whereas all mice survived N15m1 infection (data not shown). Surviving mice (N15m1 immunized) were challenged 7 weeks later with WT virus IC. All N15m1 immunized mice survived whereas all naive mice succumbed to infection.

Figure 7. EndoU Mutant MHV Stimulates IFN and Protective Immunity in Mice. (A) MHV expressing mutations in EndoU (N15m1 and N15m3) induce early production of type I IFN in bone marrow-derived macrophages (BMDMs). BMDMs were infected with WT or mutant MHV at an MOI of 0.1. At indicated time points, total RNA was extracted and analyzed for mRNA levels of IFN-α11 by qPCR. The levels of mRNA relative to β-actin mRNA were expressed as $2^{-\Delta\Delta CT}$ [$\Delta\Delta CT = CT_{(\text{gene of interest})} - CT_{(\beta-\text{actin})}$]. Values were analyzed using a two-way ANOVA test by time. (B) Representative electron micrograph images depicting induction of BMDM cell apoptosis following infection with mutant MHV or staurosporine positive control. No apoptosis is observed in mock or WT MHV-infected BMDMs. Arrows indicate nuclear chromatin condensation, a hallmark of apoptosis. (C) B6 BMDMs were infected with WT or mutant MHV at a MOI of 0.1. At indicated time points, cell lysates were collected for the detection of cleaved–caspase-3 (active form), N protein, and β-actin by Western blotting. (D) Primary challenge of C57/BL6 mice by N15m1 is protective against secondary challenge with WT virus. For the primary infection, mice were challenged intracranially (IC) with WT or N15m1 MHV. Primary WT infection was lethal by 10 days post-infection (DPI), whereas all mice survived N15m1 infection (data not shown). Surviving mice (N15m1 immunized) were challenged 7 weeks later with WT virus IC. All N15m1 immunized mice survived whereas all naive mice succumbed to infection.
**Final section remarks.** From this preliminary work on the IFN antagonism functions of a highly conserved coronavirus endoribonuclease, EndoU, and defining the multifunctional activities of FIPV PLP2, it was clear that further investigation of IFN antagonists encoded by feline coronavirus would positively benefit the field. Feline coronaviruses (FCoVs) are arguably the most important veterinary infection in young cats and infections can be highly lethal. However, vaccine design and implementation has been a perennial challenge and no effective vaccine exists. The long-term goal of the FCoV research program in the Baker Lab is to develop an effective, live-attenuated vaccine against feline coronavirus through mutations of encoded interferon antagonists such as the EndoU and PLP2. The scope of my dissertation, which will be discussed in detail below, was to establish basic virology techniques to investigate FCoV in the lab and to design genetic, tissue culture and technical tools to facilitate further study of these important pathogens.

**Feline Coronaviruses**

**Classification and Categorization.**

Feline coronaviruses (FCoV) are members of the *Alphacoronavirus* genus (*Nidovirales* order; *Coronaviridae* family; *Orthocoronavirinae* subfamily) (Figure 1) and are closely related to other important veterinary pathogens, such as canine coronavirus (CCoV) and transmissible gastroenteritis virus (TGEV), which share relatively high genetic similarity. The FCoV genome (Figure 2) is single-stranded, non-segmented, positive-sense RNA comprising approximately 29 kilobases. The genome organization and predicted RNA structures of a clinical FIPV isolate is described by Dye et al. (Dye and Siddell, 2007). Contained on the genome are 11 open reading frames, which
encode the two replicase polyproteins pp1a and pp1ab, four structural protein S, E, M and N (HE is not present), and two accessory protein regions, ORF3abc and ORF7ab. Individual strains of feline coronavirus are further defined by two criteria: serotype, based on antigenicity and sequence of the spike protein, and biotype (often referred to as “pathotype”) related to cell tropism and the disease characteristics resulting from infection (Table 5). The two FCoV biotypes include feline enteric coronavirus (FECV), which causes a mild, often sub-clinical enteritis, and feline infectious peritonitis virus (FIPV), the etiologic agent of the severe, systemic, immune-mediated disease known as feline infectious peritonitis (FIP). Both biotypes can encode either serotype I or serotype II (often referred to simply as “type I” or “type II”) spike proteins. The serotype designation was initially made on the basis of antibody binding specificity but has recently been updated to include sequence- and protease-cleavage site-specific designations (Whittaker et al., 2018).

<table>
<thead>
<tr>
<th>BIOTYPES</th>
<th>SEROTYPE</th>
<th>RECEPTOR</th>
<th>CELL TROPISM</th>
<th>DISEASE</th>
</tr>
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<tbody>
<tr>
<td>Feline enteric CoV (FECV)</td>
<td>I</td>
<td>Unknown receptor; DC-SIGN co-factor</td>
<td>Intestinal, lung and kidney epithelia</td>
<td>Subclinical enteritis; self-limiting; persistent and recurrent</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>Feline aminopeptidase N; DC-SIGN co-factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feline infectious peritonitis virus (FIPV)</td>
<td>I</td>
<td>Unknown receptor; DC-SIGN co-factor</td>
<td>Myeloid lineage cells: monocytes, dendritic cells, macrophages</td>
<td>Feline infectious peritonitis (FIP): high mortality; systemic; immune-mediated</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>Feline aminopeptidase N; DC-SIGN co-factor</td>
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</tbody>
</table>

Table 5: Feline Coronavirus Biology.
Historical Significance.

Feline coronaviruses are the most common pathogen identified in the feces of domestic cats and are widespread with upwards of 90% seropositivity in certain domestic feline populations (Hohdatsu et al., 1992; Pedersen, 2009). As a result, feline coronaviruses, more specifically FIPV, are considered some of the most significant causative agents of communicable disease in feline medicine. The connection between a coronavirus and FIP was first suggested by Ward et al. in 1970 (Ward, 1970), and the first strains were cultured in autochthonous macrophage tissue derived from an infected cat in 1976-1980 (Black, 1980; N C Pedersen, 1976). A permissive cell type, the macrophage-like Fcwf-4 cell line, was also described during this time and was used to isolate several FCoV strains including the serotype I Black strain, the progenitor to the strain used in this dissertation (Black, 1980; Jacobse-Geels and Horzinek, 1983).

The emergence of FCoV-mediated disease occurred suddenly in the United States in the 1950-60s as cases of feline infectious peritonitis (FIP), then termed an “important disorder of cats” (Holzworth, 1963), were increasingly diagnosed by veterinarians at Angell Memorial Animal Hospital in Boston, MA. Investigators often speculate on the conditions that led to the emergence of FCoVs during this time and suggest three, possibly connected factors that may have facilitated the widespread infection of cats (Pedersen et al., 2009). First is the potential for a speciation event in which a related coronavirus, such as CCoV or TGEV, which became widespread in the United States just 10 years prior, jumped into the feline population (Haelterman, 1962). Indeed, recombination between these related alphacoronaviruses is observed (Benetka et al., 2006; Herrewegh et al., 1998; Pedersen, 2009; Wesley, 1999). A prime example
of natural recombination is the serotype II FCoV spike protein, which contains sequences of both FCoV and CCoV (Herrewegh et al., 1998). Additionally, at least one CCoV strain has been demonstrated to not only infect cats, but also enhance FIPV infection (McArdle et al., 1992). A second possibility for emergence of highly pathogenic FCoVs could stem from an FECV strain variant that is more easily mutable (lower “mutagenic barrier”) to FIPV that could have arisen during that time. A final theory involves the changes in feline husbandry in the years following WWII. As purebred cats became more desirable, the density of mixed-breed cats in animal shelters increased along with the number of feral cats. The cohousing of large numbers of cats (and dogs for that matter) increases the chance of virus transmission and recombination between FCoV strains. Feline leukemia virus (FeLV), another prominent pathogen of cats, also emerged during this time and may have acted as a cofactor for FIPV: FeLV infection leads to states of immune suppression, a scenario likely to allow development of a large, genetically diverse FECV/FIPV quasispecies (Pedersen et al., 2009). It is likely that multiple factors, possibly all three introduced here, led to the widespread prevalence of feline coronavirus within the domestic cat population – a dominance still observed today.

**Biotypes.**

Feline coronaviruses are broadly grouped into two biotypes (often referred to as “pathotypes”), feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV). Traditionally, the biotype designation is based on several factors including cell tropism, disease progression or severity, and a collection of genetic markers in the S and 3c accessory gene (reviewed in Kipar and Meli, 2014; Pedersen, 2014, 2009),
although the range of disease signs and clinical outcomes are likely to extend beyond these two basic biotype definitions. Intriguingly, FIPV is not transmissible but rather, arises directly from FECV during persistent infection of intestinal epithelia.

FECV is endemic in most healthy domestic cat populations. The virus is shed in the feces and urine of infected cats beginning 1 week after introduction. Shedding can be transient, recurrent or chronic for months or years. Due to the nature of the shedding, the virus is transmitted via the fecal-oral route and is carried into the gut where it replicates in mature apical epithelial cells in the intestine. FECV infection of cats causes mild enteritis associated with loose stool and diarrhea and commonly leads to an asymptomatic, persistent infection (Addie, 2011; Addie et al., 2003; Pedersen et al., 2008). Immunity to FECV is tenuous and felines that clear the virus are often re-infected with the same FECV or a strain with high genetic similarity. Additionally, antibody titers to FECV positively correlate with increased virus shedding in the feces, and it thought that cell-mediated rather than antibody-mediated immunity is important to clear FECV infection. A subset of FECV infections (3-10%) result in progression to lethal feline infectious peritonitis (FIP) (Kipar and Meli, 2014; Pedersen, 2014a, 2009), a systemic, immune-mediated disease with high mortality rates (Addie and Jarrett, 1992; Niels C. Pedersen, 1976). This rapid-onset disease is due to genotypic and phenotypic changes in a subpopulation of FECV virions resulting in infection of myeloid-lineage cells. The infection of myeloid cells and the onset of FIP defines the second FCoV biotype, FIPV.

FIPV is the second FCoV biotype and is not horizontally transmitted despite being highly infectious if isolated and artificially introduced. The lack of transmission is
likely due to the shift in tropism away from intestinal epithelia resulting in little to no detectable FIPV shed in the feces. Rather, FIPV specifically infects myeloid lineage cells including monocytes, macrophages and dendritic cells leading to systemic, immune-mediated disease pathology and mortality rates close to 99% (Pedersen, 2014a).

**The Internal Mutation Theory.**

Characterizing the genotypic and phenotypic requirements for FECV to FIPV progression is one of the more well-studied aspects of feline coronavirus biology as it has such dramatic effects on disease and mortality. To date, the most widely-accepted model is known as the “internal mutation theory” (Poland et al., 1996; Vennema et al., 1998), which proposes that, within an individual animal, FIPV arises directly from FECV due to non-synonymous mutations in spike (S) and the group-specific protein 3C. These mutations accumulate and arise independently, not as a “set” and therefore, the phenotype resulting from these mutations is critical – mainly productive infection of myeloid-lineage cells.

Numerous factors can contribute to the accumulation of mutations in the FCoV genome during infection. All coronaviruses encode both an RNA-dependent RNA polymerase (nsp12), which has an error rate of 1 per 10,000 nucleotides, and an ExoN (nsp14) with proofreading activity to ensure sequence fidelity. Despite this, when one group compared 11 genomes each of FECV and FIPV obtained during natural infections, they found that approximately 10% of the FIPV nucleotide identity was unique, and did not occur across the FECV genomes tested (Chang et al., 2012; Pedersen, 2014a). Further, over half of these mutations occurred in spike while others
accumulated in ORF1ab and the accessory genes. The high rate of substitution suggests that advantageous mutations accumulate over the course of multiple replication cycles and, therefore, both acute and persistent FECV infection can increase the density of mutations. A second factor is immune suppression, which can allow mutations to arise that would normally be selected against due to a loss of fitness. FIP rates are higher in kittens and likely result during the primary FECV infection when virus titers are higher, and mutations are tolerated due to a less developed immune system. In adult cats with recurrent or persistent FECV infection, perturbations of the host feline immune state leading to immune deficiency can allow virus replication to flare up (Pedersen, 2009; Poland et al., 1996; Tekes and Thiel, 2016), resulting in the formation of a quasispecies and the genetic sampling required for progression to FIPV. Genetic recombination amongst co-infecting FECV is the final component that leads to accumulation of mutations. This can occur between members of a quasispecies or if a cat becomes infected with a different strain of FECV.

Mutations that accumulate specifically within spike and accessory protein 3c are thought to be the determinants of myeloid cell infection and progression to FIPV (Bank-Wolf et al., 2014; Cham et al., 2017; Chang et al., 2012; Licitra et al., 2014, 2013; Regan et al., 2008). The spike protein is the major determinant of cell tropism and critical for infection of myeloid cells. This was first demonstrated using FCoV recombinants where investigators determined that regions of the FIPV spike were absolutely required for macrophage replication (Rottier et al., 2005). Further studies done using serotype I laboratory strains determined that spike cleavage can be variable between biotypes. FECV-UCD strain recovered from experimentally-infected cats
maintained the S1/S2 furin cleavage site, while an FIPV strain, UCD1, lost this furin cleavage site exposing a heparan sulfate binding motif allowing spike interaction with polysaccharide residues (de Haan et al., 2008), which may impact cell tropism. In parallel using serotype II FCoVs, Regan et al. identified differential roles for pH and cathepsin cleavage during entry of FECV compared to FIPV (Regan et al., 2008). This study demonstrated that specific S cleavage site sequences and, by extension, specific host proteases are necessary to FCoV tropism. In an important study, another group compared the genomic sequences of 11 FECV-FIPV pairs as well as 100s of individual strains and found two mutations in the fusion peptide of spike S2, which were present in 96% of FIPV and none of the FECV strains (Chang et al., 2012). The following year, Licitra et al. identified that 66% of FIPV strains evaluated carried unique mutations in the S1/S2 cleavage site, which altered the efficiency of cleavage by host furin (Licitra et al., 2013). Interestingly, several of these mutations at or around the S1/S2 cleavage site were also observed in non-pathogenic strains of FECV suggesting that this may be an early step in acquiring myeloid tropism. Together, these reports demonstrate that mutations to S that alter either spike cleavage or fusion are associated with or directly lead to myeloid cell tropism.

In approximately 20% of FECV primary infections, FECV replication in macrophages is observed without severe disease progression (Pedersen, 2009; Poland et al., 1996). This indicates that other mutations need to occur in order to cause FIP and simple infection of macrophages is not sufficient. The accessory protein 3C, produced from the polycistronic ORF3 region, is the second component that contributes to FCoV biotype transition, although the exact function of 3c is unknown. Across several studies
investigators determined that approximately two-thirds of sequenced FIPV strains have mutations resulting in a truncated 3C protein, with the other one-third carrying extensive mutations at the 3’ end (Bálint et al., 2012; Chang et al., 2012, 2010, Pedersen et al., 2012, 2009). The authors collectively suggested that truncation of the 3C protein (or significant mutation) is critical for macrophage tropism as truncated 3c coding sequences were only observed in myeloid cell-isolated strains and not in strains derived from fecal or intestinal materials. Further, Hsieh et al. demonstrated that expression of full-length 3C in feline macrophage-like Fcwf-4 cells significantly reduced the titers of FIPV strains encoding truncated 3c genes (Hsieh et al., 2013). Interestingly, FECV intestinal isolates have been identified with collections of mutations at the 3’ end of the full-length 3C protein coding sequence and may represent a transition-state virus population.

Collectively, the comparative studies of FCoV spike and 3C support the original proposal of the “internal mutation theory” in which FIPV arises directly from FECV: i) perturbations of feline immune competency lead to bursts of FECV quasispecies replication allowing new mutations to be sampled without negative selection; ii) spike mutations accumulate in a subset of FECV virions, allowing attachment and entry into myeloid cells; iii) truncations of the 3C protein enhance replication within these myeloid cells providing a competitive advantage; iv) infected myeloid cells traffic to various tissue sites leading to systemic infection and progression to FIP.

Interestingly, following transition, the FIPV genome is relatively stable with an observed mutation rate of 5-6x10^{-6} over 50 passages (Phillips et al., 2013). This demonstrates that not only are large numbers of mutations tolerated (relative to the
parental FECV), but also illustrates the large degree of genetic disruption that can occur to the FCoV genome before significant disruptions to virus fitness occur. Finally, this observation underscores the high degree of genetic plasticity and variability observed between FCoV isolates and the various states of transition isolates may exist in.

**Serotypes.**

The feline coronavirus spike (S) glycoprotein is largely responsible for determining host range and cell tropism as it mediates both receptor binding and membrane fusion. These large, trimeric structures have extensive ectodomains, which represent the main surface antigen and binding target of FCoV-specific antibody responses. As a result, characterizing FCoVs based on antigenicity of the spike protein was an early method used to group new strains, and has revealed the existence of two such serotypes: serotype I and serotype II (Hohdatsu et al., 1991a; Pedersen et al., 1984). Thus, FECV and FIPV strains can be defined as either serotype I or serotype II.

Serotype I FCoVs, which utilize an unknown host receptor for entry, are widespread and are responsible for the majority (70-90%) of natural infections. Serotype II FCoV strains, which round out the other 10-30% of infections, express a naturally-occurring recombinant of feline and canine coronavirus type I S proteins (Addie et al., 2003; Benetka et al., 2004; Herrewegh et al., 1998; Hohdatsu et al., 1992; Kennedy et al., 2002). Serotype II viruses use feline aminopeptidase N (fAPN), a cell-surface metalloprotease distributed in intestinal, lung and kidney epithelia, as an entry receptor. Both serotypes are known to use dendritic cell (DC)–specific intercellular adhesion molecule (ICAM) grabbing nonintegrin (DC-SIGN), a C-type lectin, as an entry co-factor enabling entry into macrophages, however this factor is not sufficient for entry (Regan et
A commendable number of studies focus on type II FCoV as these viruses are much more easily propagated in cell culture than serotype I FCoVs. However, it is difficult to know how accurately type II laboratory strains reflect natural infections with type I viruses given that the bulk of what is known about type I is extrapolated from studies using a type II virus.

With the advent of inexpensive high-throughput sequencing and FCoV strain isolation it has become increasingly clear that the traditional serotype designation is not sufficient to describe the large amount of FCoV strain variation. Recently, a new method to classify FCoV strains has been proposed to update the serotype designations and improve the taxonomy of alphacoronaviruses (Whittaker et al., 2018). Whittaker et al. argue that grouping FCoV based on spike antigenicity alone inappropriately implies that only minor biologic differences separate these viruses. However, it is now widely appreciated that major biologic differences do exist between serotypes including severity of associated pathogenesis (Pedersen, 2014a) and usage of cellular components, such as cholesterol, for replication (Takano et al., 2016). Additionally, serotype I and II FCoV spike proteins engage different host cell entry receptors (Dye et al., 2007; Tresnan et al., 1996), contain unique cleavage sites on S that require different host proteases for cleavage, and can even differ in the site of virus membrane fusion: surface versus endosomal. (Licitra et al., 2013; Whittaker et al., 2018). Therefore, rather than defining FCoV simply on antigenicity of S, Whittaker et al. propose to further categorize FCoVs into two clades by combining function-based data (e.g. receptor binding; protease cleavage) with S-protein sequencing (Whittaker et al., 2018). Although this method of categorizing FCoV strains is objectively more accurate, for the sake of
continuity, this dissertation will refer to strains based on their original serotype
designations.

**FIPV Pathogenesis and FIP Clinical Features.**

FIPV primarily infects myeloid cells such as monocytes, macrophages and
dendritic cells. During artificial infection, FIPV replication in macrophages progresses
slowly over 2 weeks and is critical for determining disease progression. In rare cases,
the feline mounts a strong, rapid cell-mediated immune (CMI) response, containing the
virus within the mesenteric lymph nodes and eventually clearing it. However, in the
majority of cases the CMI is not strong enough and the virus titer, distribution and naïve
macrophage infection rate increase dramatically. Coincident with the rise in FIPV viral
load is the onset of detectable viruses-specific antibody titers in the blood between 10-
21 days post-infection. Although antibodies have been shown to neutralize virus in vitro,
during natural infection, antibody-dependent enhancement (ADE) of disease is
observed. In this case, antibodies specific to S (and commonly the E protein) prevent
engagement of the virus receptor; however, these large antibody-virus complexes are
endocytosed via FcR-mediated uptake and accumulate in the late endosome and virus
replication proceeds.

FIP can present in two forms depending on the dominant immune response to
infection. The more common “wet” disease occurs when humoral immunity is strong and
CMI is absent. This wet disease is effusive and results in inflammation of the visceral
serosa and omentum with exudation into the abdomen. This is visualized by significant
enlargement of the body cavity due to intestinal distension and ascites fluid buildup. The
wet systemic disease results from the development of so-called pyogranulomas, a
collection of protein-rich edema fluid containing T and B lymphocytes and neutrophils that surround a large number of FIPV-infected macrophages, around different organ venules. In response to pyogranuloma formation, additional macrophages are recruited, which exacerbate inflammation with the release of cytokines and the activation of the classical complement cascade. Prolonged inflammation and complement activation cause microhemorrhages and red blood cell lysis. Infected cats usually succumb to FIP several days after the development of pyogranulomas.

The rarer form of FIP, known as “dry” disease occurs when humoral immunity is strong and CMI is present, but weak. In this case, the initial infection is more contained, but not completely eliminated thus the disease progresses more slowly over weeks to years. The dry disease presents with more traditional granuloma formation and involvement of parenchymatous organs such as the kidneys, mesenteric lymph node, bowel wall, liver and most commonly the eyes and central nervous system. Granulomas form when B and T lymphocytes surround around a small number of macrophages with little to no replicating virus. Unlike the wet disease, the inflammation is low-intensity and chronic rather than massive and acute. Complications due to prolonged inflammation occur in multiple organ systems and usually involve paralysis and seizure. Although the dry form progresses more slowly, the mortality rate is the same as the wet form – nearly 99% fatal.

Due to the degree of immune suppression during FIPV infection, co-infections are common and lead to additional complications, accelerated disease and enhanced transmission of FECV. Feline leukemia virus (FeLV) infection is observed in one-half to one-third of FIPV cases and other coinfections include opportunistic bacterial pathogens
and parasites such as *Toxoplasma gondii*. Several groups have suggested that these co-infections, specifically of FeLV, which can induce immune suppression, can enhance progression to FIPV in co-housed cats.

**T and B Cell Responses and Effects on FIP Progression.**

FIP is defined as an “immune-mediated” disease meaning that the degree of associated pathology is largely due to differences in the timing strength and type of the immune response. Significant imbalances between intrinsic, T and B cell immune responses is the most widely accepted reason for the inability of cats to resist FIPV infection and the resulting > 90% mortality rate (de Groot-Mijnes et al., 2005; Kipar and Meli, 2014; Pedersen, 2014a, 2009).

Although rare, positive FIP outcomes are associated with rapid cell-mediated immunity (CMI) involving cytotoxic T lymphocytes and natural killer (NK) cells. A strong CMI-response limits virus growth through direct killing of virus-infected cells, mostly macrophages, in the mesenteric lymph node and prevents the massive upregulation of pro-inflammatory cytokines and granuloma formation that mediate disease (Pedersen, 2014a). Increased IFN-γ production, a major product of activated CD8⁺ T cells and NK cells, is associated with protection in cats with natural FIPV infection and was observed in peripheral blood mononuclear cells (PBMCs) taken from experimentally-infected cats that survived FIPV challenge (Satoh et al., 2011). Surviving cats were also found to have high levels of IL-10, an important immune-suppressing cytokine produced by T regulatory cells. Additional evidence supporting the importance of CMI comes from examination of cats that succumb to FIPV infection. In these cases, CD4⁺ and CD8⁺ T cells and NK cells were found to be depleted in peripheral blood of moribund cats with
natural FIPV infections (Vermeulen et al., 2013). T regulatory cells, which produce IL-10 and function to regulate the degree of inflammation in response to infection, were also found to be depleted suggesting that elimination of these cells may significantly contribute to unregulated inflammation associated with severe pathology (Vermeulen et al., 2013). Finally, transcriptional profiling of PBMs recovered from FIPV-infected animals demonstrated enrichment of the PD-1 / PD-L1 factor pair (Harun et al., 2013), which is involved in repression of cytotoxic T cell responses, further highlighting the importance of cell-mediated immune responses in controlling FIPV.

The majority of FIPV infections result in death of the infected animal and are associated with depletion of T cells resulting in inefficient CMI, systemic virus replication and unrestricted inflammation (Kipar et al., 2006; Pedersen, 2014a). Indeed, moribund cats naturally infected with FIPV were found to have a higher proportion of activated macrophages and neutrophils (Kipar et al., 2006; Takano et al., 2009b), increased serum levels of macrophage differentiation / stimulation factors including IL-6, GM-CSF, M-CSF and IL-1β, and reduced immune regulatory factors such as IL-10 and IL-12 (Kipar et al., 2006). Macrophages from moribund FIPV-infected animals also expressed high levels of B-cell activation markers including IL-6, CD40 and B cell activating factor (BAFF), suggesting that B cells are somehow involved in FIP progression (Takano et al., 2009a). Indeed, activation of B cells and production of FIPV-specific antibodies (humoral immunity) is strongly associated with rapid disease progression though a process called antibody-dependent enhancement (ADE) (Pedersen and Boyle, 1980; Takano et al., 2008; Weiss and Scott, 1981). FCoV-specific antibodies are observed during both FECV and FIPV infections (Pedersen, 2009) and are directed against...
surface epitopes on the FCoV S and E proteins. In tissue culture, these antibodies lead to virus neutralization; however, these same neutralizing sites were also responsible for directing ADE in animals – a fact that has complicated vaccine design for decades (Takano et al., 2011). One group demonstrated the negative impact of these antibodies experimentally by injecting subclinically-infected cats with serum obtained from cats fully infected with either FECV or FIPV (Takano et al., 2008). This passive immunization led to serotype-matched-specific ADE and rapid disease progression compared to control.

Several groups have proposed models of how ADE of FIP occurs. Studies by Dewerchin and colleagues determined that macrophages infected with FIPV expressed viral antigens on the cell surface (Dewerchin et al., 2008). When antibodies bound these antigens, the antigens were rapidly internalized (Dewerchin et al., 2008, 2006). The internalization of these cell surface antibody-antigen complexes reduces to chance of virus-infected macrophages to undergo antibody-dependent cell-mediated cytotoxicity (ADCC), a process in which infected cells are actively killed by surveying NK cells through interactions between NK cell Fc receptors (FcR) and a cell-bound antibody. Thus, FIPV-infected macrophages survive and continue to produce virus. A second, more traditional model of ADE was also proposed by this group (Dewerchin et al., 2008). During in vivo infection, it was observed that large antibody-virus complexes were endocytosed by macrophages, accumulated in mature endosomes and led to productive infection. This internalization process was dependent on FcR-γ on the surface of the macrophage, which allows internalization of viruses by CoV-receptor independent process. This provides some clarity on how the same virus epitope could lead to virus neutralization in tissue culture, but ADE in vivo – likely these “neutralized”
viruses do not require surface-level spike-receptor engagement, but rather gain entry through endocytosis of the entire complex. By either method, FIPV is able to enhance access to macrophages and maintain productive infection without triggering targeted cell death.

The complex relationship between innate and adaptive responses and the immune-consequences that lead to FIP development need to be strongly considered during vaccine design. For this reason, vaccine development had been slow and viewed more as a pipedream, and much more emphasis has been placed on development of therapeutics (see below). However, new studies from our group on generating live-attenuated CoV vaccines through generation of hyper-interferon inducing (HII) strains, and the research presented in this dissertation may overcome some of the previous challenges to designing an effective FIPV vaccine.

**Current Therapeutic Options.**

A large number of studies have looked at the efficacy of various therapeutic compounds in treating FIP. These compounds fall into three main categories: i) immune modulatory compounds targeting the rapid systemic inflammation arising during FIPV infection; ii) compounds targeting cellular pathways usurped by the virus during replication; iii) direct-acting antiviral inhibitors that specifically block virus replication.

As pathology associated with FIPV infections is mediated by an overactive inflammatory response, it is reasonable to predict that targeting these immune responses may decrease disease severity. Reduced levels of TNF-alpha, a proinflammatory host factor, were shown to be associated with positive outcomes in cats with natural FIPV infections (Kipar et al., 2006), however, treatment with the anti-
TNF-alpha inhibitor Pentoxifylline failed to reduce FIP-associated mortality (Fischer et al., 2011). Another group pre-treated cats with type I IFN, which has pleiotropic antiviral effects, in an attempt to prime antiviral innate immune responses. This prophylactic administration of type I IFN was also ineffective at reducing mortality rates following FIPV infection (Weiss et al., 1990), an observation consistent with the presence of IFN signaling and ISG antagonists present in FCoVs (Dedeurwaerder et al., 2014, 2013). Interestingly, combining IFN treatment with peptides mimicking the FIPV spike heptad repeat (HR)-domain (predicted entry inhibitors) had a synergistic antiviral effect on reducing serotype II FIPV infection (Liu et al., 2013) suggesting that type I may be effective as a treatment supplement. These experiments, however, were performed in tissue culture and it is unclear if the tandem IFN/HR-peptide treatment would be effective in vivo.

Several groups have demonstrated effective FIPV treatment by employing compounds that target cellular processes. Takano and colleagues observed that type I, but not type II FIPV replication requires the presence of plasma-membrane cholesterol (Takano et al., 2016) and predicted that this may be a cellular target of antiviral treatment. Indeed, a follow-up study demonstrated that pre-treatment of cells with U1866A, an inhibitor of cholesterol transport, caused intracellular cholesterol accumulation and strong inhibition of type I FIPV replication (Takano et al., 2017). This study also reported that the antiviral activity of U1866A was reversed by the HDAC inhibitor Vorinostat, which reverses the negative effects of U18666A on the host protein Niemann-Pick C1 (NPC1) suggesting a possible role of NPC1 in type I FCoV replication. Further evidence for the role of NPC1 was recently demonstrated (Takano
et al., 2019). This study determined that therapeutic treatment of infected cats with the antifungal drug Itraconazole, which blocks NPC1, had significant antiviral activity against serotype I, but not serotype II FIPV. These studies represent exciting new avenues of drug development and treatment of cats with FIPV; however, as they target cell-intrinsic properties, further investigation will be needed to determine the long-term safety of these drugs.

The most promising anti-FIPV therapeutics are the class of small-molecule inhibitors with direct-acting antiviral activities. Several of these compounds have been developed and specifically inhibit the FIPV 3C-like protease (3CLpro), a viral enzyme absolutely required for CoV replication. St. John et al. investigated the inhibitory potential of several peptidomimetic compounds, which impersonate the natural peptide cleavage target and non-covalently associate with the active site of 3CLpro (St. John et al., 2015). Two such compounds were found to inhibit purified recombinant FIPV 3CLpro with 50% inhibitory concentration (IC$_{50}$) values in the sub-micromolar range. An exciting study was recently published by Kim et al., which demonstrated near-full reversal of FIP upon treatment with another 3CLpro inhibitor, GC376 (Kim et al., 2016). Investigators experimentally infected cats with serotype I FIPV (m3c-2 strain) and administered doses of GC376 after development of clinical signs of FIP and observed recovery of treated cats after several weeks. It should be noted that GC376 is a small, dipeptidyl compound containing a catalytic warhead, which covalently binds the catalytic cysteine reside in the active site of 3CLpro and may have unintended off-target effects on cellular proteases. Additionally, the compound has reduced access to the CNS due to the blood-brain barrier and therefore may be ineffective at treating the dry form of
disease. Although exciting, further investigation of this inhibitor will be needed before wide-spread use is adopted by veterinary professionals.

Much progress has been made in developing effective therapeutics to treat cats infected with FIPV. However, realistic and effective treatment of sick cats would likely require near-immediate recognition of FIP-like disease symptoms, multiple injections and a substantial investment by drug companies to synthesize large quantities of compounds, all factors that drive up the total cost of treatment. Additionally, these methods do not address the high seroprevalence of FECV in domestic cat populations and cannot prevent new infections with subclinical strains. The gold standard in the field remains the development of an effective vaccine against serotype I FCoV allowing long-term and widespread immunity. Such a vaccination would be effective at preventing the spread of the virus, decrease global seroprevalence and allow maintenance of isolated FCoV disease using the more expensive antivirals. Over the decades, however, developing a vaccine for serotype I FIPV has been a significant challenge to the field and as a result of numerous factors, discussed in the following section, no such vaccine exists...yet.

The Challenges and Value to Investigating Serotype I Feline Coronavirus

Overview.

Serotype I viruses account for the bulk (80-90%) of naturally occurring FCoV infections in domestic cats and are considered one of the most important veterinary animal pathogens. Investigating type I FCoV in a controlled laboratory setting is required to understand the basic virology of natural infection, characterize clinical isolates, test novel therapeutics, and develop effective feline vaccines. Additionally,
serotype I FCoVs provide an important model system in which to study the pathogenesis, immune regulation, and basic virology of the Alphacoronavirus genus, which comprises several under-studied yet economically-important animal coronaviruses such as TGEV and PEDV. However, a number of challenges to studying serotype I FCoVs have burdened the field for decades and include inefficient methods of virus isolation and growth in tissue culture, limited permissive cell types, the lack of a known receptor and significant variation between clinical and lab-adapted virus strains. As a result, many groups have focused on the less prevalent (<10%) type II FCoV as these viruses are much more easily propagated. However, despite the utility of the type II FCoVs it is difficult to know how accurately type II laboratory strains reflect natural infections with type I viruses given that the bulk of what is known about type I is extrapolated from studies using a type II virus. Thus, more needs to be done to facilitate research of serotype I feline coronavirus in order to overcome the challenges described in this section.

**Virus Isolation and Strain Variation.**

Since the early 1980’s, a number of groups have isolated feline coronavirus strains representing both bio- and serotypes (Table 6). As mentioned, most groups study serotype II strains due to their rapid growth in tissue culture (TC). For example, the widely used FECV 79-1683 and FIPV 79-1146 viruses have been TC-adapted and readily propagate in cell lines expressing feline APN, such as Crandell Rees Feline Kidney (CRFK) cells (Miguel et al., 2002). Although there is debate on how “natural” these strains are, TC-adaptation does not appear to effect the patho-biology of the serotype II viruses as strains lead to the same illness in cats from which they were
derived (Pedersen, 2014a, 2009). In addition, several serotype II FCoV including the C1Je and NTU156 strains, have been maintained as primary clinical isolates (CI) by passage in felines (Dye and Siddell, 2007; Lin et al., 2013). This technique preserves the natural “clinical” virus phenotype, avoids any tissue-culture adaptations and allows experimental control of natural serotype II infection.
<table>
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<th>BIOTYPE</th>
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<th>SPECIMEN</th>
<th>ACC #</th>
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</table>

**Table 6: Feline Coronavirus Strains.** Abbreviations: GenBank accession number (Acc #); TN406-Low Passage (LP), TN406-High Passage (HP), DF2-temperature-sensitive strain (ts); Causes FIP without indicated severity [FIP (+)]; Clinical isolate (CI), Reverse genetics strain (Rev Gen), Tissue culture adapted strain (TC Adapt); Unpublished sequence submitted to GenBank [[(Taboni et al., 2007) unpub]].
The same cannot be said of serotype I FCoV strains, however. Serotype I FCoVs do not readily adapt to growth in cell culture and the few strains that have been successful isolated, required either numerous rounds of passage before cytopathic effects were observable [e.g. (Amer et al., 2012; Black, 1980)] or use of entry-enhancement techniques such as spinoculation (Hohdatsu et al., 1995) or addition of DEAE-dextran or trypsin during virus adsorption (Jacobse-Geels and Horzinek, 1983). As a result of forcing virus growth in an atypical environment, TC-adapted type I FIPV strains lose in vivo pathogenicity and do not cause FIP in experimentally-infected cats (Pedersen and Black, 1983; Thiel et al., 2014). This suggests two things: first— that the current isolation techniques used to obtain serotype I viruses likely select for a subset of virus genotypes that do not represent the majority; and second— significant genomic mutations must be obtained in order for serotype I FCoVs to adapt to growth in the currently available feline cell lines. Both of these factors distance the lab-adapted strains from the parental clinical isolates and therefore complicate controlled investigations of virus attenuation, vaccination and evaluation of therapeutics. At least one type I FIPV strain, mc3-2, has maintained a virulent phenotype by continuous passage in cats (Kim et al., 2016) suggesting that preserving the genotype of FIPV is achievable of cultured appropriately.

The issues surrounding type I FCoV isolation are exemplified by the prototypic serotype I FIPV strain Black, also referred to as TN406— one of the first type I FIPV isolated (Black, 1980). As this virus was derived from a cat with FIP, the low-passage (LP) Black strain remains highly pathogenic when introduced back into an animal (Black, 1980). However, as is observed with all TC-adapted type I FIPV strains, the
high-passage (HP) Black virus lost all pathogenicity following TC-adaptation (Pedersen and Black, 1983). More recent studies using variants of FIPV Black produced by reverse genetics further demonstrated that this virus was avirulent and had acquired numerous mutations in the spike and accessory genes (Thiel et al., 2014). Interestingly, all known serotype I FCoVs that replicate in TC lose in vivo virulence and accumulate significant mutations in spike and the ORF7 accessory protein region (Kipar and Meli, 2014; Pedersen, 2014a, 2009). While mutations in the spike likely affect receptor or host-protease usage, it is unclear how these and the ORF7 mutations affect pathogenesis. At least one group has suggested that ORF7 is an antagonist of type I interferon (Dedeurwaerder et al., 2014), indicating that TC-adaptation may result in viruses that are less resilient to innate immune responses.

As a final note, it is well known that natural FIPV infection produces a viral quasispecies in feline tissues comprising numerous genetic variations and stages of progression from FECV to FIPV. As a result, virus isolation offers only a fraction of the total FIPV strain diversity and, further, results in large strain-to-strain variation between isolates. This, paired with the mutations required for TC-adaptation, make investigating type I FCoV complicated and ultimately inconsistent. Thus, better isolation techniques and permissive cell types need to be identified in order to replicate clinical serotype I FIPV isolates without the requirements of TC adaptation.
Permissive Cell Types.

The cell types available to study serotype I FCoV are limited and this has been one of the biggest hurdles to studying these viruses in the lab (Table 7). Unlike serotype II FCoV, which replicate in cells that naturally or ectopically express feline APN, the receptor for type I FCoVs is not known, thus permissive cell types are difficult to identify, and the receptor cannot be simply expressed in common cell lines such as BHK or HEK 293Ts. Several TC-adapted strains of type I FIPV, particularly the Black strain, replicate in two continuous cell lines, the feline airway epithelial cell line, AK-D, and the feline macrophage-like cell line *felis catus* whole fetus 4 (Fcwf-4). As epithelial cells are an unnatural replication site for FIPVs, the immediate issue with AK-D cells is that they do

<table>
<thead>
<tr>
<th>CELL NAME</th>
<th>CELL TYPE</th>
<th>TYPE I FIPV</th>
<th>TYPE II FIPV</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crandell Rees Feline Kidney (CRFK)</td>
<td>Immortalized feline kidney</td>
<td>No</td>
<td>Yes</td>
<td>(Miguel et al., 2002)</td>
</tr>
<tr>
<td>AK-D</td>
<td>Feline airway epithelia</td>
<td>Yes; Low titer</td>
<td>-</td>
<td>(Millet and Whittaker, 2015; Benjamin W. Neuman et al., 2006; Pedersen, 2009)</td>
</tr>
<tr>
<td>Fcwf-4</td>
<td>Feline macrophage-like</td>
<td>Yes; Low titer</td>
<td>Yes</td>
<td>(Alazawy et al., 2011; Jacobse-Geels and Horzinek, 1983)</td>
</tr>
<tr>
<td>Peripheral blood mononuclear cells (PBMCs)</td>
<td>Feline Primary cells; monocytes, lymphocytes, macrophages, others</td>
<td>Yes</td>
<td>Yes</td>
<td>(Harun et al., 2013)</td>
</tr>
<tr>
<td>Macrophages</td>
<td>Feline Primary; Bone-marrow isolated or stem cell-derived</td>
<td>Yes</td>
<td>Yes</td>
<td>(Gow et al., 2013; O’Brien et al., 2018; Rottier et al., 2005; Sprague et al., 2005)</td>
</tr>
<tr>
<td>Dendritic Cells</td>
<td>Feline Primary; Bone-marrow isolated or stem cell-derived</td>
<td>Yes</td>
<td>Yes</td>
<td>(Bienzle et al., 2003; van Berne, 2008)</td>
</tr>
</tbody>
</table>

Table 7: Cell Types Permissive to FIPV Infection.
not reflect a normal infection route and it is likely that TC-adaptations that allow type I viruses to replicate in these cells have adverse effects on virus pathology and shift viruses away from a clinically-important phenotype. Fcwf-4, another other permissive cell line, was originally isolated from the peritoneal cavity of a cat infected with FCoV. This continuous cell line was further characterized as “macrophage-like” due to active phagocytic activity, and the presence of Fc receptors and non-specific esterases (Jacobse-Geels and Horzinek, 1983). Compared to AK-Ds, Fcwf-4 cells represent a more biologically-relevant cell type in which to study serotype I FCoVs. Indeed, many groups have used these cells to study both type I and type II FCoVs and they have been an important tool for recovering type I FIPV strains and modeling feline coronavirus infection in macrophages. The type I FIPV Black strain, for example, was originally isolated using Fcwf-4 cells (Black, 1980), and others, such as strain DF2, replicate in Fcwf-4 cells as well. However, these cells have been used to study type I FCoVs more out of necessity than utility and have a number of associated issues. First, previous studies report that type I FIPV grows to low titers ($10^4 - 10^5$ pfu/mL) in these cells relative to type II ($>10^6$ pfu/mL) (Tekes et al., 2012) and can be measured by determining either the 50% tissue culture infectious dose (TCID$_{50}$) (Ramakrishnan, 2016; Reed and Muench, 1938) or by plaque assay (Takano et al., 2017; Tekes et al., 2012, 2010). Second, type I virus growth kinetics are variable in Fcwf-4 cells, requiring between 15-96 h to achieve maximum titer (Jacobse-Geels and Horzinek, 1983; O’Brien et al., 2018; Tekes et al., 2012, 2008)). Third, some reports suggest that type I viruses produced in Fcwf-4 cells are highly cell-associated (Jacobse-Geels and Horzinek, 1983; Pedersen et al., 1984) and multiple freeze-thaw cycles may be required to recover virus,
which further reduces the overall titer. Finally, Fcwf-4 cell doubling time is slow (>31 h) (American Type Culture Collection, 2013), cells do not grow to high density and begin to die after several passages. These growth issues make advanced genetic techniques such as Crispr/Cas gene editing, which will be critical to evaluating attenuated FCoV strains, unrealistic. Thus, although serotype I FCoVs grow in Fcwf-4 cells, additional cell types are needed that grow viruses to high titer and are amenable to genetic manipulation.

Feline primary cells including total PBMCs, macrophages, and dendritic cells are the most natural cell types in which to grow and isolate serotype I FIPV strains. As these are a natural cell type, limited TC adaptations are required for sustained virus replication and the high virulence FIPV genotype is maintained. Certain type I strains such as UCD1, -2, and -3 have been successfully cultured in these ex vivo feline tissues and re-introduced into cats to cause FIP (Pedersen et al., 1981a; Pedersen and Floyd, 1985). However, use of these cells is limited by several factors: first, due to high seroprevalence, acquiring FCoV-free tissue samples is challenging without an established colony of specific pathogen (SPF)-free cats and deriving immune cells from bone marrow can take up to two weeks; second, primary cells usually cannot be passaged and new cells need to be plated for each experiment leading to increased variability, and finally, many genetic techniques such as gene expression or Crispr/Cas gene editing cannot be performed in primary cell further limiting the scope of available experiments.

While each cell type discussed here is important for different aspects of FCoV research, ultimately a biologically-relevant, genetically tractable, continuous cell line that
replicates high titers of type I FCoVs is needed. Although this seems like a lofty challenge, such a cell line is critical for the discovery of the type I FCoV entry receptor, recovery of clinical isolates without TC-adaptation, determining the function of FCoV proteins that antagonize innate immunity and for intensive study of vaccine strains developed through rational design. One of the goals of this dissertation was to identify and characterize such a cell line to enable future research of these important animal pathogens.

**Objective of Dissertation Research.**

Since the 1980’s FCoVs have been predominantly studied in veterinary settings where the focus was on diagnosis of sick cats and describing the pathogenesis of strains isolated in-house using any cells available. In this environment, researchers emphasized use of protocols and cells that allowed growth of the virus and as a result, the nearly three decades of reports and reviews contain a high degree of variability amongst the basic virology techniques, cell lines and virus strains characterized. This issue of variability has led to much confusion and over-complication of the literature making study of serotype I FCoVs an exercise in extrapolation of data collected from either highly-diverged type I lab strains or serotype II viruses rather than well-controlled experimentation of biologically-relevant type I viruses in a natural environment.

The long sought-after goal of FCoV research is the development of a broadly-effective type I vaccine to protect young kittens from developing FIP. Immunization against serotype I FIPV is likely to be the most effective method to prevent this disease as this represents the majority of circulating virus (Pedersen, 2009), but due to the issues discussed in this section, development of a type I FIPV vaccine has been
challenging and ultimately unsuccessful (Kiss et al., 2004; Pedersen, 2014b, 2009). Furthermore, conventional vaccination methods using inactivated, high-passage or temperature-sensitive viruses have been restricted to serotype II FCoV [e.g. (Haijema et al., 2004)]. Indeed, Felocell® FIP, the only currently available FIP vaccine, contains a temperature-sensitive version of the serotype II virus, DF2. However, this vaccine fell out of favor as it failed to protect against the dominant, circulating serotype I virus. Thus, the challenge posed to researchers in the field, and the ultimate goal of this dissertation, is to develop a set of “tools” – methods, reagents, genetically-tractable cell types, that will facilitate efficient investigation of serotype I FCoV. Here, I describe my efforts to i) characterize a new variant of Fcwf-4 cells that rapidly produce high-titers of cell-free FCoVs comprising both bio- and serotypes and use these cells to establish a uniform plaque assay to determine cell titer; ii) demonstrate the genetic malleability of these new Fcwf-4 cells through Crispr/Cas guided disruption of the type I IFN receptor and the expression of a feline surface protease, TMPRSS2. The ultimate goal of this dissertation is to facilitate the generation of live-attenuated type I FCoV vaccine strains through investigation and mutagenesis of IFN antagonists. Thus, in the final section, I describe iii) establishing a reverse genetics system to produce a lab-sequenced strain of FIPV Black, which can be specifically attenuated through direct mutation. Together, the contribution of this dissertation is to provide several basic virology tools to bolster basic science research of type I FCoVs and help develop an effective type I FIPV vaccine.
CHAPTER TWO
MATERIALS AND METHODS

Viruses and Cells

Viruses.

Feline coronavirus strains including type I feline infectious peritonitis virus (FIPV) Black (TN406), type II FIPV WSU 79-1146, and type II feline enteric coronavirus (FECV) WSU 79-1683 were kindly provided by Dr. Fred Scott, Cornell University College of Veterinary Medicine, Ithaca, NY. Viruses were propagated in Fcwf-4 Cornell University (CU) cells and recovered from supernatants for stock virus preparation. Briefly, sub-confluent T-75 flasks of Fcwf-4 CU cells were infected with virus at 1 multiplicity of infection for 1 hour in 2 mL serum-free media at 37°C and 5% CO₂. Infectious media was replaced with 8 mL EMEM (see below) containing 5% FCS, and the monolayer was incubated at 37°C and 5% CO₂. After 24 hours, supernatants were collected and clarified by centrifuging at 1,000 xg for 10 minutes at 4°C. Virus-containing cell-free supernatants were then aliquoted (100 – 300 μL) and stored at -80°C. Titers of stock virus were determined by plaque assay on AK-D or Fcwf-4 CU cells. Virus aliquots that were thawed more than twice were pooled and a new titer was determined.

HEK 293T and 293T/17 Cells.

Human embryonic kidney (HEK) 293 T cells were maintained in DMEM (#10-017-CV, Corning) containing 10% FCS, supplemented with 1% nonessential amino
acids, 1% HEPES, 1% L-glutamine, 1% sodium pyruvate, and 1% pen/strep (penicillin/streptomycin). For generation of lentivirus transducing particles, a second line of HEK 293T/17 (ATCC® CRL-11268™) was purchased from ATCC and maintained in the same media formula. When cells were confluent, the monolayer was washed with PBS, then cells were removed by addition of 1.5 mL 0.25% trypsin-versene solution for 1-2 min at room temperature. For routine passaging, cells were transferred (1:10 split) to a new T-75 flask every 2 days.

**Felis catus Whole Fetus (Fcwf-4) ATCC Cells.**

Fcwf-4 cells were purchased from the ATCC (ATCC® CRL-2787™), and designated Fcw-4 ATCC cells. Fcw-4 ATCC cells were maintained in Minimal Essential Medium Eagle (EMEM) (Sigma, #M0268) containing 10% FBS, supplemented with 1.5 g/L sodium bicarbonate, 1% non-essential amino acids, 1% HEPES, 1% sodium pyruvate, 1% L-glutamine and 1% penicillin/streptomycin. As described by the ATCC, the doubling time for these cells is >31 h. When cells were confluent in T-25 flask, the monolayer was washed with PBS, then cells were removed by addition of 1.5 mL 0.25% trypsin-versene solution for 2-3 min at room temperature. For routine passaging, cells were transferred (1:3 split) to a new T-25 flask every 3 days.

**Felis catus Whole Fetus (Fcwf-4) Cornell University Cells.**

A second source of Felis catus whole fetus cells were provided by Dr. Edward J. Dubovi, Cornell University College of Veterinary Medicine, Ithaca, NY, designated Fcw-4 CU, and maintained in the same medium as the Fcw-4 ATCC cells. When cells were confluent, the monolayer was washed with PBS, then cells were removed by addition of 2 mL 0.25% trypsin-versene solution for 1-2 min at room temperature. For routine
passaging, approximately $3.3 \times 10^6$ cells were transferred (1:3 split) to a new T-75 flask in a total of 20 mL complete media every 2-3 days.

**Generation and Maintenance of Fcwf-4 CU IRN Cells.**

To generate the Fcwf-4 CU IFNαR null (IRN) cell line, a modified Crispr-Cas protocol, based on the GeCKO system (Shalem et al., 2014) was used to disrupt the type I IFN alpha receptor 2 (IFNαR2) in Fcwf-4 CU parental cells. Guide DNAs were designed using Benchling (Benchling, Inc.) to target the second and third exons (felis_catus_6.2; range = cgrC2: 11959183-11979720) of the feline ifnar2 gene (Accession NM_001278859.1). Single guide DNA sequences are listed in **Appendix B** (**Primer Index 2**). For each exon target, guide DNAs were phosphorylated and annealed by combining 10 μM of the forward and reverse guide DNA oligos, 1x T4 DNA ligase buffer containing ATP, T4 PNK ligase (NEB; M0201S) and nuclease-free H$_2$O in a total reaction volume of 10 μL. The reactions were then incubated at 37°C for 30 min; 95°C for 5 min; 95-25°C ramping down at 5°C intervals holding at each temperature for 5 min. In parallel, the lentiCrisprV2-puroR$^+$ plasmid (Addgene #52961) was linearized with BsmBI (NEB), the 13 kb band was extracted from an agarose gel then column purified using a SV gel and PCR clean-up system (Promega). Annealed and phosphorylated oligo sets were diluted 1:200 in nuclease-free H$_2$O then inserted into the linearized lentiCrisprV2-puroR$^+$ cassette between flaking BsmBI sites using T4 DNA ligase (NEB) at 16°C overnight. Following transformation into Top10 *E. coli* and subsequent miniprep purification (Promega), plasmid DNAs were double digested with AgeI (NEB) and KpnI (NEB) to reveal a fragment of 621 bp confirming proper insertion of guide DNA sequences; plasmids were confirmed by sequencing using the U6
universal forward primer 5'-GGG CAG GAA GAG GGC CTA T-3'. To disrupt the ifnar2 gene, transducing particles (TPs) carrying the lentiCrisprV2-puroR+ plasmids, encoding guide DNA, cas9 endonuclease and puromycin resistance cassettes, were generated. Briefly, 3.3 μg each of lentiCrisprV2-puroR+ (empty [EV] or carrying guide DNA sequences), psPax2 (lentiviral packaging plasmid) and pHEF-VSV-G (vesicular stomatitis virus glycoprotein) plasmids (generously supplied by Edward M. Campbell, Department of Microbiology and Immunology, Loyola University Chicago, Stritch School of Medicine, Maywood, IL) were co-transfected into a 10 cm dish containing 5x10^6 HEK-293T/17 cells using polyethylenimine (PEI) (5 μg PEI / 1 μg DNA) in antibiotic-free DMEM 10% FCS for 24 h followed by replacement with complete DMEM containing 10% FCS with antibiotics. Alternatively, the 10 μg of total plasmid DNA was transfected into 5x10^6 HEK-293T/17 cells using TransIT LT1 (Mirus) (3 μL LT1 / 1 μg DNA) without media replacement in DMEM 10% FCS with antibiotics. For either method, after 48 h, supernatants containing TPs were collected and stored at 4°C and cells were replenished with 10 mL DMEM containing 5% FCS with antibiotics. After an additional 24-hour incubation (72 hours total) at 37°C and 5% CO₂, supernatants were collected and combined with the previously collected lot. Total supernatants were centrifuged 1000 xg at 4°C for 10 min to remove cell debris, then centrifuged overnight (16+ hours) at 5,000 xg and 4°C. To concentrate, supernatants were poured off and pellets were suspended in 1/10 – 1/100th the original volume with EMEM (-FCS). A minimal volume of concentrated TPs was added to parental Fcwf-4 CU cells for 1 hour at 37°C and 5% CO₂ followed by addition of EMEM 10% FCS and incubation for 48 h. Transduced cells were positively-selected by replacing media with EMEM containing 10% FCS and 10
µg/mL puromycin (InvivoGen) for 96 h. Puromycin-selected cells were grown as a heterologous population designated Fcwf-4 CU 2.2 (for the guide DNA sequence specific for the second target on the second ifnar2 exon). A clone of the Fcwf-4 CU 2.2 cells, confirmed for reduction in responsiveness to type I IFN (see CHAPTER THREE: EXPERIMENTAL RESULTS), was isolated and designated Fcwf-4 CU IRN (Interferon alpha Receptor Null). Briefly, >10,000 Fcwf-4 CU 2.2 cells were sorted using a FACS Aria Cell Sorter (BD) into a 96-well plate at 1 cell/well. Clones were grown over several weeks and expanded into 24- then 6-well plates in EMEM containing 10% FCS and 10 µg/mL puromycin. Clones that were positive for permissively to FIPV Black infection were evaluated for IFN responsiveness (see CHAPTER THREE: EXPERIMENTAL RESULTS). The Fcwf-4 CU IRN cell line was isolated in this manner and confirmed to be both permissive to FIPV Black infection and have low IFN-responsiveness. The sequence of the IFNαR mRNA expressed in the Fcwf-4 CU IRN cell line was determined by sequencing 15 PCR clones of the region (Appendix D). Briefly, total RNA was isolated from Fcwf-4 CU or IRN cell monolayers and cDNA was generated with random hexamer primers via the RevertAID First-Strand cDNA Synthesis kit (Thermo Fisher). The full IFNαR region was amplified from cDNA using the F IFNaR2 Full FWD and F IFNaR2 Full REV primer set (Appendix B; Primer Index 1) in a thermocycler set at 92°C for 2 min; 35 cycles at 92°C for 1 min, 50°C for 1 min and 68°C for 100 min; and 68°C for 7 min. The resulting 1600 bp band was gel extracted from a 0.8% SeaPlaque GTG™ Agarose (Lonza) gel and column purified using an SV gel and PCR clean-up system (Promega) prior to insertion into TA cloning pCR-XL-TOPO® plasmids using the TOPO® XL PCR Cloning Kit (Life Technologies). 15 plasmids for
each cell type were then transformed into Top10 *E. coli* and purified by MiniPrep (Promega) and sequenced using M13 FWD, M13 REV, f IFNaR2 SEQ 1 FWD and f IFNaR2 SEQ 1 REV sequencing primers (*Appendix B; Primer Index 1*). Consensus sequences were compared using Clone Manager 9 (Sci-Ed Software) revealing a 14-nucleotide deletion in the cas9 target region of the IFNαR Fcwf-4 CU IRN cells resulting in a truncated protein sequence. These cells were maintained in the same medium as the parental Fcwf-4 CU cells. For routine passaging, confluent monolayers were washed with PBS, cells were removed by addition of 2 mL 0.25% trypsin-versene solution for 1-2 min at room temperature, and approximately 3.3x10^6 cells were transferred (1:3 split) to a new T-75 flask in a total of 20 mL complete media every 2-3 days.

**Feline Airway Epithelial (AK-D) Cells.**

AK-D cells were purchased from the American Type Culture Collection (ATCC) (ATCC® CCL-150™) and maintained in Dulbecco’s Modified Eagle Medium (DMEM; Gibco, #12100-046) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, #S11150), supplemented with 2.2 g/L of sodium bicarbonate (Sigma, #S5761), 1% non-essential amino acids (HyClone, #SH30238.01), 1% HEPES (HyClone, #SH30237.01), 1% sodium pyruvate (Corning, #25-000-Cl), 1% L-glutamine (HyClone, #SH30034.01), and 1% penicillin/ streptomycin (Corning, #30-002-Cl). When cells grew to a confluent monolayer, the medium was removed, and the monolayer was rinsed with PBS. The cells were removed by addition of 2 mL of 0.25% trypsin (Gibco, #15090-046) in versene solution (0.48 mM EDTA in PBS) for 1-2 min at room temperature. For routine passaging cells were transferred (1:5 split) to a new T-75 flask every 3 days.
**Generation and Maintenance of AK-D 2.2 (IFNαR-disrupted) Population.**

A heterologous population of AK-D cells with reduced responsiveness to type I interferon, termed AK-D 2.2, were recovered after transduction with Crispr/Cas guide RNAs targeting the second exon of the interferon alpha receptor using the same protocol as the Fcwf-4 CU IRN cells, however a clone was not obtained. Briefly, concentrated TPs were diluted 3:1 in DMEM containing 10% FCS and applied to parental AK-D cells for 48 h followed by selection in DMEM containing 10% FCS and 10 µg/mL puromycin (InvivoGen) for 96 h. Puromycin-selected cells were grown as a heterologous population designated AK-D 2.2 cells and verified for permissively to FIPV Black infection and reduced IFNα responsiveness. AK-D 2.2 cells were maintained in the same media as the parental AK-D cells. For routine passaging, cells were transferred (1:5 split) to a new T-75 flask every 3 days after a PBS wash and disruption with 0.25% trypsin-versene.

**Felis catus Bone Marrow-derived Macrophages (fBMDMs).**

For generation and culture of feline bone marrow-derived macrophages, feline femurs were harvested by Cornell University Veterinary Biobank from specific pathogen-free cats euthanized according to IACUC approved protocols, and total bone marrow content was collected. Red blood cells and fatty tissue were removed by lysis in ACK lysis buffer and straining through a 0.70 µm filter (Falcon). The remaining cells, predominantly hematopoietic stem cells, were cryopreserved at 5.0x10^7 cells/mL in 90% FBS and 10% DMSO. Feline bone marrow derived macrophages (fBMDMs) were differentiated as previously reported (Gow et al., 2013) with slight modification. Briefly, 5.0x10^7 bone marrow cells were plated in 100 x 26 mm petri dishes (VWR, #25387-030)
in DMEM (Corning, #10-017-CV) supplemented with 20% FBS, 10,000 IU/mL recombinant human (rh) M-CSF (PeproTech, #300-25) and 50 μM β-mercaptoethanol then incubated at 37°C and 5% CO₂. At day 3, supernatant was removed, clarified of cells, diluted 1:2 with fresh DMEM supplemented with 20% FCS and 10,000 IU/mL rhM-CSF, and returned to cells. fBMDMs were recovered on day 6 by gentle aspiration in PBS following 30 min, 4°C incubation in PBS.

**Mycoplasma Detection and Removal from Tissue Culture.**

All cells used in this study were monitored for mycoplasma contamination using a PCR-based assay. Cell culture supernatants were routinely collected after 3 days of culture and then heat-inactivated at 95°C for 10 min. PCR amplification for mycoplasma detection was performed using a forward primer: 5’- GGC GAA TGG GTG AGT AAC ACG -3’ and a reverse primer: 5’- CGG ATA ACG CTT GCG ACC TAT G -3’. Thermocycler settings were as follows: initial denaturation at 95°C for 10 min; 35 cycles consisting of denaturation at 95°C for 45 sec, annealing at 60°C for 45 sec, and extension at 72°C for 60 sec; and a final extension at 72°C for 10 min. The PCR products were analyzed on 1% (w/v) agarose gel. DNA fragments were visualized with a UV transilluminator after being stained with ethidium bromide. If mycoplasma was detected, the cells were treated for 7 days with 0.5 μg/mL of mycoplasma removal agent (Bio-Rad, #BUF035). All results shown here are from mycoplasma negative cells.

**FIPV PLP2 Functional Assays**

**Transcleavage Assay.**

FIPV PLP2 protease activity (targeting and cleavage of the LXGG motif) was determined using a transcleavage assay. Briefly, 2x10⁵ HEK 293T cells/mL were plated
in 12-well plates 24 h prior to transfection. 500 ng of plasmids encoding recFIPV-PLP2(V5) wild-type, catalytic mutant or empty vector were combined with 25 ng of plasmid expressing nsp2/nsp3-GFP substrate and co-transfected into cells using TransIT LT1 (Mirus) (3 μL LT1 / 1 μg DNA). After 16-18 h, cells were lysed in 100 μL IκB lysis buffer (20 mM Tris at pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 12.5 mM Na pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na orthovanadate, 1 mg/mL leupeptin, 1 mM PMSF) on ice then centrifuged at 14,000 xg for 10 min at 4°C to pellet nuclei. Lysate supernatants were separated using a 10% SDS-PAGE gel then transferred to a PVDF membrane using a semi-dry transfer apparatus (Bio-Rad). Membranes were blocked overnight in 5% milk-TBST at 4°C then incubated with primary antibody diluted in 5% milk-TBST for 1 hour at room temperature: to detect the nsp3-GFP cleavage product, a 1:2500 dilution of rabbit-anti GFP (ThermoFisher Scientific) was used; to determine PLP2 expression, a 1:5000 dilution of mouse-anti-V5 tag (Bio-Rad) was applied; a 1:5000 dilution of mouse-anti β-actin (Bio-Rad) was used to determine equivalence in protein loading. Membranes were washed three times for 15 min in TBST buffer, then incubated with donkey anti-rabbit-HRP (1:2500) or goat-anti-mouse-HRP (1:2500) secondary antibody (SouthernBiotech) for 1 h at room temperature. Three more 15 min washes in TBST were performed prior to addition of Western Lightning® Plus-ECL (PerkinElmer, Inc.) chemiluminescent substrate and visualization on a FluorChem™ E (Protein Simple) imaging system. Band sizes were determined relative to PageRuler™ Prestained protein ladder (Thermo Fisher).

**Deubiquitinase (DUB) and DeISGylase Assays.**

The ability of FIPV PLP2 to deconjugate ubiquitin or ISG15 molecules from total
proteins was determined using DUB or DeISGylase assays. Briefly, 2×10^5 HEK 293T cells/mL were plated in 12-well plates 24 h prior to assay. Cells were co-transfected with 500 ng of FLAG-ubiquitin (Flag-Ub) for DUB assay or 500 ng myc-ISG15 and 250 ng each of pUbcH8, pUbe1L, pHerc5 for DeISGylase assay, along with 250, 500 or 1000 ng of plasmid encoding recFIPV-PLP2(V5) wild-type, catalytic mutant or empty vector. At 16-18 h, cells were lysed in 100 μL IκB lysis buffer on ice then centrifuged at 14,000 xg for 10 min at 4°C to pellet nuclei. Lysate supernatants were separated using a 10% SDS-PAGE gel, transferred to a PVDF membrane using a semi-dry transfer apparatus (Bio-Rad) and blocked overnight in 5% milk-TBST at 4°C. Primary antibodies were diluted in 5% milk-TBST as follows: mouse-anti-FLAG (SigmaAldrich) at 1:2500 (DUB assay), mouse-anti-myc (Developmental Studies Data Bank, University of Iowa) at 1:250 (DeISGylase assay), and mouse-anti-V5 tag (Bio-Rad) at 1:2500 or a 1:5000 dilution of mouse-anti β-actin (Bio-Rad). Antibody dilutions were applied to membranes for 1 hour at room temperature, which were then washed three times for 15 min in TBST buffer and incubated in a 1:5000 dilution of goat-anti-mouse-HRP secondary antibody (SouthernBiotech) for 1 h at room temperature. Three more 15 min washes in TBST were performed prior to addition of Western Lightning® Plus-ECL (PerkinElmer, Inc.) chemiluminescent substrate and visualization on a FluorChem™ E (Protein Simple) imaging system. Deconjugation is visualized by a reduction in the relative intensities of the protein smear. Band sizes were determined relative to PageRuler™ Prestained protein ladder (Thermo Fisher).

**IFN Antagonism Assay.**

To determine if recFIPV-PLP2 is a type I IFN antagonist, the following assay was
performed as previously described (Niemeyer et al., 2018) with modification. Briefly, 2x10^5 HEK-293T cells/mL were transfected with plasmids encoding Renilla (RL) and Firefly (FF) luciferases (luc) along with WT or catalytic mutant rec-FIPV-PLP2. An IFN-β promoter (p125-luc) controls FF luc gene expression, while RL luciferase gene expression is determined by a constitutive herpes simplex thymidine-kinase (TK) promoter. Stimulation of the IFN-β promoter triggered by overexpressed, constitutively-active MDA5 leads to an auto-activation of the IFN pathway and increased FF luc. Plasmid amounts for a given 24-well plate format are as follows: pRL-TK (5 ng), p125-luc (250 ng), pEF- BOS-MDA5-3xFLAG His10 (100 ng) and 100 to 200 ng of PLP2-expressing plasmids. Cells were lysed at 18 hpt and 20 μL of luc-containing lysate was transferred to white-opaque 96-well plates and used for assessment of luc activity using a luminometer (Veritas). Relative IFN promotion is determined by calculating the ratio of ifn(FF)/luc(RL). Each condition was performed in triplicate in at least three experiments.

**Plaque Assay**

The plaque assay technique was established using AK-D, AK-D 2.2 and Fcwf-4 CU cells. 6.5 x 10^5 cells per well were plated in 6-well plates or 3.0-x10^5 cells per well were plated in 12-well plates. Cells were washed in PBS, then infected with 300 μL of 10-fold serial dilutions of viral samples in FCS-free media for 1 h at 37°C. Cells were overlaid with a 0.5% Oxoid agar (Oxoid LTD, #LP0028)-DMEM containing 1% FBS mixture. Plates were incubated at 37°C for 48 h (or the indicated time) and fixed using 3.7% formaldehyde-PBS solution for 30 min. Viral plaques were visualized by staining with 0.1% crystal violet for 30 min and photographed. We note plaques were clearly evident when we used Oxoid agar, but not if we used Noble agar.
**Virus Growth Kinetics**

We analyzed the growth kinetics of type I FIPV Black in four cell lines: AK-D, AK-D 2.2, Fcwf-4 CU, and Fcwf-4 ATCC. Type II FCoV titers were evaluated in Fcwf-4 CU cells. For all cell types, 1.5 x 10^5 cells were plated in 24-well plates or 3.0 x 10^5 cells in 12-well plates. After incubating for 24 h, cells were infected with FIPV in serum-free media at a multiplicity of infection (MOI) of 0.1 at 37°C. After a 1 h incubation, the infectious media were replaced with fresh media containing 2% FBS. At the indicated time points, cell-free supernatants and/or infected cells were harvested and used for titration by plaque assay on AK-D (FIPV Black) or Fcwf-4 CU (type II FCoVs) cells.

**Evaluating Virus Titers from Cell-free and Cell-associated Samples**

We compared the cell-free and cell-associated growth kinetics of FIPV in several cell lines: AK-D, AK-D 2.2, Fcwf-4 CU and Fcwf-4 ATCC cells. For all cell types, 1.2 x 10^6 cells were plated in a 60 mm^2 dish, 1.5 x 10^5 cells were plated in 24-well plates or 3.0 x 10^5 cells in 12-well plates in DMEM or EMEM containing 10% FBS. After incubating for 24 h, cells were infected with FIPV Black in serum-free media at a multiplicity of infection (MOI) = 0.1 at 37°C. After a 1 h incubation, the infectious media were replaced with fresh complete media. Cell supernatants and infected cells were harvested at indicated time points and used to prepare cell-free and cell-associated samples, respectively, for titration by plaque assay on Fcwf-4 CU cells as described above. The supernatant was collected from the cultures at the times indicated and subjected to centrifugation at 2,200 xg for 10 min at 4°C to remove any dead cells. This cell free supernatant was aliquoted and frozen at -80°C until use. To prepare the cell--associated sample, 0.5 mL of medium was added to the infected cells, and the entire plate was
frozen at -80°C. The infected cells were then subjected to two additional rounds of freezing and thawing (37°C for 1-2 min). After the third thaw, the cells and medium were transferred to a centrifuge tube and centrifuged at 2,200-3,000 xg for 10 min at 4°C to remove cell debris. The supernatant containing the viruses released from the cells during the freeze-thaw process was designated the cell-associated virus sample. Cell-associated samples were stored at -80°C. To determine FIPV titers, plaque assays were performed on Fcwf-4 CU cells as described above.

**Type I Interferon Responsiveness Assay**

To determine feline cell responsiveness to type I interferon, quantification of ISG54 transcript production by RT-qPCR following stimulation with feline IFN was performed. Cells (1.0 x 10^5 in a 24-well plate) were treated with 10, 100, or 1000 U/mL of purified feline IFN-alpha (PBL Assay Science, #15100-1) for 6 h. To determine ISG54 and feline β-actin mRNA production, total RNA was extracted using a RNeasy Mini Kit (Qiagen) and an equal amount of RNA (1000 ng) was used for cDNA synthesis using RT² HT First-Strand Kit (Qiagen, #330401). qPCR was performed with specific primers (Appendix B; Primer Index 1) for feline β-actin transcript (FWD 5’- CAA CCG TGA GAA GAT GAC TCA GA -3’; REV 5’- CCC AGA GTC CAT GAC AAT ACC A -3’) (Kessler et al., 2009) or ISG54 transcript (FWD 5’- CCT GAG CTG CAT GAC AAT ACC A -3’; REV 5’- CAC GTG AAA TGG CAT TTA AGT TGC CGC AG -3’) using RT² SYBR Green qPCR Mastermix (Qiagen, #330502). A Bio-Rad CFX96 thermocycler was set as follows: one step at 95°C (10 min); 40 cycles of 95°C (15 s), 60°C (1 min), and plate read; one step at 95°C (10 s); and a melt curve from 65°C to 95°C at increments of 0.5°C/0.5 s. Samples were evaluated in triplicate and data are representative of three
independent experiments. The levels of mRNA are reported relative to β-actin mRNA and expressed as $2^{-\Delta CT} = C_{T(gene \ of \ interest)} - C_{T(\beta-actin)}$.

**Immunofluorescence Detection of FIPV Nucleocapsid Protein**

Monolayers of $1.0 \times 10^5$ AK-D and Fcwf-4 CU cells were cultured in 8-well chamber slides (Nalge Nunc International, #177445) at 37°C for 24 h. Cells were infected with FIPV Black at a MOI of 0.1 for 1 h at 37°C. At times indicated, the infected cells were fixed with 3.7% formaldehyde-PBS solution for 30 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and then incubated with blocking solution containing 5% normal goat serum and 0.1% Triton X-100 in PBS at 4°C overnight. For immunofluorescence staining of FIPV Black-infected cells, the cells were incubated with mouse monoclonal anti-FIPV nucleocapsid (CCV2-2) (Poncelet et al., 2008) (Bio-Rad, #MCA2594B) as a primary antibody at a dilution of 1:500 at room temperature for 1 h. Cells were then incubated with a secondary antibody, Alexa Fluor 568-conjugated goat anti-mouse IgG (Thermo Fisher Scientific, #A11004) at a dilution of 1:1000 in the presence of Hoechst33342 (Thermo Fisher Scientific, #H1399) at a dilution of 1:1000 for nucleus stain. After 30 min incubation with the secondary antibody at room temperature, the cells were then washed with PBS, mounted, and examined under a fluorescence microscope.

**Morphologic Staining**

Feline bone marrow-derived macrophages, Fcwf-4 CU, or Fcwf-4 ATCC cells, grown for 24 h in 100 x 26 mm petri dishes (VWR, #25387-030), were washed with PBS, incubated in PBS for 30 min at 4°C, then collected with gentle pipetting. Using a Cytospin (Shandon), 200 µL of cells (10,000 cells/mL) suspended in PBS supplemented
with 2% BSA were spun onto glass coverslips (pre-treated with 2% BSA in PBS) at 115 xg for 6 min. Cells were dried, rinsed in PBS, then fixed in absolute methanol for 1 min. Wright-Giemsa (Thermo Fisher Scientific, #9990710) staining was performed per the manufacturer’s instructions using a 3 min primary stain and a 2 min counter stain, and washed with 1 mL rinse solution. Slides were dried and imaged under oil immersion at 100x magnification.

**Cloning and Expression of Feline TMPRSS2 in Fcwf-4 CU IRN Cells**

A dsDNA gBlocks® (IDT) fragment containing the coding sequence of feline TMPRSS2 variant 1 (GenBank accession # XM_023238709.1) was designed with several modifications for cloning into the pLVX-Puro vector. The 5’ end was designed to contain a pLVX complementarity region (5’-TAC TAG AGG ATC ACC GGA TCT AGA T-3’), followed by an XhoI cleavage site and a Kozak consensus sequence (5’-ACC ATG G-3’). The 3’ end contains a spacer sequence (5’-GGT-3’) followed by an in-frame V5 tag coding region (5’-GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG-3’), translation termination sequence (5’-TAG-3’), BamHI cleavage site and a pLVX complementarity region (5’-ACC GGA TCT AGA TAA CTG ATC ATA A-3’). pLVX-Puro vector was double-digested with XhoI (NEB) and BamHI (NEB) and the feline TMPRSS2 gBlocks fragment was inserted using NEBuilder® HiFi DNA Assembly (NEB) using a 1:5 vector to insert ratio as per the manufacturer’s instructions. The assembled plasmid was transformed into Top10 E. coli, purified by MaxiPrep (Promega) and sequenced using CMV FWD, fTMPRSS2 SEQ F1, and fTMPRSS2 SEQ F2 sequencing primers (Appendix B; Primer Index 1). Transducing particles (TPs) carrying the pLVX-fTMPRSS2(V5)-puroR+ plasmids, encoding lentivirus 5’ LTR, psi (ψ) packaging signal,
rev-response element (RRE) and feline TMPRSS2(V5) were generated under the direction of Dr. Michael Hantak (Loyola University Chicago). Briefly, 3.3 μg each of pLVX-fTMPRSS2(V5)-puroR+, psPax2 (lentiviral packaging plasmid) and pHEF-VSV-G (vesicular stomatitis virus glycoprotein) plasmids (generously supplied by Edward M. Campbell, Loyola University Chicago) were co-transfected into 10 cm dishes containing 5x10^6 HEK-293T/17 cells using polyethylenimine (PEI) (5 μg PEI / 1 μg DNA) in antibiotic-free DMEM 10% FCS for 24 h followed by replacement with complete DMEM containing 10% FCS with antibiotics. After 48 h, supernatants containing TPs were collected and stored at 4°C and cells were replenished with 10 mL DMEM containing 5% FCS with antibiotics. After an additional 24-hour incubation (72 hours total) at 37°C and 5% CO₂, supernatants were collected and combined with the previously collected lot. Total supernatants were centrifuged 1000 xg at 4°C for 10 min to remove cell debris, then centrifuged overnight (16+ hours) at 5,000 xg and 4°C. To concentrate, supernatants were poured off and pellets were suspended in 1/10 – 1/100th the original volume with EMEM (-FCS). For expression of feline TMPRSS2(V5), transducing particles were diluted 1:5 in serum-free EMEM and a minimal volume was incubated with Fcwf-4 CU IRN cells for 1 hour at 37°C and 5% CO₂ followed by addition of EMEM containing 6.25% FCS. After 48 hours, cells were lysed in either lysis buffer A (4% sodium dodecyl sulfate, 3% dithiothreitol, 40% glycerol, and 0.065 M Tris-HCl pH 6.8 in H₂O) for evaluation of TMPRSS2(V5) by western blot, or lysed in RLT buffer (Qiagen) for evaluation of TMPRSS2 mRNA levels by qPCR. For western blot, lysates were sonicated, and 50,000 cell-equivalents were loaded and electrophoretically separated through 7-15% acrylamide Mini-PROTEAN® TGX™ precast SDS-PAGE gels (Bio-Rad)
in 1x Running Buffer (0.19 M glycine, 0.025 M trizma base, and 1% sodium dodecyl sulfate in H₂O). The acrylamide gels, methanol-activated Immun-Blot® PVDF membranes (Bio-Rad) and filter paper (Bio-Rad) were soaked for 10 min in semi-dry transfer buffer (48 mM trizma base, 39 mM glycine, 1.3 mM sodium dodecyl sulfate and 20% methanol in H₂O at pH 9.2) and proteins were transferred from acrylamide gels to the PVDF membranes using a semi-dry Trans-Blot® Turbo™ transfer system (Bio-Rad). Membranes were blocked at 4°C overnight in 5% milk in TBST (0.02 M trizma base, 0.15 M NaCl and 1% Tween 20 in H₂O) followed by 90 min room temperature incubation in primary antibodies specific for TMPRSS2(V5) tag (murine monoclonal IgG; Genscript) or β-actin (murine monoclonal IgG; Genscript) diluted 1:2500 and 1:5000, respectively, in 5% milk-TBST. The membranes were washed three times for 15 min each in TBST followed incubation for 1 hour at room temperature in goat-anti-mouse-HRP (SouthernBiotech) secondary antibody diluted 1:5000 in 5% milk-TBST. Three more 15 min washes in TBST were performed prior to addition of Western Lightning® Plus-ECL (PerkinElmer, Inc.) chemiluminescent substrate and visualization on a FluorChem™ E (Protein Simple) imaging system. Band sizes were determined relative to PageRuler™ Prestained protein ladder (Thermo Fisher). For detection of feline TMPRSS2 mRNA transcript levels, quantitative PCRs were performed. Briefly, total RNA was extracted from RLT lysates using a RNeasy Mini Kit (Qiagen) and an equal amount of RNA (500 ng) was used for cDNA synthesis using RT² HT First-Strand Kit (Qiagen, #330401). qPCR was performed with specific primers for feline TMPRSS2 (feline tmprss2 FWD / feline tmprss2 REV) or feline β-actin transcript (fβ-Actin FWD / fβ-Actin Actin REV) (Kessler et al., 2009) (Appendix B; Primer Index 1) using RT²
SYBR Green qPCR Mastermix (Qiagen, #330502). A Bio-Rad CFX96 thermocycler was set as follows: one step at 95°C (10 min); 40 cycles of 95°C (15 s), 58°C (1 min), and plate read; one step at 95°C (10 s); and a melt curve from 65°C to 95°C at increments of 0.5°C/0.5 s. Samples were evaluated in triplicate and data are representative of three independent experiments. The levels of mRNA are reported relative to β-actin mRNA and expressed as $2^{-\Delta CT} [\Delta CT = C_T(\text{gene of interest}) - C_T(\beta-\text{actin})]$.

**Determining IFNaR2 Expression and Amplicon Melt Temperature**

Expression and amplicon melt temperature of feline ifnar gene exons 1-4 were determined by qPCR. Briefly, confluent monolayers of Fcwf-4 CU and Fcwf-4 CU IRN cells were grown overnight in complete EMEM, lysed in RLT (Qiagen) and total RNA was isolated using the RNeasy RNA isolation kit (Qiagen). Equivalent amounts of RNA were used to generate cDNA using RevertAID cDNA synthesis kit (ThermoFisher). Feline β-actin (primers: fβ-Actin FWD / fβ-Actin REV) and IFNaR2 exon 1-4 (primers: Fel ifnar mRNA Exon1-4 RT FWD / Fel ifnar mRNA Exon1-4 RT REV) transcript levels were determined by qPCR using Sybr Green Master Mix (Qiagen) (Appendix B; Primer Index 1). qPCRs were performed in a CFX96 thermocycler (Bio-Rad) using the following settings: 95°C for 10 min; 40 cycles of 95°C for 15 s, 58°C for 1 min and SYBR read; 95°C for 10 s; melt curve from 65°C to 95°C at 0.5°C/0.5 s. For qPCR, samples were evaluated in triplicate and data represent three independent experiments. The levels of mRNA are reported relative to β-actin mRNA and expressed as $2^{-\Delta CT} [\Delta CT = C_T(\text{gene of interest}) - C_T(\beta-\text{actin})]$. Melt temperatures were determined by plotting the change in fluorescence with temperature \(-d(\text{RFU})/dT\) versus temperature (°C) then calculating the point at which the largest change in fluorescence occurs (Krenke et al., 2005).
Detecting FIPV N gene in FIPV-infected Fcwf-4 CU IRN Cells Expressing Tmprss2

To determine effects of TMPRSS2 on FIPV replication, Fcwf-4 CU IRN cells were transduced with increasing dilutions of lentiviruses expressing TMPRSS2 then infected with FIPV Black. N gene expression was determined by qPCR. Briefly, 24-well Cell-Bind plates (Corning) were seeded with 0.5 mL Fcwf-4 CU IRN cells at 3.0 x 10^5 cells/mL in complete EMEM and incubated overnight at 37°C and 5% CO₂. Media was replaced with 100 uL EMEM (-FCS) (mock), or cells were transduced with 100 uL fTMPRSS2(V5) lentivirus TPs left undiluted, or diluted 1:20, 1:10, 1:5 and 1:2.5 in EMEM (-FCS). After 1 h incubation, 400 uL EMEM (6.25 % FCS) was added to each well (without media replacement) to give 500 uL and 5% FCS. Cells were incubated for 48 h to allow expression of TMPRSS2 then infected with FIPV Black at MOI of 0.1 or left uninfected (mock; mock expressing TMPRSS2). After an additional 18 h infection, cells were lysed in RLT (Qiagen) and RNA was isolated using RNeasy Mini Kit (Qiagen). Equivalent amounts of RNA were used to generate cDNA using the RevertAID cDNA Synthesis Kit (ThermoFisher). FIPV Black N gene (primers: FIPV-N-qpcr-F / FIPV-N-qpcr-R) and β-actin (primers: fβ-Actin FWD / fβ-Actin REV) transcript levels were determined by qPCR using Sybr Green Master Mix (Qiagen) (Appendix B; Primer Index 1). qPCRs were performed in a CFX96 thermocycler (Bio-Rad) set to: 95°C for 10 min; 40 cycles of 95°C for 15 s, 58°C for 1 min and SYBR read; 95°C for 10 s; and a melt curve from 65°C to 95°C at increments of 0.5°C/0.5 s. Samples were evaluated in triplicate and data are representative of three independent experiments. The levels of mRNA are reported relative to β-actin mRNA and expressed as 2^-ΔCT [ΔCT = CT(gene of interest) - CT(β-actin)].
Reverse Genetics Methods

Genomic RNA Isolation from FCoV Virions.

Genomic RNA (gRNA) was isolated from 600 μL cell-free supernatants containing FCoV virions at concentrations greater than 1.0x10^5 PFU/mL. 100 μL aliquots of supernatant were mixed with TriReagent (MRC, Inc.) at a ratio of 1:10 in 1.5 mL eppitubes. The mixture was incubated for 5 min at room temperature to dissociate the nucleoprotein complexes. 0.25 mL of chloroform (Sigma-Aldrich) was added per 0.75 mL of TriReagent used in the initial homogenization, the mixtures were shaken vigorously by hand for 1 min, incubated at room temperature for 15 min then centrifuged at 12,000 xg for 10 min at 4°C for phase separation. The top aqueous layers containing RNA were extracted into new RNase-free 1.5 mL eppitubes and 5 μg of glycogen was added (1 μL at 5 mg/mL). Room temperature 200 proof isopropanol (Sigma-Aldrich) was added at a 1:1 ratio and the six tubes were inverted to mix, incubated at room temperature for 10 min then centrifuged 12,000 xg for 60 min at 4°C. The supernatants were discarded, the pellets were washed in 1.25 mL 75% ethanol then centrifuged 7,500 xg for 5 min at 4°C. The RNA was washed in 75% ethanol three times total. The final ethanol washes were discarded, and the pellets were allowed to air dry. To dissolve, 10-20 μL RNase-free water was added directly to the pellets and tubes were incubated at 55°C for 15 min. The six dissolved pellets were then combined, and concentrations were determined by spectrophotometer (NanoDrop).

Deep-sequencing, Cloning and Modification of a Lab Strain of FIPV Black.

The FIPV Black (TN406) strain used as the backbone for the reverse genetics system was kindly provided by Dr. Fred Scott, Cornell University College of Veterinary
Medicine, Ithaca, NY. The genomic sequence of this specific FIPV Black laboratory strain was determined by deep sequencing. Briefly, AK-D cells were infected with FIPV Black for 24 hours, the cell monolayer and supernatant were freeze-thawed by alternating -80°C and 37°C incubations, then centrifuged for 10 min at 2,000 xg and 4°C to remove cell debris. The supernatant was sent to the Veterinary Diagnostic Laboratory (Department of Diagnostic Medicine and Pathology, College of Veterinary Medicine, Kansas State University) and subject to Ion Torrent Next Generation Sequencing. The resulting sequencing reads were aligned to a reference FIPV Black genome (GenBank Accession #EU186072.1) and a consensus sequence of this laboratory strain of FIPV Black was generated (Appendix C). The concept for the contiguous cDNA FIPV Black reverse genetics system was based on the previously described method for generating murine hepatitis virus (Yount et al., 2002) and involves sub-cloning cDNA segments of the coronavirus genome onto individual plasmids, ligation and transcription of the full-length cDNA into genomic RNA followed by electroporation into cells. Based on the deep-sequenced genome of the FIPV Black lab strain, seven contiguous cDNA segments (A-G) (Table 8), comprising the entire genome, were designed, amplified using specific primer sets from genomic RNA and sub-cloned onto individual plasmids. Each cDNA fragment contains unique restriction sites, which overlap only with the adjacent fragment and ensures that the seven excised fragments ligate in a specific A to G order and reflect a complete cDNA version the original FIPV genomic sequence. To generate the seven cDNA plasmids, 1.5 μg of FIPV Black genomic RNA was used as a template for cDNA synthesis with random hexamer primers via the RevertAID First-Strand cDNA Synthesis kit (Thermo Fisher) as per the manufacturer’s specifications.
Using the resulting cDNA as a template, PCR reactions were set up using the Expand™ Long Range dNTPack (Roche via Sigma-Aldrich) kit and segment-specific primer sets (Appendix B; Primer Index 3) were included to amplify cDNA segments B-F using the following specifications: 0.2 mM dNTP mix, 0.2 uM forward primer, 0.2 uM reverse primer, 3.75% DMSO, 1x reaction buffer, 1.5 U enzyme mix and 2 μL cDNA per 50 μL reaction. The amplified cDNA segments were gel extracted from a 0.8% SeaPlaque GTG™ Agarose (Lonza) gel and column purified using an SV gel and PCR clean-up system (Promega) prior to insertion into TA cloning pCR-XL-TOPO® plasmids using the TOPO® XL PCR Cloning Kit (Life Technologies). Plasmids were then transformed into Top10 E. coli and purified by MaxiPrep (Promega). cDNA segments A and G were individually synthesized and cloned into pUC57(AmpR⁺) vectors by BioBasic Inc. (Markham, ON, Canada). The A segment sequence was modified from the genomic FIPV Black sequence in the following ways: a T7 polymerase promoter sequence 5’-TAA TAC GAC TCA CTA TAG-3’ was added at position -21 relative to the start of the FIPV Black genome and a natural BsmBI cut site was removed at position 3806 (GAG to GAA). The G segment sequence was modified to include a stretch of 21 consecutive adenine nucleotides at the 3’ end. Additionally, the nucleocapsid (N) gene sequence was amplified from the FIPV Black total cDNA using specific primers (Appendix B; Primer Index 3) and cloned into pcDNA-3.1 using NEBuilder® HiFi DNA Assembly (NEB) as per the manufacturer’s specifications. Large-scale preparations of the cDNA plasmids A-G, and N gene were obtained using a MaxiPrep kit (Promega) then sequenced (Appendix B; Primer Index 4) prior to long-term storage at -80°C.
Assembly of Full-length FIPV Black Infectious Clone cDNA.

The method of assembling the full-length infectious clone of the FIPV Black sequenced laboratory strain was based on the previously described reverse genetics method for murine hepatitis virus (Yount et al., 2002). Similarly, our design for the reverse genetics system for FIPV involves ligation of the seven contiguous cDNA fragments at specific junctions in order to generate the full-length cDNA infectious clone. First, each of the plasmid DNAs containing specific cDNA segments of the FIPV genome, (FIPV plasmids A-G) was grown to a high concentration, isolated by Maxiprep (Promega), and 40 μg was digested with BsmBI, or double digested with BsmBI and BamHI, or SfiI according to the manufacturer’s specifications (NEB) (Table 8) FIPV cDNA plasmids D and G were singly digested with BsmBI resulting in desired fragment sizes of 2,339 and 4,385 bp respectively, while the FIPV Black A plasmid was double-digested with BsmBI and BamHI resulting in a desired fragment size of 4,810 bp. FIPV plasmids B, C, E and F were double digested with BsmBI and SfiI resulting in desired fragment sizes of 4,064 bp for B plasmid, 4,609 bp for C plasmid, 4,762 bp for E plasmid and 4,272 bp for F plasmid. The SfiI cut site is located on the pCR-XL-TOPO® vector and was included to allow clearer band separation when purifying the fragments. Additionally, 40 μg of the N gene plasmid (pcDNA-3.1 encoding the FIPV Black nucleocapsid gene) was linearized by DraIII digestion. The digests were then diluted in 6x loading dye (Ambion) and run through a 0.8% SeaPlaque GTG™ (Lonza) low-melt agarose gel in TAE (tris-acetate-EDTA) buffer. The desired bands (FIPV Fragments A-G and linearized N plasmid) were visualized on a DarkReader transilluminator (Clare Chemical Research) to prevent UV-induced nucleotide cross-linking, cut from the
agarose gel, purified using an SV gel and PCR clean-up system (Promega), and eluted in 120 μL nuclease-free H₂O. To remove excess agarose, the fragments were further purified by addition of chloroform at a ratio of 1:1. The chloroform-DNA solutions were mixed by vortex for 1 min, centrifuged 5 min at 15,000 rpm (21,130 xg) and the top aqueous layers containing DNA were recovered and placed into new 0.6 mL eppitubes. The concentration of each purified cDNA fragment was determined, and aliquots were stored at -80°C. To generate the full-length infectious clone cDNA, molar equivalents (0.02-0.05 pmol) of each fragment were combined, totaling approximately 6 μg DNA, and ligated using 6,000 U of T4 DNA Ligase (NEB) and 1x T4 ligase buffer (50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT) in a 200 μL reaction for 16-24 hours at 16°C. The full-length ligation product (~29.2 Kb) was purified using phenol-chloroform-isoamyl alcohol extraction then precipitated with isopropanol. Briefly, 20 μL (1:10) of 3M NaOAc at pH 5.2 and 220 μL (1:2) phenol-chloroform-isoamyl alcohol (25:24:1 ratio) at pH 8.0 were added to the 200 μL ligation reaction. The solution was shaken vigorously by hand for 1 min and centrifuged at room temperature for 2 min at 14,000 rpm (18,407 xg) for phase separation. The top aqueous phase containing DNA was extracted into a new tube, followed by addition of room temperature 200 proof isopropanol (1:1 ratio) and inversion to mix. The DNA was allowed to precipitate for 10 min at room temperature before centrifuging at 14,000 (18,407 xg) for 10 min at room temperature. The pellet was washed two times in 500 μL of 75% ethanol (chilled in -80°C) and once in 500 μL 95% ethanol (chilled in -80°C) with pelleting at 14,000 (18,407 xg) for 3 min in between washes. The pellet was allowed to air dry until translucent and 11.5 μL nuclease-free H₂O was added. To dissolve the pellet, the tube was held at room
temperature for 1 hour with intermittent flicking. The dissolved pellet was used immediately for *in vitro* transcription without storage.

**Full-length Ligation Junction PCR.**

To determine if ligation of the seven cDNA fragments was contiguous, PCRs across each junction were performed. Briefly, molar equivalents of purified fragments A-G were ligated overnight at 16°C using T4 Ligase (NEB). Using 10 ng of ligation product as template, PCRs were performed using Expand Long-Range Taq Polymerase (Roche via Sigma) to amplify each junction using junction-specific primers (AB, A5/B1R [761 bp]; BC, B5/C1R [903 bp]; CD, C6/D1R [900 bp]; DE, D3/E1R [730 bp]; EF, E7/F1R [1057 bp]; FG, F6/G1R [1154 bp]) (*Appendix B; Primer Index 4*). Reactions were performed in an Eppendorf thermocycler set at 92°C (2 min); 25 cycles at 92°C (10 s), 52 or 55°C (15 s), and 66°C (80 s); and held at 66°C for 7 min. PCR products were diluted in 6x loading dye (NEB) and electrophoretically separated through a 0.9% agarose gel. Bands were visualized under UV due to intercalation of ethidium bromide.

**In Vitro Transcription and RNA Electroporation.**

Full-length mRNA copies of the FIPV infectious clone cDNA and additional mRNA copies of the FIPV N gene were synthesized using the *mMessage mMachine T7* (Ambion) *in vitro* transcription kit as per the manufacturer’s specifications with the following modifications. To generate full-length infectious clone mRNAs, the 11.5 μL purified cDNA ligation (see above) was combined with 3.5 μL of 30 mM GTP (2.1 mM final), 25 μL 2x NTP/CAP (1x final), 5 μL 10x reaction buffer (1x final) and 5 μL T7 polymerase/enzyme mix. N gene transcripts were generated by combining 500 ng purified linearized N gene plasmid with 12.5 μL 2x NTP/Cap (1x final), 2.5 μL 10x
reaction buffer (1x final), 2.5 μL T7 polymerase/enzyme mix and nuclease-free H2O in a 25 μL reaction. The reactions were incubated at 40°C for 25 min, 37°C for 50 min, 40.5°C for 25 min then held at 4°C in the thermocycler. During the in vitro transcription incubation time, Fcwf-4 CU cells were prepared for electroporation. Briefly, Fcwf-4 CU cells were grown to confluence in T-75 flasks (Corning) in complete EMEM over 24-48 hours prior to electroporation. The Fcwf-4 CU cells were washed in 10 mL 1x PBS, dissociated from the plate with 2 mL 0.25% trypsin-verseine at 37°C for 2 min then diluted 1:10 in complete EMEM. The cells were pelleted at 4°C for 7 min at 760 rpm (104 xg), washed twice in 10 mL cold 1x PBS with centrifuge cycles at 4°C for 7 min at 760 rpm (104 xg) to pellet in between, and counted on a hemocytometer using a 1:10 dilution of cells in 0.4% trypan blue-PBS. Washed and pelleted Fcwf-4 CU cells were suspended to 1x10^7 cells/mL in cold Opti-MEM® I (Gibco, #11058-021) and held on ice. Completed in vitro transcription reactions were used directly for electroporation without additional purification. Briefly, 22 μL N gene transcript, 45 μL full-length infectious clone transcript and 600 μL Fcwf-4 CU cells (1x10^7 cell/mL) were combined, in order, in a chilled 0.4 cm gap Gene Pulser® cuvette (Bio-Rad, #1652081) and electroporated with 3 pulses of 850 V, 25 μF capacitance and ∞ resistance at 5 sec intervals using a Gene Pulser Xcell™ (Bio-Rad) apparatus. As a positive control, 500 ng of purified FIPV Black genomic RNA and 250 μg N gene transcript were combined and electroporated into 600 μL Fcwf-4 CU cells (1x10^7 cell/mL) using the same conditions as above. As a negative control, 600 μL Fcwf-4 CU cells (1x10^7 cell/mL) with no added RNA were electroporated using the same conditions as above. All electroporated cells were allowed to recover at room temperature for 10 minutes then diluted in 10 mL complete EMEM and plated into
T-75 flasks. After 3-5 hours, when the cells had settled and begun attaching to the flask, media was gently removed and replaced with 10 mL complete EMEM. The cells were then incubated for 24-72 hours at 37°C and 5% CO₂, or until cell cytopathic effects were observable in the positive control.

**Detection of FIPV Black N gene Subgenomic RNA.**

Virus nucleocapsid (N) gene subgenomic (sg) RNAs containing the FIPV Black leader sequence were detected by reverse-transcriptase PCR (RT-PCR). Briefly, cell monolayers containing electroporated FIPV RNA were lysed in RLT buffer (proprietary lysis and binding buffer containing guanidinium thiocyanate) as per the manufacturer’s instructions (Qiagen) at room temperature between 24- and 72-hours post-electroporation. Total RNA was extracted using a RNeasy Mini kit (74104, Qiagen) and eluted in 30 μL RNase-free water. Equivalent amounts of RNA, up to 4 μg, were converted to cDNA using the RevertAid First Strand Synthesis kit (Thermo Scientific, K1621) using random hexamer primers. PCR to amplify N gene sgRNA was carried out using Expand Long-Range dNTPack (Roche) with the following specifications: 0.2 mM dNTP mix, 0.2 uM FWD primer, 0.2 uM REV primer, 3.75% DMSO, 1x reaction buffer, 1.5 U enzyme mix and 1 μL cDNA per 20 μL reaction. To amplify N gene, the following primers were used: Forward FIPV Black Leader Primer 5’- GTG CTA GAT TTG TCT TCG GAC ACC -3’; N Gene Reverse 5’- CAT CAG CAT GAG GTC CTG TAC CTG A -3’ (**Appendix B; Primer Index 1**) resulting in a 331 bp amplicon. PCR reactions were heated to 92°C for 2 min, cycled 35 times between 92°C for 20 s, 61°C for 20 s, and 68°C for 20 s, then incubated at 68°C for 7 min for a final extension. PCR products were electrophoretically separated on a 0.9% agarose gel and visualized under UV light.
Generation of Figures, Graphics, Tables and Reference Management

The open source illustration program INKSCAPE (Inkscape.org) was used to create original graphic figures. All other figures that incorporate experimental data and statistical analyses were generated using GraphPad Prism 6 software (Graphpad.com). Tables were created using Microsoft Word for Mac version 16.22 (Microsoft.com). All reference materials were organized and incorporated using Mendeley software version 1.19.3 (©Mendeley Ltd.; Mendeley.com).
CHAPTER THREE

EXPERIMENTAL RESULTS

Section 1: Characterizing Replication Kinetics and Plaque Production of Serotype I Feline Infectious Peritonitis Virus in Three Feline Cell Lines

Rationale.

It is critical that we investigate type I FIPV in laboratory cell culture in order to understand the basic virology of natural infection, characterize type I clinical isolates, test novel therapeutics, and develop effective feline vaccines with broader coverage. However, this has been challenging because type I FCoVs cannot be easily adapted to laboratory cell culture; furthermore, the receptor for type I is not known (Cham et al., 2017; Dye et al., 2007; Hohdatsu et al., 1998), making the identification of highly permissive cell types difficult. Select type I isolates, such as the FIPV Black strain used in this study, have been adapted to growth in tissue culture at the cost of reduced in vivo virulence (Black, 1980; Pedersen, 2009; Tekes et al., 2008; Thiel et al., 2014). Feline airway epithelial (AK-D) cells propagate FIPV Black (Regan et al., 2012); however, these cells do not represent natural tropism for FIPV. Felis catus whole fetus 4 (Fcwf-4) cells are a more physiologically-relevant feline macrophage-like cell line (Jacobse-Geels and Horzinek, 1983), but these cells come with several technical drawbacks for studying type I FIPV: Typical replication of serotype I FIPV in Fcwf-4 cells takes between 15 and 96 hours to reach maximum titers, which usually range between $10^3$ and $10^4$ pfu/mL. Additionally, FIPV produced in these cells tends to be very cell-
associated and requires multiple freeze-thaw cycles to release – a technique that further reduces the final titer. Together, these factors have made investigation of type I FIPV challenging.

As part of this study, we characterized three feline cell lines—two from the American Type Culture Collection (ATCC) and one from Cornell University—and evaluated the replication kinetics, efficiency of plaque formation, and responsiveness of these cells to type I interferon in order to identify the optimal cell culture conditions for serotype I FIPV Black. The experiments performed here were done in collaboration with Dr. Amornrat O’Brien (Loyola University Chicago) and culminated in a co-first author manuscript: O’Brien and Mettelman et al., 2018. In this study, we found that an Fcwf-4 cell line established at Cornell University College of Veterinary Medicine, designated Fcwf-4 CU, propagates type I FIPV to significantly higher titers in cell-free supernatant and with more rapid kinetics compared to commercially available Fcwf-4 cells. We show that Fcwf-4 CU cells are less responsive to exogenous type I interferon than Fcwf-4 cells from the ATCC and are permissive to infection by both biotypes of type II FCoV. To facilitate quantitation of FIPV Black, we established a standardized plaque assay method using Fcwf-4 CU cells and commercially available AK-D cells and show that both cell types permit rapid and consistent quantitation of infectious titers of type I FIPV as well as type II FIPV and FECV from cell-free supernatants.

**Results.**

**Type I FIPV Black replication kinetics vary between feline cell types.** To determine the optimal cell type and conditions required to grow the type I FIPV Black strain, we evaluated virus growth kinetics using a standard infection time course. Cells
were infected at a multiplicity of infection (MOI) of 0.1 and virus titer was determined by plaque assay from cell-free supernatants over 96 hours. FIPV Black, a distinct type I lab strain that replicates in feline epithelial cells, replicated as expected in AK-D cells reaching a maximum titer \(>10^6\) pfu/mL at 36 hours post-infection (hpi) (Figure 8A). In our hands, using Fcwf-4 cells purchased from the ATCC, the replication of FIPV Black reached a maximum titer \(>10^4\) pfu/mL over 72-96 hpi (Figure 8A). Strikingly, FIPV Black replication kinetics and maximum titer were drastically different in an Fcwf-4 cell line established at Cornell University College of Veterinary Medicine (Fcwf-4 CU). Using these cells, the virus reached a significantly higher maximum titer of \(>10^6\) pfu/mL at 20 hpi. In other words, nearly 100 times more virus was produced from the Fcwf-4 CU cells a full 2-3 days faster than in Fcwf-4 ATCC cells.

To address whether the differences in titer observed between AK-D, Fcwf-4 CU, and Fcwf-4 ATCC cells were due to differences in cell-free and cell-associated virus, we compared the cell-associated and cell-free virus titers from each cell type at the time points around the respective maximum titers. Surprisingly, the cell-free virus titers were higher than the cell-associated titers at all time points and in all cell types assayed (Figure 8B). This indicates that FIPV Black virions are released into cell supernatant during infection of cell culture and freeze-thaw cycles are not necessary to obtain high virus titers.
Although the maximum titers of FIPV Black were comparable between AK-D and Fcwf-4 CU cells, the progression of cell cytopathic effects (CPE) induced by the virus...
differed. FIPV Black formed large, uniform syncytia in Fcwf-4 CU cells, while individual cell-death-induced clearings were observed in infected AK-D cells (Figure 9A). Of note, maximum titers from both cell types were obtained just prior to the appearance of major CPE, allowing a visual guide to virus collection. To further demonstrate this point, Fcwf-4 CU and AK-D cells infected with FIPV Black were labeled with an anti-nucleocapsid antibody (CCV2-2) (Poncelet et al., 2008) and visualized by immunofluorescence prior to the induction of major observable CPE. As expected, the majority of cells were positive for virus antigen (Figure 9B) and the differences in CPE are clearly shown: note the syncytial membrane fusion in infected Fcwf-4 CU cells and the maintenance of distinct cell membranes in infected AK-D cells (Figure 9B). Together, these results demonstrate the ability of the Fcwf-4 CU cells to rapidly produce high levels of type I FIPV Black in cell-free supernatants.
A.

**Fcwf-4 CU**

<table>
<thead>
<tr>
<th>Mock</th>
<th>16 hpi</th>
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<th>24 hpi</th>
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**AK-D**

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<th>36 hpi</th>
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<th>72 hpi</th>
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B.

**Fcwf-4 CU**

<table>
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**AK-D**

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<th>Mock</th>
<th>+ FIPV/24 hpi</th>
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**Figure 9: FIPV Black-induced Cell Cytopathic Effect Varies Between Infected Cell Types.** A) Progression of cell cytopathic effects (CPE) induced by FIPV Black over time in Fcwf-4 and AK-D cells (MOI=0.1). Localized cell syncytia formation (black, closed arrows) and discrete cell death (white, open arrows) indicated on brightfield images taken at 20x magnification. B) Immunofluorescence staining of FIPV Black-infected Fcwf-4 CU and AK-D cells (MOI=0.1) at indicated hours post-infection (hpi). Nucleocapsid protein staining (Red; CCV2-2) indicates infected cells; nuclei stained in blue (Hoesch3342). Images taken at 60x magnification.

AK-D and Fcwf-4 CU cells facilitate rapid and consistent plaque assays for FIPV Black. After observing the rapid and uniform development of CPE and release of virus into cell supernatants during infection of AK-D and Fcwf-4 CU cells, we reasoned
that these cells would be employable in a standardized plaque assay to consistently
determine FIPV Black titer. To this end, we calculated the endpoint titer and compared
the size, uniformity, and timing of virus plaque development over time in AK-D, Fcwf-4
ATCC, and Fcwf-4 CU cells following infection with 10-fold dilutions of the same FIPV
Black virus stock initially grown on Fcwf-4 CU cells. A detailed description of the plaque
assay is provided in the Materials and Methods; we note here that Oxoid agar is critical
for visualizing clear plaques. At 2 days post-infection (dpi), FIPV Black formed
enumerable plaques on both AK-D and Fcwf-4 CU cells with the latter cells producing
more numerous and larger plaques at higher dilutions (**Figure 10 top**). Plaques were
not observed in Fcwf-4 ATCC cells at 2 dpi (**Figure 10 top**). Plaques were detected at 3
dpi (**Figure 10 middle**); and were clearer at 4 dpi in Fcwf-4 ATCC cells (**Figure 10
bottom**). Calculated titers overall were higher in Fcwf-4 CU (>10^7 pfu/mL) than in AK-D
cells (≥10^6 pfu/mL); however, we report that both cell types are useful for determining
virus titer, whereas the Fcwf-4 ATCC cells are not ideal for use in this assay.
Figure 10: Evaluating FIPV Black Plaque Development and Titer Over Time in Different Feline Cell Lines. Tenfold serial dilutions ($10^{-3}$-$10^{-6}$) of virus inoculum, derived from 24-hour cell-free supernatant of Fcwf-4 CU cells infected with FIPV Black at a MOI=0.1, were applied to AK-D, Fcwf-4 ATCC, and Fcwf-4 CU indicator cells. Following Oxoid agar/media overlay, plates were incubated for 2 (top), 3 (middle), or 4 (bottom) days before cells were fixed with 3.7% formaldehyde and stained with 0.1% crystal violet. Titers were calculated by plaque counts and are presented as plaque-forming units (pfu)/mL. ND indicates that titers were not determined.
**Fcwf-4 CU cells are distinct in morphology and IFN-responsiveness.** The apparent differences in production and kinetics of FIPV Black virus in two Fcwf-4 cell lines led us to ask if there are morphologic or functional differences between the two cell types. To answer this question, we first compared the single-cell morphologies of Fcwf-4 ATCC, Fcwf-4 CU cells, and primary feline bone marrow-derived macrophages (fBMDMs) by Wright-Giemsa staining. The typical morphologic characteristics of feline macrophages (large cytoplasmic inclusions, a non-dominant nucleus, a non-ruffled cell membrane) (Bienzle et al., 2003) were observed for the fBMDMs (Figure 11A). Comparison of the two Fcwf-4 cell lines revealed stark differences in morphology. Fcwf-4 ATCC cells were large with a smooth cell membrane and had large and abundant cytoplasmic inclusions comparable to the fBMDMs (Figure 11A). Fcwf-4 CU cells were more similar in size to fBMDMs; however, the Fcwf-4 CU cell line exhibited fewer cytoplasmic inclusions and more cell membrane ruffling (Figure 11A) than either the fBMDMs or Fcwf-4 ATCC cells. Neither Fcwf-4 cell line had a “true” macrophage morphology further corroborating their original “macrophage-like” description (Jacobse-Geels and Horzinek, 1983). As macrophages are innate immune cells that restrict virus replication through production of interferon-stimulated genes (ISGs) in response to type I IFN, we reasoned that differences in virus replication may be due to variation in cell IFN-responsiveness. Therefore, we asked if Fcwf-4 ATCC and CU cell lines differed in responsiveness to exogenous type I IFN by measuring the resulting ISG54 transcript production following treatment with IFN. Remarkably, Fcwf-4 ATCC cells produced significantly higher ISG54 transcripts in response to IFN stimulation compared to Fcwf-4 CU cells (Figure 11B), suggesting that the Fcwf-4 CU cells are much less responsive to
IFN. This is not to say, however, that Fcwf-4 CU cells are insensitive to IFN since they also exhibit significant, dose-dependent ISG54 transcript production. Together, these data highlight the distinct morphology of the Fcwf-4 CU cells and suggest that enhanced virus replication in these cells may be due, at least in part, to reduced IFN-responsiveness.
Figure 11: Fcwf-4 CU Cells Differ in Morphology and IFN-responsiveness From Fcwf-4 ATCC. A) Single-cell morphology of feline bone marrow-derived macrophages (fBMDMs), Fcwf-4 CU, and Fcwf-4 ATCC cells determined by Wright-Giemsa stain following cytospin preparation. Images were taken at 100x. B) Interferon-stimulated gene (ISG) 54 transcript levels from Fcwf-4 ATCC (grey) and Fcwf-4 CU (black) cell lines stimulated with increasing concentrations of feline type I interferon (IFNα). After 6 hours, total RNA was extracted and analyzed by qPCR for ISG54 and feline beta-actin. ISG54 mRNA expression was normalized to β-actin via the delta Ct method $(2^{-\Delta Ct} [\Delta Ct=Ct(\text{gene of interest})-Ct(\beta-\text{actin})])$, then presented as relative expression over corresponding mock (basal) expression. Data are representative of three independent experiments performed in triplicate and presented as means ±SD. Values were analyzed by unpaired t-test. ** P < 0.01; *** P < 0.001; **** P < 0.0001.
Fcwf-4 CU cells replicate both biotypes of type II FCoV. Due to the high titer and rapid kinetics of type I FIPV Black replication in the Fcwf-4 CU cells, we next addressed whether these cells are permissive to type II FCoV infection. Fcwf-4 CU cells were infected with type I FIPV Black or one of two type II viruses, FIPV 79-1146 or FECV 79-1683. Cell cytopathic effects were observed over time in these cells, with the viruses all forming similar, large syncytia (Figure 12A). Cell-free virus titers were determined at 12 and 24 hpi. All three viruses accumulated to titers >10^6 pfu/mL in supernatants by 24 hpi (Figure 12B), with FIPV 79-1146 reaching the highest titer. The kinetics of the type II viruses were faster than the type I FIPV Black strain, producing higher titers by 12 hpi (Figure 12B) and inducing more substantial syncytial CPE by 16 hpi (Figure 12A). Next, we asked if the Fcwf-4 CU cells could be utilized in a plaque assay for determining the titer of type II FCoV. Indeed, both FIPV 79-1146 and FECV 79-1683 formed clear, uniform, enumerable plaques at 24 hpi (Figure 12C). Thus, we have demonstrated that the Fcwf-4 CU cells replicate both FCoV types and biotypes to high titers and are employable in a plaque assay to consistently determine the titers of all viruses assayed.
Figure 12: Fcwf-4 CU Cells and are Permissive to Both Biotypes of Type II FCoV. A) Cell cytopathic effect at 12- and 24-hours post-infection (hpi) of Fcwf-4 CU cells infected with indicated FCoV strains (MOI=0.1). B) FCoV growth kinetics in Fcwf-4 CU cells (MOI=0.1) determined by C) plaque assay on Fcwf-4 CU indicator cells. Tenfold serial dilutions of cell-free virus inoculum were applied to cells. Following Oxoid agar/media overlay, cells were fixed in 3.7% formaldehyde and stained using 0.1% crystal violet after 24 hours. FCoV used were the type I strain FIPV Black and the type II strains FIPV WSU 79-1146 and FECV WSU 79-1683. Plaque assay performed in triplicate; error bars ±SD.
Section 2: Generation of Feline Cell Lines to Study Attenuated or Clinically-important FCoV Strains

Rationale.

Immunization against serotype I FIPV is likely to be the most effective vaccination method to protect against FIP (Pedersen, 2009). To date, implementing an effective serotype I FCoV vaccine has been unsuccessful and new strategies and approaches are needed in order to achieve wide-spread protection. Targeted elimination of coronavirus type I IFN antagonists resulting in a hyper interferon-inducing (HII) phenotype has been a successful method to generate live-attenuated coronavirus strains and successful vaccines (Deng et al., 2019b, 2017; Kindler et al., 2017). In these studies, two conducted by our group, permissive cells deficient in type I IFN signaling were a key component allowing for recovery and characterization of IFN antagonist-deficient viruses. The lack of the type I IFN receptor (IFNαR) allows recovery, propagation and accurate determination of virus titer of mutant viruses produced by reverse genetics without negative selective pressure or induction of suppressive mutations in the viral genome. I hypothesized that feline cell lines that are i) highly permissive to serotype I FCoV and ii) deficient in IFNαR signaling would allow for the development and investigation of IFN antagonist-deficient FIPV viruses and could be employed in future studies to recover vaccine candidate strains using reverse genetics. A feline IFNαR-deficient cell type, however, is not currently available. In this section of my dissertation, I proposed to eliminate IFN responsiveness in two serotype I FCoV-permissive cell lines: feline airway epithelial (AK-D) cells and Fcwf-4 CU cells by employing the Crispr/Cas gene-editing system to disrupt domain 2 of the type I IFN
receptor (IFNαR2). First, I demonstrate that a polyclonal population of Crispr/Cas-transduced AK-D cells (termed AK-D 2.2) display a significantly reduced response to exogenous IFNα stimulation. Infection of AK-D 2.2 cells with serotype I FIPV induces rapid development of cytopathic effect, while maintaining production of infectious virus. Further, I demonstrate the utility of the AK-D 2.2 cells in determining the titer of serotype I FIPV by plaque assay. Second, using parental Fcwf-4 CU cells, which we demonstrated grow high titers of FIPV Black and had reduced IFN-responsiveness (above) (O’Brien et al., 2018), I again used Crispr/Cas9 gene editing to disrupt IFNαR2 and expanded clones from the resulting total population of transductants. One isolate, termed IFNαR2 null (IRN), was found to have significantly reduced IFN-responsiveness. Comparison of the amino acid sequences of the parental (Fcwf-4 CU) and clone (IRN) interferon alpha receptor indicates that the reduction in IFN-responsiveness observed in the clone is likely due to early termination of the protein. Growth kinetics of serotype I FIPV Black was similar in both cell lines.

As the overall goal of this dissertation is to develop tools to investigate clinically-important serotype I FCoV strains, I proposed to further modify the Fcwf-4 IRN cells to express host proteases to enhance replication efficiency of type I FCoVs. The presence and density of specific host cell proteases are important determinants of coronavirus entry (Millet and Whittaker, 2015) and several groups have reported that ectopic expression of proteases, such as the transmembrane serine protease 2 (TMPRSS2), can enhance coronavirus entry and replication (Glowacka et al., 2011; Park et al., 2016; Regan et al., 2008). Serotype I FCoV spikes contain two protease cleavage sites, which can be cleaved to induce membrane fusion. The S1/S2 site contains a multibasic
cleavage sequence (RRXRR), which is processed by cell-surface proteases including furin and furin-like proteases such as TMPRSS2. Cleavage at the S1/S2 site mediates viral entry at the cell surface. The downstream S2' site is cleaved within host endosomes by cathepsin-L, allowing virus entry at this later stage. The serotype I spike protein encoded by the TC-adapted TN406 (Black) strain used in our experiments contains a multibasic S1/S2 cleavage (RSRR) site (Whittaker et al., 2018) and therefore we predicted that expression of TMPRSS2 would enhance FIPV Black replication. Indeed, using the parental Fcwf-4 IRN cells, which are already highly permissive to FIPV Black and deficient in type I IFN signaling, I cloned and expressed feline TMPRSS2 and demonstrate increased production of virus over time.

Together, the data presented in this section describe the generation of two feline cell lines that are deficient in IFN-signaling and one that expresses TMPRSS2, both characteristics that will be useful in recovery of live-attenuate type I FIPV strains and clinical isolates from infected cats.

Results.

Evaluating the interferon response in AK-D and IFNαR2-deficient AK-D 2.2 cells. Wild type feline airway epithelial (AK-D) cells were used as the first parental cell type to knock down the feline type I interferon receptor (IFNαR2). AK-D cells were chosen because they are permissive to serotype I feline coronavirus and have a defined genotype. To knock down the IFNαR2, wild type AK-D cells were transduced with pLentiCRISPRv2 encoding a specific guide RNA targeting the second exon of the feline ifnar2 gene (2.2). Positive selection was performed using puromycin and cells were cultivated as a heterogeneous population termed AK-D 2.2. As there is no available
IFNαR-specific antibody to detect the presence of feline IFNαR2 protein, we used a functional assay to evaluate the AK-D 2.2 cells for the IFNαR2-deficient phenotype. Increasing concentrations of purified feline IFNα were exogenously applied to AK-D or AK-D 2.2 cells and RNA levels of IFN stimulated gene (ISG) 54 were determined by quantitative PCR (qPCR). ISG54 expression is prominently upregulated following activation of the type I IFN receptor (Fensterl and Sen, 2011) and is used here to report on the functionality of the IFNαR. As expected, wild type AK-D cells responded to exogenous IFNα with a dose-dependent increase in ISG54 expression. The AK-D 2.2 cell population, however, produced significantly fewer ISG54 transcripts than wild type (Figure 13), indicating a disruption of the IFN-I response-pathway. The subtle but concentration-dependent ISG54 transcription observed from AK-D 2.2 cells may be attributed to the presence of an additional receptor, or incomplete silencing in some cells in the population. Nonetheless, these results indicate that the AK-D 2.2 cells have a diminished total response to IFNα, which is likely due to disruption of the IFNαR2.
Evaluating growth kinetics of serotype I FIPV Black in AK-D 2.2 cells. To determine if knocking down the IFNαR2 had any effect on replication of FIPV Black we evaluated the growth kinetics of FIPV Black in AK-D and AK-D 2.2 cells. We infected cells with FIPV Black at a MOI = 0.1 and titers were evaluated over time by plaque assays. FIPV Black achieved a maximum titer >10^6 plaque-forming units (PFU)/mL in all cell types tested. The kinetics of FIPV Black were similar in AK-D and AK-D 2.2 cells (Figure 14A), with both cell types producing peak titers at 42 hours post-infection (hpi), suggesting that the IFNαR2 does not significantly impact virus growth in this cell type. This is likely due to the multiple IFN antagonists expressed by FIPV, which permit
replication in AK-D cells independent of an IFN response (Dedeurwaerder et al., 2014, 2013).

While evaluating the virus kinetics, we also observed that high virus titers were obtained from the cell-free supernatants. This was intriguing as it has previously been reported that FIPV is highly cell-associated and that multiple freeze-thaw cycles are required to release infectious virus (Jacobse-Geels and Horzinek, 1983). Therefore, we asked if FIPV Black virus titer was comparable in supernatant (cell-free) and freeze-thawed cell monolayers (cell-associated) in AK-D or AK-D 2.2 cells. We report that, between 30-42 hpi when maximum virus titers are achieved, FIPV Black titers are higher in cell-free supernatants than in the cell-associated fractions (Figure 14B) for both cell types. These results demonstrate that high virus titers can be derived from the supernatants alone and that further processing of cell monolayers by freeze-thaw cycles may even be detrimental to virus titer.
Determined the utility of AK-D 2.2 cells in visualizing FIPV Black plaques.

Next, we asked if AK-D 2.2 cells can be used as an indicator cell type to accurately determine FIPV-Black titers by plaque assay. An ideal indicator cell type allows a virus
to form well-defined and easily enumerable plaques; therefore, we sought to determine if plaque size is affected by the absence of IFNαR2 in AK-D 2.2 cells. We infected AK-D or AK-D 2.2 cells with serotype I FIPV-Black at a MOI = 0.1 and evaluated how the reduced IFNα response in AK-D 2.2 cells affected virus-induced cytopathic effect (CPE). Over 48 h, we observed increased CPE in AK-D 2.2 cells compared to wild type AK-D cells (Figure 15A), suggesting an increase in virus spread that we speculate is due to the reduction in induced ISGs in AK-D 2.2 cells. A plaque assay utilizing AK-D 2.2 cells resulted in large and enumerable plaques that allowed for more accurate determination of FIPV Black titer compared to wild type AK-D cells (Figure 15B). In AK-D 2.2 cells, plaques were more easily identified at 72 hpi, and plaque sizes were comparable to those on Fcwf-4 CU cells at 48 hpi. It was difficult to accurately determine virus titer using wild type AK-D cells, as the plaques were too small to count accurately at both time points and led to an underestimation of PFU/mL. Our results highlight the utility of the AK-D 2.2 cells in determining the titer of serotype I FIPV and indicate that this cell line will be critical in future studies investigating IFN antagonist-mutant strains of FIPV.
Figure 15: AK-D 2.2 Cells Can Be Used to Determine Serotype I FIPV Titer. A) Representative images of AK-D (top row) and AK-D 2.2 (bottom row) of uninfected (Mock) cells or cells infected with FIPV Black (MOI = 0.1) taken at indicated hours post-infection (HPI). Mock images were taken at 24 h post-plating. B) Fcwf-4 CU cells were infected with MOI = 0.1 serotype I FIPV Black and supernatants were collected at 24 HPI after which plaque assay analysis was performed on AK-D, AK-D 2.2, or Fcwf-4 CU indicator cells. Images were taken at 48 (top) and 72 (bottom) HPI. (A-B) Images are representative of three independent experiments.
Evaluating the IFN-responsiveness and FIPV Black replication kinetics in a clone of Fcwf-4 2.2 Poly cells. In addition to disrupting IFN signaling in AK-D cells, I also targeted the IFNαR2 in the feline macrophage cell line, Fcwf-4 CU. These cells are a more physiologically relevant cell type in which to study FIPV and grow Black to high, cell-free titers (O’Brien et al., 2018). To knock down the IFNαR2, wild type Fcwf-4 CU cells were transduced with pLentiCRISPRv2 targeting the second exon of the feline ifnar2 gene (2.2), the same sequence targeted in AK-D cells. Positive transductants were selected using puromycin and cells were cultivated as a heterogeneous population termed Fcwf-4 2.2 Poly (polyclonal). To evaluate the IFN-responsiveness of the 2.2 Poly cells, cultures were treated with 1000 U of feline type I IFN for 6 hours and evaluated for expression of ISG54. Compared to the parental Fcwf-4 CU cells and a population transduced with an empty pLevtiCRISPRv2 (EV), the Fcwf-4 2.2 Poly cells had reduced ISG54 production suggesting positive disruption of the ifnar2 gene (Figure 16A). Further, FIPV Black replication kinetics were similar in parental and 2.2 Poly cells (Figure 16B). As this polyclonal population of cells had reduced IFN-responsiveness and no negative impact on virus replication, I decided to isolate and evaluate several clonal cell lines to decrease genetic variability. A total population of Fcwf-4 CU 2.2 Poly cells were sorted into a 96-well plate at 1-2 cells per well then grown over 30 days. Three clones, CL1-3, were evaluated for IFN-responsiveness and, as before, by first stimulating with 1000 U of IFN then evaluating the ISG54 response (Figure 16C). Clone 3 had the most subtle ISG54 response, which was further demonstrated using increasing concentrations of IFN stimulation (Figure 16D). The Fcwf-4 CU 2.2 Clone 3 cells were termed “IRN” for IFNα Receptor Null. FIPV Black replication kinetics were
similar in the parental Fcwf-4 CU and Fcwf-4 IRN clonal cell line as determined by plaque assay measuring infectious particles (Figure 16E) and by qPCR measuring production of N gene transcript (Figure 16F). Cell cytopathic effects induced by the virus were also similar between the Fcwf-4 CU and IRN cell lines, although CPE was more robust late in infection, possibly due to slightly higher virus titers observed at 30 hpi (Figure 16G). Together, these results describe the successful generation and isolation of an IFNα response-deficient Fcwf-4 CU cell line, which will be highly useful in the recovery and study of IFN-antagonist mutant FCoVs in future studies.
Figure 16: Fcwf-4 IRN Cells Have Reduced IFN-responsiveness and Replicate FIPV Black to High Titer. A, C, D) Fcwf-4 CU (CU), empty vector-transduced (EV), Fcwf-4 CU 2.2 Poly, Clones CL1 and CL2 or Fcwf-4 CU IRN cells treated with 0, 100 or 1000 U feline IFNα for 6 h and ISG54 and β-actin detected by qPCR. B, E) FIPV Black growth kinetics (MOI=0.1) in given cell type over time. >2 replicate plaque assays done at each time using Fcwf-4 CU indicator cells. Values reported as mean plaque forming units (PFU) / mL. F-G) N gene expression by qPCR (F) and CPE (outlined) (G) taken over time during FIPV Black infection (MOI = 0.1) of Fcwf-4 CU or IRN cells. mRNA expression normalized to β-actin and presented as relative expression over mock. Data represent >2 independent experiments in triplicate. Mean ± SD analyzed by unpaired t-tests. * P < 0.05; ** P < 0.01; *** P < 0.001; non-significant (ns).
Determining how IFN signaling is disrupted in Fcwf-4 IRN cells. The Crispr/Cas gene editing system targets and cleaves a specific sequence leading to non-homologous repair and in/del mutations that can lead to disruption in gene transcription, point mutations or early termination of the protein. To determine how the IFNαR gene was disrupted in the Fcwf-4 IRN cell line, I first isolated total RNA from these cells and compared the expression of IFNαR2 by qPCR. In these experiments, the expression of IFNαR2 was actually higher than the parental cells (Figure 17A) indicating that the disruption did not occur at the level of transcription. I also compared the melting temperatures of the amplicons produced during qPCR (Krenke et al., 2005) and found that they differed, on average, by 1°C (Figure 17B) suggesting a possible difference in the nucleotide composition. To determine what the difference in sequence was, I cloned and sequenced the IFNαR2 mRNAs produced in Fcwf-4 CU and IRN cells (Appendix D). From the sequencing, I found that the IRN IFNαR mRNA encoded a 14-nucleotide deletion at the intended Cas9 cleavage site (Figure 17C), which translated to a frame-shift and an early termination at residue 27 (Figure 17C) resulting in a shortened, non-functional IFNαR.
Figure 17: Fcwf-4 IRN Cells Encode a Shortened, Null Mutant IFNαR Protein. A) Feline IFNαR2 exon 1-4 expression determined from total RNAs collected from Fcwf-4 CU and Fcwf-4 CU IRN confluent monolayers. mRNA expression normalized to β-actin (2^-ΔCt [ΔCt=C(gene of interest)-C(β-actin)]) and presented as such. Data represent two independent experiments in triplicate. Mean ± SD analyzed by unpaired t-tests. ** P < 0.01. B) Melt curves and maximum melt temperatures of IFNαR2 exon 1-4 amplicons produced during qPCR of RNAs obtained from Fcwf-4 CU or Fcwf-4 CU IRN cells. Melt temps determined by plotting change in fluorescence with temperature [-d(RFU)/dT] versus temperature (°C) then calculating the point at which the largest change in fluorescence occurs (Krenke et al., 2005). C) Amino acid sequence alignment of the IFNαR2 region targeted by Crispr/Cas9 in Fcwf-4 CU and Fcwf-4 CU IRN cells. Disruption of the IFNαR2 in the Fcwf-4 CU IRN cells due to a 14 nt deletion resulting in a nonsense mutation, a shortened protein and a null phenotype. Nucleotide site targeted by Cas9 cleavage indicated by arrow; asterisk indicates termination codon; blue highlights indicate positional differences between the amino acid sequences.
Expression of feline TMPRSS2 improves FIPV Black replication. Host-cell proteases are critical to coronavirus entry and replication. As the goal of this dissertation is to develop a number of tools to aid in investigating feline coronavirus, I determined if of a host protease, TMPRSS2, may enhance FCoV replication. The feline TMPRSS2 protein carrying a C-terminal V5 tag was cloned into a pLVX lentivirus backbone vector and termed pLVX-fTMPRSS2(V5). To demonstrate subcellular localization and to visualize expression, HEK 293T cells were transfected with the pLVX-fTMPRSS2(V5). The V5 antibody was detected by immunofluorescence within the cell cytoplasm and at the cell surface (Figure 18A) and both the full-length (55 kDa) and the cleavage product (>25 kDa), indicating an active protein, were detected from these cells by western blot (Figure 18B, left). Fcwf-4 CU IRN cells that were transduced with pLVX-fTMPRSS2(V5) lentivirus also had detectable levels of the full-length and active forms of feline TMPRSS2 (Figure 18B, right) demonstrating that expression of an active feline protease was achievable in this cell line. Therefore, I next sought to determine if TMPRSS2 had an impact on replication of FCoV. Fcwf-4 CU IRN cells were transduced with increasing dilutions of pLVX-fTMPRSS2(V5) lentivirus for 48 hours then infected with FIPV Black at 0.1 multiplicity of infection (MOI) for 18 hours. Expression of TMPRSS2 at dilutions below 1:20 (Figure 18C) resulted in significant increases in FIPV N gene transcript (Figure 18D) compared to cells that did not express TMPRSS2, suggesting that addition of a feline protease can enhance virus replication. Interestingly, N gene expression was reduced at lower dilutions of TMPRSS2 (1:2.5) suggesting that expression of these proteases needs to be carefully titrated and too much protein can result in reduced titers. It should be noted that uninfected Fcwf-4 CU IRN cells that were
transduced with the 1:2.5 dilution of lentivirus had significant morphologic differences and were more detached from the plate compared to cells transduced with higher dilutions (data not shown) indicating that the lower N gene expression observed at higher expression of TMPRSS2 likely impacts the ability of the virus to enter cells.

Together, these data describe the successful generation of two IFNαR-null cell lines by Crispr/Cas, the isolation of an Fcwf-4 CU IFNαR-null clone and, further, demonstrate that expression of a feline protease in these cells can enhance replication of serotype I FIPV.
Figure 18: Feline TMPRSS2 Improves FIPV Black Replication in Fcwf-4 IRN Cells.

A) Immunofluorescence detection of feline TMPRSS2 (V5) in HEK 293T cells. 200 ng of pLVX-fTMPRSS2(V5) or pLVX (mock) was transfected into HEK 293T/17 cells for 18 h. Cells were fixed with 3.7% PFA, permeabilized with 0.1% Triton X-100, blocked in 5% NGS / 0.3% Triton X-100 and stained with mouse-anti-V5 (1:500). 1:1000 goat-anti-mouse AlexaFlor568 (TRITC) was used to visualize TMPRSS2(V5); Hoesch 3342 (1:1000) was used to stain nuclei.

B) Detection of feline TMPRSS2(V5) by western blot following transfection of HEK 293T cells (left) or transduction of Fcwf-4 CU IRN cells with pLVX lentiviruses encoding TMPRSS2(V5) (right). The full length (< 55 kDa) and cleavage product (> 25 kDa) of TMPRSS2 are indicated. Feline β-actin used to visualize protein loading.

C-D) The impact of feline TMPRSS2 expression on FIPV replication evaluated in Fcwf-4 CU IRN cells. Indicated dilutions of pLVX-fTMPRSS2(V5) or Mock (empty) transducing particles were applied to Fcwf-4 CU IRN cells for 48 hours prior to infection with FIPV Black (MOI = 0.1). RNA was isolated after 18 h infection and qPCRs were performed to detect TMPRSS2 (C), N gene (D), and β-actin transcripts. mRNA expression normalized to β-actin \(2^{-ΔCt} [ΔCt=Ct(gene \ of \ interest)-Ct(β-actin)]\) and presented as such. Data represent two independent experiments in triplicate. Mean ± SD analyzed by unpaired t-tests. ** P < 0.01; *** P < 0.001; non-significant (ns).
Section 3: Progress Toward Developing an Infectious Clone Reverse Genetics System of Serotype I FIPV Black

Rationale.

One consideration for serotype I FIPV vaccine design is the strength of the innate immune response to immunization. A strong innate immune response, characterized by high levels of type I interferon (IFN), typically results in robust adaptive responses and development of immune memory (Hoebe et al., 2004; Siegrist, 2013). Coronaviruses including FCoV, however, prevent or subvert innate signaling using a number of virus-encoded type I IFN antagonists (Dedeurwaerder et al., 2014; Deng et al., 2017; Rose and Weiss, 2009; Totura and Baric, 2012) that limit development of an appropriate inflammatory response (Channappanavar et al., 2016). In the case of FIPV infection, a large, unregulated inflammatory response is mounted, which ultimately leads to the immune-mediated pathology observed in FIP. The adaptive responses, including cytotoxic T cell and humoral B cell stimulation/recruitment are generally weak and do not typically result in long-lasting immune memory (Tang et al., 2011). As a further complication, antibodies that are produced against the spike and envelope proteins of FIPV mediate antibody-dependent enhancement of disease (Corapi et al., 1992; Olsen et al., 1992). Although it is not known how ADE affects circulating FCoV, it remains a significant concern for vaccine development (Huisman et al., 2009).

Strong cytotoxic T cell responses during FIPV infection are associated with positive disease outcomes (de Groot-Mijnes et al., 2005; Pedersen, 2014a; Satoh et al., 2011) as are increased levels of IL-12, IL-21, IL-10 and type II IFN gamma (Dedeurwaerder et al., 2013; Kipar et al., 2006; Kipar and Meli, 2014). One group even
argued that a lack of IL-12, which functions to promote T cell differentiation and activation, may be the major determinant for progression to FIP (Kipar et al., 2006).

Importantly, type I IFN signaling has been shown to enhance cytotoxic T cell responses through promotion of IL-12, IL-21, MHC-I, and IFN-γ [reviewed in (Meager, 2006; Tough, 2012)] as well as promote memory T cell development [reviewed in (Huber and Farrar, 2011)]. Thus, enhancing the type I IFN response early during FIPV infection may lead to development of long-lasting T cell responses, which may effectively clear subsequent infections.

Several groups have shown that the elimination of coronavirus IFN antagonists, therefore stimulating production of type I IFN, has been a successful method of generating attenuated coronavirus strains (Deng et al., 2019b, 2017; Haijema et al., 2004; Kindler et al., 2017). Our group and others have demonstrated that functional elimination of the conserved endoribonuclease (EndoU) in mouse hepatitis virus (MHV) leads to the stimulation of multiple IFN-I signaling pathways, rapid cellular apoptosis in macrophages, and complete protection of mice against lethal challenge. Therefore, an EndoU FIPV mutant virus that stimulates high type I IFN and causes apoptosis in macrophages may be considered a vaccine candidate. The FIPV group-specific regions ORF7 and ORF3 have also been implicated in innate immune antagonism, and are thought to act on interferon stimulated genes (ISGs) rather than type I IFN signaling directly (Dedeurwaerder et al., 2014, 2013). Removal of these group-specific regions or individual proteins from serotype II FIPV leads to reduced virus replication in macrophage-like cells. Haijema and colleagues demonstrated that individual deletion of ORF3, or to a lesser extent ORF7, was attenuating to a serotype II FIPV and led to
protection against a second challenge without ADE (Haijema et al., 2004). These studies suggest that elimination of one set of the group-specific genes can be used to attenuate the more dominant serotype I FIPV. Thus, deletion of a set of group-specific genes may enhance the attenuation phenotype of an EndoU-mutant FIPV, thereby producing strong innate and adaptive T cell responses.

To facilitate investigation of FCoV interferon antagonists for attenuation studies, I designed a reverse genetics system based on a sequenced laboratory strain of the model type I feline coronavirus FIPV Black. Similar to the MHV, SARS- and MERS-CoV systems (Scobey et al., 2013; Yount et al., 2003, 2002), our approach utilizes contiguous cDNA plasmids to produce full-length copies of the FIPV genomic RNA. The cDNA plasmids can be prepared and purified to high concentration while maintaining sequence fidelity and allow more straightforward mutagenesis. Together, this system facilitates genetic manipulation of targeted genes and allows for production of infectious virions with a defined genotype. The FIPV Black reverse genetics system described here complements previously-established systems for feline coronavirus (Tekes et al., 2008).

**Results.**

**Designing, constructing and evaluating a contiguous plasmid reverse genetics system of FIPV Black.** The reverse genetics system described here was developed based on the genomic sequence of an in-lab strain of FIPV Black generously provided by Fred Scott (Cornell University). To determine the genomic sequence of the FIPV Black laboratory strain, cell-free and cell-associated fractions obtained from infected AK-D cells were combined and sent to the Kansas State Veterinary Diagnostic
Laboratory for deep-sequencing. A consensus sequence of our in-lab FIPV Black was generated by aligning the deep-sequencing reads to a known FIPV Black genome (EU186072.1). The resulting genomic sequence of the in-lab FIPV Black genome was then used as a template off of which the reverse genetics system was design and from here will simply be referred to as FIPV Black. The design of the FIPV Black contiguous cDNA plasmid reverse genetics system, based on work from Yount and colleagues (Yount et al., 2002), is diagrammed in Figure 19.
Figure 19: FIPV Black Infectious Clone Reverse Genetics Schematic. (1) RNA genome of lab-seq FIPV Black sub-cloned on 7 cDNAs, each containing two unique terminal restriction sites. A T7 promoter was added to the 5’ end of A frag and a poly-A tail was added to the 3’ end of G frag; (2) Large-scale plasmid preps digested to release frags, which were gel isolated, column purified, and chloroform extracted. Molar equivalents of frags ligated using T4. (3) Ligation product and linear plasmid encoding the Black nucleocapsid (N) gene in vitro transcribed using T7 into full-length, capped mRNAs and (4) electroporated into Fcwf-4 CU. (5) After 24-72 h, CPE visualized. RNA isolated for detection of subgenomic (sg) RNA by RT-PCR, or virus recovered.
First, the consensus FIPV Black genome was divided into seven sections (A-G) \textit{in silico} (Table 8). For segments B-F, PCR fragments were amplified from total cDNA, generated using isolated FIPV Black gRNA as a template, using specific primer sets (Appendix B; Primer Index 3). These primers appended two terminal BsmBI sites on each segment that produce unique 4 nt overhangs upon digestion and only base-pair with the next contiguous fragment (Table 8). PCRs were performed using a high-fidelity Taq Polymerase (Roche), which appends a T/A residue on the terminal ends of each segment and allows insertion of each segment into pCR-XL-TOPO® using TA cloning. The A and G segments could not be PCR amplified (without mutation) due to the complexity of the 5’ and 3’ ends of the gRNA, which contain low GC%, multiple repeating nucleotide stretches and secondary structures. Instead, these segments were synthesized by BioBasic, Inc and inserted into pUC57 vectors. The sequence of the A segment was modified at the 5’ end to encode an in-frame T7 RNA transcription promoter sequence (5’- TAA TAC GAC TCA CTA TAG -3’) upstream of the genomic sequence, and a natural BsmBI site was eliminates at position 3,806. The G segment was modified to carry a 21-nt poly-A tail. All other segments were unmodified and large-scale DNA preparations were purified and sequenced to confirm sequence fidelity (Appendix B; Primer Index 4). Thus, each of these seven cDNA subclones encodes a specific cDNA segment of the FIPV Black gRNA. The sequence of the FIPV Black nucleocapsid protein was also amplified and cloned onto pCDNA-3.1 with a 5’ T7 promoter sequence.
Following subcloning, the seven cDNA segments were excised from the cloning vectors using restriction enzyme digest (Table 8) and purified by separation through a low-melt agarose gel, cutting out the appropriate cDNA fragment and cleaning on a DNA-binding column. In addition to BsmBI, cDNA clones B, C, E and F were also digested with SfiI, which cleaves the cloning vector into smaller fragments allowing better visualization of the intended cDNA fragments on an agarose gel. The plasmid encoding the N gene was linearized by digestion with DralI. The seven eluted segments of cDNA, now referred to as “A-G Fragment”, and the linearized N gene were further purified by chloroform extraction in order to concentrate the DNA and remove excess agarose gel. The size and purity of each cDNA fragment was confirmed by separation on an agarose gel (Figure 20A).

Next, to generate a full-length cDNA copy of the FIPV Black genome, molar equivalents of each fragment were combined and ligated overnight using T4 Ligase. In
order to confirm that fragments aligned in the proper order, PCRs were performed to amplify each junction using junction-specific primers (AB, A5/B1R; BC, B5/C1R; CD, C6/D1R; DE, D3/E1R; EF, E7/F1R; FG, F6/G1R) (Appendix B; Primer Index 4) using the full-length cDNA as template. The PCR products of each junction were successfully amplified (Figure 20B) and the size of full-length cDNA was confirmed (Figure 20C, second lane). Once confirmed, the ligation product was extracted using phenol-chloroform-isoamyl alcohol, precipitated using isopropanol and washed with ethanol.

In the third, fourth steps of the reverse genetics process (Figure 19), the purified ligation product and the linearized N gene DNAs were in vitro transcribed using T7 polymerase into 5’-capped mRNAs encoding the FIPV Black infectious clone (ic) or the N gene (Figure 20C). These mRNAs can be directly co-electroporated into Fcwf-4 cells to produce infectious virus and are detectable visually by CPE development and by amplifying subgenomic RNA by RT-PCR.
Figure 20: Experimental Evaluation of FIPV Reverse Genetics Steps. A) cDNA frags (A-G) electrophoretically separated on an agarose gel observed using ethidium bromide under UV. B) PCR amplification of full-length ic cDNA ligation junctions visualized on an agarose gel under UV. C) Agarose gel depicting the full-length cDNA ligation (LIGATION), and the in vitro transcribed N gene (NG TSC.) and the full-length infectious clone (IC FV TSC.) RNAs. D) Prototypic “syncytial” and “melted” cell cytopathic effects (CPE) observed after 24 h electroporation of Fcwf-4 CU cells with purified genomic (g) RNA from FIPV Black. E) Determining the optimal electroporation voltage needed to introduce gRNA and N gene transcript into Fcwf-4 CU cells. 600 uL Fcwf-4 CU cells at 1x10^7 cells/mL in Opti MEM pulsed three times at indicated voltage (V) and plated in 10 cm dishes. Brightfield images taken at 24-48 h at 40x magnification.
To determine the optimal electroporation conditions needed to introduce RNA into Fcwf-4 CU cells, I used purified gRNA isolated from lab-stocks of FIPV Black. Fcwf-4 CU cells were washed twice in PBS and diluted to 1x10^7 cells/mL in OptiMEM. In a cuvette, 600 uL of Fcwf-4 CU cells were added to 500 ng of FIPV Black gRNA and 250 ng of in vitro transcribed N gene RNA. Cells and RNA were pulsed three times at increasing voltages (450-1200 V), with constant 25 μF capacitance and ∞ resistance, then plated into 100 cm dishes for 24 and 48 hours. Cells electroporated at all voltages developed CPE by 48 hours-post electroporation (hpe) (**Figure 20E**), however, optimal conditions were achieved at 850 V as cells were healthy, and CPE was the most robust (**Figure 20E**). Two types of CPE were observed including the classic syncytial formation and a “melted cell” phenotype (**Figure 20E**) and were used as first indicators of successful generation of infectious FIPV Black.

The use of IFNα signaling-deficient cells is critical to recovery IFN-antagonist-deficient FIPV Black mutant viruses. As this is the primary future use of this reverse genetics system, it was important to determine if the IFNαR-deficient cell line, Fcwf-4 CU IRN cells, could also be used to successfully recover wild-type FIPV Black from electroporated gRNA. Indeed, substantial CPE was observed over 44 hpe in Fcwf-4 CU IRN cells electroporated with gRNA and N gene RNAs (**Figure 21B**). Interestingly, the CPE was more robust in the IRN cells compared to the CU cells (**Figure 21A**), corroborating previous results obtained during traditional infection (**Figure 16 E-G**).
Figure 21: FIPV gRNA Electroporation Produces Infectious Virus in Fcwf-4 CU and IRN Cells. A-B) FIPV Black gRNA (500 ng) was combined with N gene transcript (250 ng) and electroporated into $6 \times 10^6$ Fcwf-4 CU (A) or Fcwf-4 CU IRN (B) cells at 850 V, 25 μF C and $\infty$ R. Images taken at indicated hours post-electroporation (hpe). Arrows indicate CPE development. Mock cells electroporated without RNA.
Together, these data demonstrate that all steps of the reverse genetics process have been successfully optimized and describe the successful and reproducible generation of FIPV Black from electroporated gRNA in both Fcwf-4 CU and Fcwf-4 CU IRN cells as a positive control.

**Generation and detection of a wild-type infectious clone FIPV Black.** To determine if this reverse genetics system could produce infectious virus, a full-length, mRNA infectious clone (ic) of the FIPV Black lab strain was constructed by digestion and ligation of fragments A-G and subsequent *in vitro* transcription. The icFIPV mRNA and N gene transcripts were co-electroporated into Fcwf-4 CU cells using optimized electroporation conditions. Fcwf-4 CU cells electroporated with 500 ng of purified FIPV Black gRNA and 250 ng N gene transcript was used as a positive control; Fcwf-4 CU cells electroporated without RNA (mock) were used as a negative control. The development of syncytial CPE was observed at 24 hpe in cells electroporated with both the icFIPV and FIPV gRNA (**Figure 22A**) suggesting that virus was produced in both cases. These syncytia were not observed in the mock-electroporated cells indicating that this cell morphology was not a side-effect of electroporation and was a result of virus replication. The detection of subgenomic (sg) RNAs, which are only produced during active CoV replication, was used in order to confirm the presence of FIPV. Mock, icFIPV- and FIPV gRNA-electroporated Fcwf-4 CU cells with were lysed at 24 or 48 hpe (depending on CPE development in the positive control) and I used RT-PCR to determine if L-containing subgenomic N gene mRNA was present. Indeed, sgRNA was detectable in both the positive control and the icFIPV (331 bp) (**Figure 22B**) confirming successful generation of an ic of FIPV Black using our reverse genetics system.
Figure 22: FIPV Infectious Clone Produces Syncytial CPE and N gene Subgenomic RNA. A) Development of syncytial cell cytopathic effect (black arrows) at 24 hours post-electroporation (hpe) of Fcwf-4 CU cells with mock (0 ng RNA), genomic (g) RNA (500 ng + 250 ng N gene transcript) or infectious clone (ic) RNA (total transcription product with 350 ng N gene transcript), respectively. Representative brightfield images taken at 60 x magnification. B) Detection of nucleocapsid (N) gene subgenomic (sg) RNA by RT-PCR. Total RNA was isolated from Fcwf-4 CU cells electroporated with mock, gRNA, or icFIPV RNA, as in (A), at 24-48 hpe. cDNA was generated using random hexamer primers. RT-PCRs using primers specific to the leader sequence (forward) and the N gene (reverse) were run using the cDNAs from each cell type as templates. The products were separated on an agarose gel and visualized with ethidium bromide under UV. Positive amplicons present as a 331 bp band. Image representative of > 3 experiments.
CHAPTER FOUR
DISCUSSION
Summary and Implications

Overview.

A number of issues related to the growth and characterization of serotype I feline coronavirus have slowed investigation of these important animal pathogens and hindered the development of effective vaccines. The goal of the research presented in this dissertation was to begin to establish a “tool box” containing protocols, reagents, cell lines and genetic techniques to facilitate the study of clinically relevant and mutant serotype I feline coronaviruses. In the first section, colleagues and I identified and characterized the growth kinetics of FIPV Black in a new, physiologically-relevant feline cell line, termed Fcwf-4 CU, and found that these cells were capable of rapidly producing high titers of cell-free virus. In the next section, I successfully disrupted IFN signaling in these Fcwf-4 CU cells and a second cell line, AK-D, using Crispr/Cas demonstrating that advanced genetic manipulations could be successfully performed in either cell type. A clonal cell line, termed Fcwf-4 CU IRN was isolated and found to be both IFN insensitive and also permit growth of high titers of FIPV Black. Expression of feline TMPRSS2 in these cells also increased FIPV replication indicating that these cells may be useful for isolation of serotype I strains directly from animal tissues and further highlights the importance of having a genetically plastic cell line. Finally, using the sequence of a laboratory strain of FIPV Black, I generated an infectious clone reverse
Characterizing Replication Kinetics and Plaque Production of Serotype I FIPV in Three Cell Lines.

**Summary.** Investigating type I feline coronaviruses (FCoVs) in tissue culture is critical for understanding the basic virology, pathogenesis, and virus-host interactome of these important veterinary pathogens. This has been a perennial challenge as type I FCoV strains do not easily adapt to cell culture and viruses that do adapt, lose pathogenicity. We characterized the replication kinetics and plaque formation of a model type I strain FIPV Black (TN406) in Fcwf-4 cells established at Cornell University (Fcwf-4 CU). We determined that maximum virus titers (>10^7 pfu/mL) were recoverable from infected Fcwf-4 CU cell-free supernatant at 20 hours post-infection. Type I FIPV Black and both biotypes of type II FCoV formed uniform and enumerable plaques on Fcwf-4 CU cells. Therefore, these cells were employable in a standardized plaque assay. Finally, we determined that the Fcwf-4 CU cells were morphologically distinct from feline bone marrow-derived macrophages and were less sensitive to exogenous type I interferon than were Fcwf-4 cells purchased from ATCC.

**Implications.** Since the isolation of the FIPV Black strain in 1980 (Black, 1980) it has remained a predominant model of type I FIPV because it is cultivatable in commercially available Fcwf-4 cells. However, different groups have reported major
variations in the growth kinetics, maximum obtainable titer, and techniques for recovery of this virus from tissue culture (Jacobse-Geels and Horzinek, 1983; Pedersen et al., 1984; Tekes et al., 2012, 2008; Thiel et al., 2014). For example, maximal titers measured by determining the TCID$_{50}$ can range between $>10^3$ (Jacobse-Geels and Horzinek, 1983) and $10^4$ TCID$_{50}$/mL (Takano et al., 2015) at 24-48 hpi, or measured by plaque assay between $>10^4$ (36 hpi) (Tekes et al., 2012, 2008) and $>10^7$ pfu/mL (20 hpi) (O’Brien et al., 2018). This suggests either high variability in the Fcwf-4 cell lines used or co-adaptation between a particular virus and Fcwf-4 cell line used during laboratory cultivation. Indeed, we report significant differences in FIPV Black replication properties between an Fcwf-4 cell line that was newly purchased from the ATCC, and the Fcwf-4 cells that were established at Cornell University (CU). The enhanced rate of FIPV Black virus growth and increased maximum titer ($>10^7$ pfu/mL by 20 hpi) obtained from infected Fcwf-4 CU cells, however, may not be due to co-adaptation with our particular strain of FIPV Black, as these cells also replicated both biotypes of serotype II viruses. Instead, the Fcwf-4 CU cells may be highly susceptible to FCoV infection in general and therefore may be particularly useful in generating highly-permissive cell types to isolate and grow clinical type I FCoVs. The increased infectibility of Fcwf-4 CU cells could be due to any number of cellular factors including increased abundance of specific entry co-factors (e.g. DC-SIGN) or host proteases, which have been shown to enhance coronavirus entry (Millet and Whittaker, 2015; Regan et al., 2010). However, as infection by both serotype I and serotype II viruses were enhanced, it is tempting to speculate that the reduced IFN-responsiveness of the Fcwf-4 CU cells relative to the Fcwf-4 ATCC cells may also significantly enhance infection in the former. Further, these
Fcwf-4 CU cells may express a higher density of the yet unknown serotype I virus receptor (Cham et al., 2017; Dye et al., 2007) and therefore may be critical in identifying the receptor or other cellular characteristics that allow for enhanced type I virus replication.

FIPV Black infection of Fcwf-4 cells has also been reported to be highly cell-associated (Jacobse-Geels and Horzinek, 1983; Pedersen et al., 1984), requiring suspension and freeze-thaw cycling to release infectious virus. In contrast, we found significantly higher titers of virus in cell-free supernatants and speculate that multiple freeze-thaw cycles may actually decrease virus titer by damaging the virus envelope. The release of virus into cell supernatants and the uniform CPE observed in AK-D and Fcwf-4 CU cells were critical in establishing a standardized plaque assay using either cell type. Further, we report that the stage of CPE development can be used as an indicator of when maximal virus titers can be recovered. These findings are an important step in standardizing how the FCoV field measures and reports serotype I virus titer and may reduce the overall variability in the literature.

One possible explanation for the varied reports on FIPV Black replication kinetics and maximum titer could be that laboratory lines of Fcwf-4 cells have deviated from the original ATCC stock. The Fcwf-4 cells deposited with the ATCC in the 1980’s were characterized as “macrophage-like” (Jacobse-Geels and Horzinek, 1983), which hints that these original cells were not terminally differentiated and may further develop distinct characteristics under different growth conditions. Further, as per the ATCC, Fcwf-4 cells grow as a “dual population” of adherent and suspended cells that need to be pelleted during routine passaging (ATCC personal communication) – a technique...
that, if not followed, may significantly impact the characteristics of these cells in culture over time. Subtle changes in Fcwf-4 characteristics and morphology is likely what occurred at Cornell University to produce the Fcwf-4 CU cell line, given that Fcwf-4 cells were obtained from the ATCC and then passaged for many years. To our knowledge, O’Brien and Mettelman et al. (2018) is the first report describing the phenotypic differences between the original Fcwf-4 cells available from the ATCC and a distinct lineage that was derived from the original cells.

Finally, as these Fcwf-4 CU cells are “macrophage-like” they represent a more physiologically relevant cell type in which to propagate serotype I FIPV strains, not just tissue culture (TC)-adapted strains. One of the major issues in serotype I FCoV research is attempting to extrapolate information about clinical viruses from studies using TC-adapted strains, which harbor many genetic mutations. The majority of type I FIPV strains that grow well in tissue culture, including the Black strain used here, have lost in vivo pathogenicity due to TC adaptation posing the question of biologic relevance when investigating such strains. It is likely that forcing serotype I FCoVs to grow in sub-optimal conditions selects for adapted virus genotypes at the cost of virulence traits such as the sequence of S and the presence of accessory genes 3c and 7b. These specific gene regions have been shown to be altered or lost during TC adaptation and demonstrate that the genetic cost of TC adaptation is loss of virulence (Pedersen, 2014a; Thiel et al., 2014). The characterization of the Fcwf-4 CU cells is an important step away from forcing viruses to adapt to sub-optimal conditions as these cells are highly permissive to serotype I FIPV. Future studies will be needed to determine if the Fcwf-4 CU cells can replicate new clinical isolates of type I FIPV without forcing genetic
mutations and allow propagation and investigation of more clinically relevant viruses in lab without the use of live animals.

Many challenges are still associated with the growth of type I FCoV in tissue culture, including the lack of a known cell-entry receptor and no highly-permissive cell type that rapidly grows clinical samples of these viruses. However, our studies with the Fcwf-4 CU cell line demonstrate that rapid, high titers of type I FIPV Black can be recovered from cell-free supernatants and enumerated using a standardized plaque assay. It is our hope that the Fcwf-4 CU cells will alleviate some of the technical hardships associated with the growth of type I FCoV and expedite investigation of a wider range of type I FCoV strains. The Fcwf-4 CU cells, due to their distinct growth kinetics and enhanced replication of FIPV Black virus, will be deposited at the ATCC to facilitate their distribution to the research community.

**Generation of Feline Cell Lines to Study Attenuated or Clinically-important FCoV Strains.**

**Summary and implications.** The development of live-attenuated serotype I FCoV vaccine strains may be possible through mutation of encoded IFN antagonists. Generating and subsequent characterization of these so-called “hyper interferon-inducing” (HII) FCoVs requires cell lines that lack IFN signaling in order to avoid selective pressures and determine the mechanism(s) behind the virus attenuation. To my knowledge, such IFN-nonresponsive feline cell lines were not available and studies using gene-modifying tools such as Crispr/Cas had not been performed in feline cells permissive to FCoVs. In the previous section, my colleagues and I characterized the growth kinetics of FIPV Black in AK-D and Fcwf-4 CU cells and demonstrated that rapid
growth of wild-type serotype I FIPV could be achieved given the right cell type and conditions. However, these cells still produced IFN upon stimulation suggesting that neither cell line would not be appropriate for recovery of HII viruses by reverse genetics. Previous studies from our lab using mouse hepatitis virus (MHV), a model murine coronavirus, strongly imply that production of IFN antagonist-deficient CoVs by reverse genetics absolutely requires the use of an IFN-nonresponsive cell type (Deng et al., 2017). In these studies, we determined by deep-sequencing that mutant MHV strains produced in IFN-responsive cells acquired additional mutations that suppressed the original IFN antagonist mutation. This phenomenon likely occurred in response to selection imposed by the antiviral activity of type I interferon, which selected against the attenuated HII phenotype. Cells lacking the type I IFN response allowed for recovery of “clean” mutant viruses that contained only the desired mutation. Subsequent characterization of novel MHV IFN antagonists, as well as the relevant mechanisms underlying their activities, were undertaken using IFNαR−/− cells, highlighting the need of such cell lines in investigating attenuated feline coronavirus strains.

Building on this idea, the goal of this section of my research was to genetically modify AK-D and Fcwf-4 CU cells to generate feline cell lines that are i) highly permissive to serotype I FCoV and ii) deficient in IFNαR signaling. I predict that feline cells with these two characteristics will allow for future investigation of not only IFN antagonist-deficient HII viruses, but also isolation of clinically-important FIPV strains. To this end, I first disrupted the type I interferon alpha receptor domain 2 (IFNαR2) in two feline cell lines, AK-D and Fcwf-4 CU using the Crispr/Cas gene editing system, referring to the total population of positive transductants as AK-D 2.2 and Fcwf-4 2.2
Poly, respectively. Both populations of total positive transductants had diminished IFN-responsiveness and replicated FIPV Black to high titers. Although I was unable to isolate clone of the AK-D 2.2, I showed that the polyclonal population of these cells produced higher FIPV Black virus titers from cell-free compared to cell-associated fractions of infected cells and were employable in a standardized plaque assay, a technique that will be useful in future studies for determining the titers of HII mutant FCoV strains without the negative impact of IFN on virus growth. From the Fcwf-4 2.2 Poly cell line, I isolated a clonal cell line, termed Fcwf-4 CU IRN, which I determined to have significantly diminished IFN-responsiveness. Further, I sequenced the IFNaR from these cells and determined that the “null” phenotype was likely due to a 14-nucleotide deletion in the Crispr/Cas-targeted region of the IFNaR mRNA, which led to early termination in the second exon and a shortened protein product. Replication of FIPV Black in this cell line was comparable to kinetics of the same virus in Fcwf-4 CU cells and rapidly produced high titers of cell-free virus.

Interestingly, isolation of clones that both maintained the reduced IFN-responsiveness phenotype and remained permissive to FIPV Black was difficult in Fcwf-4 2.2 Poly- and ultimately unsuccessful in AK-D 2.2 cells. For both cell populations, the majority of isolated clones were non-permissive to FIPV Black infection regardless of the degree of IFN-responsiveness. This suggests that, within a given population of AK-D or Fcwf-4 cells, a portion of cells are non-permissive to FIPV, or can become non-permissive in different culture or selection conditions such as those used in the Crispr/Cas protocol. Further, this supports the speculation that continuous passage and inadvertent selection of Fcwf-4 cells in tissue culture may alter expression of host
proteases, entry co-factors, or virus receptor over time and that infectability of these cells can change.

FIPV Black replication in parental and IFNαR-null (IRN) cells was relatively equivalent and only slightly higher virus titers were observed in IFN-unresponsive cells. Initially, I expected that virus growth would be significantly enhanced in the AK-D 2.2 and Fcwf-4 CU IRN cells as these cells are not stimulated to express high levels of ISGs in response to IFN signaling. However, the observed growth kinetic results are consistent with the function of the IFN antagonists encoded on the FIPV genome, which allow replication of the virus in the wild-type cells without significant triggering of the IFN response. In future studies, it will be very intriguing to compare the replication kinetics of WT FIPV to HII mutants in Fcwf-4 CU and IRN cells. Here, I would expect to see significantly reduced titers of HII viruses and production of type I IFN in the wild-type CU cells, but equivalent titers and no IFN production in the IRN cells. This will be a highly critical set of experiments moving forward and requires the Fcwf-4 CU IRN to demonstrate that IFN is responsible for difference in virus replication.

TMPRSS2, a transmembrane serine protease, has been shown to mediate or enhance cell-surface entry of a number of coronaviruses including HCoV 229E (Bertram et al., 2013; Shirato et al., 2017), MERS-CoV (Park et al., 2016; Shirato et al., 2013) and SARS-CoV (Glowacka et al., 2011; Reinke et al., 2017). To further demonstrate the utility of the clonal Fcwf-4 CU IRN cell line in investigation of type I FCoVs, I transduced these cells with feline TMPRSS2, and observed a significant, though modest, increase FIPV Black replication as detected by an increase in N gene expression. This is an important observation as it demonstrates that Fcwf-4 CU IRN cells can be transduced to
express functional proteins, and that addition of host factors is a viable way of enhancing serotype I FIPV infection in these cells. It should be noted that the TC-adapted Black strain used here contains a polybasic TMPRSS2 S1/S2 cleavage site (RSRR). Interestingly, the N gene expression dropped when expression of TMPRSS2 was further increased and significantly altered the morphology of the cells in culture causing cell rounding and detachment from the surface, which may have prevented efficient access to these cells by the virus. The increased presence of active TMPRSS2 could also be affecting the virus entry/exit by over-processing of the spike protein. The pre-fusion form of the CoV spike must be processed at the appropriate spatiotemporal stage of infection in order to mediate efficient membrane fusion, and it is possible that excess TMPRSS2 may have pre-maturely cleaved S leading to reduced replication efficiency. Ultimately, a stable, inducible cell line is needed to temporally control TMPRSS2 expression. As Fcwf-4 CU cells have been shown to be amenable to genetic modification by Crispr/Cas, inserting a Tet-On inducible TMPRSS2 should not be too technically challenging.

Coronavirus S-mediated membrane fusion and subsequent entry into host cells has been shown to occur at both the cell surface and within endosomes depending on the availability of host proteases (Belouzard et al., 2012; Millet and Whittaker, 2015) (Figure 23, top). For feline coronaviruses, cleavage of spike at the S1/S2 site by transmembrane serine proteases (e.g. furin; TMPRSS2) typically mediates entry at the cell-surface, while cleavage at the S2’ site by cathepsins typically mediates entry within endosomes. The route of FCoV entry can differ between serotypes. Most all serotype II viruses do not contain an S1/S2 site and rely on the S2’ for mediating membrane fusion,
while most serotype I FCoV spikes contain both the S1/S2 and the S2' and can enter by either route (Belouzard et al., 2012; Whittaker et al., 2018). For FIPV Black, we consider that the presence of a trypsin-like protease (e.g. TMPRSS2) at the cell surface can functionally replace the usual endogenous protease to promote virus replication.

Importantly, entry of serotype I FCoV can be further impacted by tissue culture adaptation. de Haan and colleagues demonstrated that the spike protein encoded on a Fcwf-4 TC-adapted serotype I FCoV strain, FIPV-UCD1, had reduced cleavage at the S1/S2 site, but exhibited increased binding to heparan sulfate (de Haan et al., 2008). Interestingly, the serotype I FCoV clinical isolate, FECV-UCD, contains a full furin cleavage site at S1/S2 and did not bind heparan sulfate, resulting in no replication of this virus in Fcwf-4 cells. This suggests that tissue culture-adapted strains may forfeit the S1/S2 cleavage site in favor of binding to heparan sulfate, which may be a more abundant entry receptor on Fcwf-4 cells compared to the yet unknown natural receptor in these cells (Figure 23, left).
The concept that TC-adaptation can alter the preferred virus entry pathway may be common amongst lab-adapted CoV strains. A recent study by Shirato et al. showed that clinical isolates of HCoV 229E, much like serotype I FCoV clinical strains, favored TMPRSS2-mediated cell-surface entry while high passage, TC-adapted strains preferentially adopted cathepsin-mediated entry in endosomes (Shirato et al., 2017).

These results have a major impact on the recovery of clinical serotype I FCoV. If...
serotype I FCoV growth in Fcwf-4 cells positively selects for the use of heparan sulfate as an entry receptor, and by extension the loss of the furin/TMPRSS2 site, then this may explain why tissue culture-adapted strains lose pathogenicity. Cell-surface level entry, mediated by S1/S2 cleavage by furin or TMPRSS2, has been shown to be involved in pathogenicity and spread of coronaviruses in vivo (Park et al., 2016; Shirato et al., 2013) possibly by increasing the kinetics of virus entry (Earnest et al., 2017). Therefore, the loss of the S1/S2 cleavage site in tissue culture-adapted strains may significantly contribute to both the loss of pathogenicity of tissue culture-adapted strains and the inability to cultivate true clinical isolates without several rounds of passage. Interestingly, serotype II FCoVs do not contain an S1/S2 cleavage site, do not apparently utilize heparan sulfate, and use feline aminopeptidase N (fAPN) - a different entry receptor than serotype I FCoV (Hohdatsu et al., 1998). As such, tissue culture adaptation of serotype II FIPV strains does not typically correspond with a loss in pathogenicity (Tekes et al., 2012; Thiel et al., 2014) suggesting that maintenance of the natural receptor/entry route may allow recovery of pathogenic type I strains. Thus, increasing the expression of S1/S2-targeting host enzymes such as furin or TMPRSS2 along with expression of other host factors that have been shown to enhance entry at the surface-level including tetraspanins, as has been shown with other coronaviruses (Earnest et al., 2017; Hantak et al., 2019), may lead to more efficient recovery of clinical type I FIPV strains (Figure 23, right). Other host proteases, such as cathepsins, have been shown to be important for FCoV entry within endosomes (Regan et al., 2008) and could also be expressed in Fcwf-4 CU or Fcwf-4 CU IRN cells using a similar system described here for TMPRSS2.
Together, the results in this section describe the successful disruption of the IFNαR in two feline cell lines and suggest that overexpression of feline proteases, in at least one of these cell lines, may be useful for enhancing infection of serotype I FCoVs. Importantly these data also demonstrate the genetic malleability of the Fcwf-4 CU cells and, to my knowledge, represent the first genetically-modified Fcwf-4 cell line. Thus, both the AK-D 2.2 and the Fcwf-4 CU IRN cells will be useful in future studies evaluating feline viruses that lack interferon antagonists and may be an important tool in isolating serotype I clinical strains from infected cats.

**Progress Toward Developing an Infectious Clone Reverse Genetics System of Serotype I FIPV Black.**

**Summary.** In this final section, I worked to establish a reverse genetics system based on a deep-sequenced laboratory strain of the model type I FCoV, FIPV Black. Using this FIPV Black sequence as a template, the genome was subcloned as seven cDNA segments (A-G) into cloning vectors. I demonstrated that each fragment could be excised from the vector, purified and ligated into a full-length cDNA version of the genomic RNA and, using *in vitro* transcription, that mRNAs of the full-length icFIPV and nucleocapsid gene could be produced. Using purified WT gRNA isolated from FIPV Black virions, I optimized the electroporation protocol and established the positive control conditions necessary to reproducibly recover infectious virus from Fcwf-4 CU or IRN cells electroporated with gRNA. Electroporation of Fcwf-4 cells with WT gRNA or icFIPV resulted in the development of CPE over 24-48 hours. Finally, sgRNA, a positive indicator of ongoing virus replication, was detectable by RT-PCR from these cells further demonstrating that viable FIPV was produced from our reverse genetics system.
**Implications.** One of the major challenges with developing the FIPV Black reverse genetics system was the high degree of sequence variability within the S and 7b regions observed following deep-sequencing. These two regions, which are critical for virus replication and cell tropism, varied greatly within the sequenced population of viruses obtained from the same supernatant and resulted in stretches of “n” reads denoting the lack of consensus. This suggested that the in-lab FIPV Black strain was likely a quasispecies populated by several dominant strains. Initially, this complicated the reverse genetics design as the system must be based on a single, consensus genome derived by giving the most abundant nucleotide at any given position. In addition to the “n” reads in S and 7b, it was also difficult to determine if the consensus generated for the rest of the genome represented a viable virus strain or a sequence mix between dominant sub-strains. To overcome this, I compared deep-sequencing results of two independent FIPV Black stocks, obtained from different sequencing facilities (Kansas State and University of Chicago) with a published sequence (EU186072.1) to construct a consensus genome that represented the population. Ultimately, this FIPV Black genome (**Appendix C**) resulted in detectable replication of infectious clone FIPV Black as determined by the presence of CPE and N gene sgRNA following electroporation.

Additional challenges were confronted when attempting to propagate the infectious clone FIPV Black strain. Unlike the positive control gRNA, syncytia induced by icFIPV did not progress after 48 hpe. Further, passage of supernatants containing icFIPV did not cause CPE nor produce sgRNAs in new Fcwf-4 CU cells. This lack of second-round infectivity suggests that the icFIPV may harbor a sub-optimal genomic
sequence preventing the *de novo* construction of infectious virions. Indeed, there are numerous possible sequence or assembly defects that could lead to non-infectious virions, several of which we addressed. First, we can detect the presence of subgenomic nucleocapsid mRNA, the production of which requires both the translation of the replicase polyprotein and the activity of the nonstructural proteins encoded on pp1a and pp1ab. This suggests that the defect is likely not within the 5′ UTR, as this structure directs translation, nor the components of the replicase polyprotein necessary to produce subgenomic mRNA transcripts. Second, the electroporated material produces syncytia, a process mediated by spike-receptor engagement. This suggests that, at the very least, the spike protein is produced, engages a receptor and is able to mediate membrane fusion. However, it does not necessarily rule out the possibility that the spike sequence is sub-optimal or mediates low-efficiency fusion, which may impact virus entry. To determine if the spike sequence was limiting the infectivity of the ic virions, I replaced our ic S with the S sequence obtained from the published serotype I sequence (EU186072.1), which was shown to be infectious (Tekes et al., 2008). However, this sequence did not produce infectious, passable virus (*data not shown*) and we concluded that the spike was likely not the issue. Finally, we looked at the 7b accessory region to determine if variability in this region could be contributing to the infection defect. Indeed, after deep-sequencing we observed a large amount of variability in the 7b accessory protein region resulting in an approximately 35 nt stretch of “n” residues indicating the lack of consensus. In our ic genome, this region was deleted entirely. While the loss of the 7b protein is commonly observed with TC-adapted FCoV strains (Thiel et al., 2014) and likely not the direct cause of the defect, the ORF7
sequence directly precedes the 3’ UTR region, which is critical for efficient initiation of subgenomic mRNA. Thus, if the sequence of 7b is not correct in the consensus, it may impose changes to the loop-structure of the downstream 3’ UTR thereby affecting the efficiency of subgenomic mRNA transcription. To attempt to answer this question, we replaced the 7b sequence of our infectious clone with the published 7b sequence (EU186072.1). Again, this did not solve the problem and no infectious virions were produced (data not shown) allowing us to conclude that the 7b region is likely not the issue.

Thus, I speculate that a possible defect preventing the production of infectious virions may be housed within the E and/or M structural proteins, which influence the formation of the virions but do not impact the production of sgRNAs or syncytia formation. The M protein is the main structural component of the coronavirus outer membrane and, along with the E protein, directs the formation of the mature virion (de Haan and Rottier, 2005). If one or both of these proteins have an incorrect sequence or are not produced at high enough levels, this may impact the formation of infectious virus. Future experiments are needed to detect production of E and M sgRNAs by RT-PCR and to attempt to visualize the completely-formed, mature virions by electron microscopy. Ultimately, the genomes of several “Baker-Lab strain” FIPV populations need to be compared following multiple rounds of plaque purification to determine the most common genomic RNA sequences and eliminate variability arising from the viral quasispecies. In parallel, individual syncytia could be isolated after icFIPV electroporation and deep-sequenced to determine if these infected cells contain a more optimal genomic sequence. Together, these experiments are the most direct means of
rectifying the issues with the consensus sequence and may help produce a fully-infectious virus by reverse genetics.

Interestingly, a recent study by Ehmann and colleges offers a different perspective on the inability of our icFIPV to passage. This group constructed the first-ever reverse genetics system for a field strain of serotype I FECV (Ehmann et al., 2018). During production of this FECV, however, they also found that virions obtained directly from electroporated cells could not be passaged, even though they were observable by EM and produced sgRNAs. Interestingly, infectious virus could only be recovered following direct injection of concentrated electroporated cell-supernatant into live felines. I speculate that infection efficiency of the icFECV was enhanced in vivo due to the presence of high concentrations of host cell proteases or other infection-enhancing cell surface molecules not present in tissue culture. Intriguingly, this same phenomenon was observed during generation of another coronavirus, porcine epidemic diarrhea virus (PEDV), by reverse genetics. Like the Ehmann study, icPEDV could only be recovered following in vivo injection, in this case of porcine intestine (Beall et al., 2016). Thus, several groups have demonstrated that production of some CoV strains by reverse genetics might require additional factors present in whole animal tissue in order to replicate. The observations made by these two groups may, in part, explain the lack of passage in my FIPV Black system – although injection of live cats is outside the scope of our current research protocol. In future studies, Fcwf-4 CU IRN cells expressing additional feline proteases, like TMPRSS2, may provide a compromise to in vivo injection and may facilitate recovery of FIPV Black by reverse genetics.
The goal of the reverse genetics system described above was to have a platform on which to generate live-attenuated serotype I FIPV strains by non-functional mutation of interferon antagonists. Investigation of interferon antagonists encoded by alphacoronaviruses, including FCoVs, have been limited to studies involving group-specific accessory proteins (Cruz et al., 2013, 2011; Dedeurwaerder et al., 2014). Early work using recombinant FCoV demonstrated that attenuation of serotype II FIPV (strain 79-1146) could be achieved through single deletion of group-specific protein clusters 3abc or 7ab, respectively (Haijema et al., 2004). Cats vaccinated with these mutant serotype II strains generated protective humoral immunity and survived subsequent challenge with homotypic strains. Although this group did not evaluate IFN production in these animals, both accessory regions 3abc and 7ab have since been shown to be involved in IFN antagonism (Dedeurwaerder, 2014; Dedeurwaerder et al., 2014, 2013). Further investigation of FCoV nonstructural proteins (e.g. PLP2 or EndoU), as has been described extensively in betacoronaviruses (Deng et al., 2019b, 2017; Frieman et al., 2009; Kindler et al., 2017; Mielech et al., 2014; Niemeyer et al., 2013; Sun et al., 2012), may also result in the discovery of new FCoV IFN antagonists. It is tempting to speculate that combined deletion of individual accessory regions with mutation in nonstructural protein IFN antagonists (e.g. PLP2 or EndoU) may lead to a HII virus phenotype and, ultimately, stimulation of immune memory.

Final Thoughts

As this research program progresses, it is my hope that the tools described in this dissertation will aid in the generation, recovery and characterization of IFN antagonist-deficient serotype I feline coronaviruses and stand as a successful means of creating
live-attenuated vaccine strains. To this end, we have recently begun a dual
collaboration with the Tekes group at Justus-Liebig University (Giessen, Germany), and
the Whittaker group at Cornell University (Ithaca, NY). Our combined research efforts
aim to produce serotype I FIPV HII viruses using an established serotype I FIPV reverse
genetics system in the Tekes lab (Tekes et al., 2008), to characterize these strains in
our lab using the tools and techniques described in this dissertation and, finally, to
evaluate attenuated strains as vaccine candidates in the Whittaker lab who have
capacity to do experimental work on felines. Together, we hope to develop a successful
means to prevent this highly pathogenic feline illness.
APPENDIX A

ANTIBODIES USED TO DETECT FELINE CORONAVIRUS
**Antibody Detection of FIPV Black Nsp15, dsRNA and N Gene Production.** A-B) Affinity-purified rabbit-anti-FIPV nsp15 (EndoU) polyclonal antibodies were generated by GenScript against a purified H227A catalytic mutant FIPV Black nsp15 protein immunogen. This antibody is effective at detecting FIPV Black nsp15 by immunofluorescence or by western blot. A) Immunofluorescence detection of FIPV Black nsp15 (FITC) and double-stranded (ds) RNA (TRITC) following 18 h infection (MOI = 0.1) in Fcwf-4 CU cells. Composite image shows localization of nsp15 to replication complexes. Images representative of two independent experiments. B-C) Western blot analysis of FIPV Black (B) nsp15 or (C) N protein from Fcwf-4 CU cell lysates following 16 h mock or FIPV Black infection (MOI = 0.1). The black arrows indicate the ~40 kDa nsp15 protein and the ~42 kDa N protein. The CCV2-2 biotinylated monoclonal antibody (Bio-Rad) detects FIPV N protein and has been previously described (Poncelet et al., 2008).
### Antibody Application for Immunofluorescence.

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### Antibody Application for Western Blot.

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<td>d-α-r-HRP (1:2500)</td>
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APPENDIX B

PRIMER INDICIES
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Primer Index 2: IFNαR2 Single Guide DNA Primers.

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Primer Index 4: Reverse Genetics cDNA Segment Sequencing.
APPENDIX C

FIPV BLACK LABORATORY SEQUENCE
ORGANISM  Feline infectious peritonitis virus Baker Laboratory Strain (Origin: AK-D Cells; Consensus)
REFERENCE  Bases 1 to 29198
AUTHORS  Robert C. Mettelman, Susan C. Baker
TITLE  Generating biologic and genetic research tools to investigate serotype I feline coronaviruses
JOURNAL  Ph.D. Dissertation; Loyola University Chicago; 2019
FEATURES  (1..29198)

[A_SEGMENT]  1..4810
5'UTR.        1..311
PP1AB.       312..20407
PP1A.         312..12407

[B_SEGMENT]  4811..8875

[C_SEGMENT]  8876..13485

[D_SEGMENT]  13486..15825

[E_SEGMENT]  15826..20588
SPIKE.     20404..24792

[F_SEGMENT]  20589..24861
ORF3ABC.  24293..25819

[G_SEGMENT]  24862..29198
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MEMBRANE.  26079..26870
NUCLEOCAPSID.  26883..28016
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7B.           28331..28905
3'UTR         28906..29198

ORIGIN

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APPENDIX D

FELINE IFNAR2 SEQUENCING ALIGNMENT
### Feline IFNaR2 Amino Acid Sequence Alignment

Amino acid sequence alignment [CLUSTAL W (1.7)] of feline interferon alpha receptor domain 2 (IFNaR2) comparing a published sequence (GenBank #NM_001278859.1) with the sequences determined from Fcwf-4 CU and Fcwf-4 CU IRN cells.
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VITA

Robert Christian Mettelman was born in Elm Grove, Wisconsin on March 12, 1991 to Douglas and Laura Mettelman. He attended the University of Wisconsin-Madison, where he earned a Bachelor of Science degree in Medical Microbiology and Immunology and a Certificate in African Studies in May 2013. During his undergraduate studies, Robert worked with JM Ané, Ph.D., studying bacterial-fungal-plant symbioses, and with BS Klein, M.D., investigating pathogenic yeast replication in immune cells.

In August 2013, Robert matriculated into the Interdisciplinary Program in Biomedical Sciences at Loyola University Chicago and joined the Department of Microbiology and Immunology. He completed his doctoral work in the laboratory of Susan C. Baker, Ph.D., where he focused on generating biologic and genetic research tools to investigate attenuated and clinical strains serotype I feline coronaviruses. Robert’s work was supported by the Arthur J. Schmitt Dissertation Fellowship in Leadership and Service, a Winn Feline Foundation Grant awarded through the Bria Fund for FIP Research, an Intramural Grant awarded by the Research Funding Committee of Loyola University Chicago, an NIH T32 Training Grant in Experimental Immunology awarded to KL Knight, Ph.D., and an NIH R01 awarded to SC Baker, Ph.D. Robert will continue his research endeavors as a Postdoctoral Fellow in the laboratory of PG Thomas, Ph.D., at St. Jude Children’s Research Hospital in Memphis, TN. There Robert will study the human infant immune response to influenza virus infection.