2020

Investigating the Mechanism of the Coronavirus Endoribonuclease in Antagonizing Innate Immune Signaling

Matthew Hackbart

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LOYOLA UNIVERSITY CHICAGO

INVESTIGATING THE MECHANISM OF THE CORONAVIRUS ENDORIBONUCLEASE IN ANTAGONIZING INNATE IMMUNE SIGNALING

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY
MATTHEW STEVEN HACKBART
CHICAGO, ILLINOIS
MAY 2020
ACKNOWLEDGEMENTS

I would like to start by thanking my future wife, Chelsea, and my family for providing their support and encouragement throughout this process. I would like to thank my mentor, Dr. Susan C. Baker for giving me the opportunity to be a scientist in her lab. I am grateful for mentorship, the independence, and the guidance from Susan that allowed me to develop as not only a scientist, but also as a writer, speaker, and mentor for others. I am also very grateful to all the members of the Baker lab including Dr. Anna Mielech, Dr. Xufang Deng, Dr. Amornrat O’Bien, Dr. Robert Mettelman, Amani Eddins, Aaron Volk, Yazmin Cruz-Pulido, and Monika Evdokimova. They all made working in the lab a great experience. They provided great help with experiments and many thoughtful discussions to help me along the way. Amornrat also kept me well-fed with a steady diet of cookies and chocolate. I would also like to thank Dr. Katherine Knight and all the students, postdocs, technicians, faculty, and members of the front office in the Department of Microbiology and Immunology at Loyola University Chicago. I could not stress enough how helpful sharing my data at Friday meetings helped with the development of my project. I am thankful for anyone who provided any new experimental ideas or hypotheses, as well as anyone who generously gifted some reagents so I could secretly do an experiment without Susan knowing. Finally, I would like to thank all my funding sources including the NIH T32 Training Grant in Experimental Immunology awarded to Dr. Katherine Knight and the NIH RO1 grant awarded to Dr. Susan C. Baker.
For the Stars, who will forever be with me.
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<th>Definition</th>
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<tbody>
<tr>
<td>(-)</td>
<td>negative-sense</td>
</tr>
<tr>
<td>(+)</td>
<td>positive-sense</td>
</tr>
<tr>
<td>2’5’-OAS</td>
<td>2’5’-oligoadenylate synthase</td>
</tr>
<tr>
<td>3’UTR</td>
<td>3’ untranslated region</td>
</tr>
<tr>
<td>3CLpro</td>
<td>3C-like protease</td>
</tr>
<tr>
<td>5’UTR</td>
<td>5’ untranslated region</td>
</tr>
<tr>
<td>A</td>
<td>area</td>
</tr>
<tr>
<td>ACE2</td>
<td>angiotension-converting enzyme 2</td>
</tr>
<tr>
<td>APN</td>
<td>aminopeptidase N</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>BMDMs</td>
<td>bone marrow-derived macrophages</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase activation and recruitment domain</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR associated protein 9</td>
</tr>
<tr>
<td>CD9</td>
<td>cluster of differentiation 9</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CEACAM-1</td>
<td>carcinoembryonic antigen-related cell adhesion molecule 1</td>
</tr>
<tr>
<td>CM</td>
<td>convoluted membranes</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CoV</td>
<td>coronavirus</td>
</tr>
<tr>
<td>CPSF</td>
<td>cytoplasmic polyadenylation specific factor</td>
</tr>
<tr>
<td>CRISPR</td>
<td>clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>Cryo-EM</td>
<td>cryogenic electron microscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CTD</td>
<td>carboxyl terminal domain</td>
</tr>
<tr>
<td>DBT</td>
<td>delayed brain tumor</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMV</td>
<td>double-membrane vesicle</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxyribonucleic acid triphosphates</td>
</tr>
<tr>
<td>DPI</td>
<td>days post infection</td>
</tr>
<tr>
<td>DPP4</td>
<td>dipeptidyl-peptidase 4</td>
</tr>
<tr>
<td>ds</td>
<td>double-stranded</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
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<td>envelope</td>
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<tr>
<td>eIF2α</td>
<td>eukaryotic translation initiation factor 2A</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>EndoU</td>
<td>endoribonuclease of uridines</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERGIC</td>
<td>ER-golgi intermediate compartment</td>
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<tr>
<td>EtOH</td>
<td>ethanol</td>
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<tr>
<td>ExoN</td>
<td>exonuclease</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas Associated Death Domain</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GMP</td>
<td>guanosine monophosphate</td>
</tr>
<tr>
<td>gRNA</td>
<td>genomic RNA</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin stain</td>
</tr>
<tr>
<td>HE</td>
<td>hemagglutinin-esterase</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HPI</td>
<td>hours post infection</td>
</tr>
<tr>
<td>HPT</td>
<td>hours post transfection</td>
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</table>
hr  hour
HRP  horseradish peroxidase
IBV  infectious bronchitis virus
IC  intracranial
IFN  interferon
IFNAR  interferon alpha receptor
IFNGR  interferon gamma receptor
IFNLR  interferon lambda receptor
ISG  interferon-stimulated gene
ISGF3  IFN-stimulated gene factor 3
Jak1  Janus kinase 1
kb  kilobase
kDa  kilodalton
L  liter
LGP2  Laboratory of Genetics and Physiology 2
LiCl  lithium chloride
M  membrane protein
m-csf  macrophage-colony stimulating factor
mAbs  monoclonal antibodies
MAVS  mitochondrial antiviral signaling protein
MDA5  melanoma differentiation-associated gene 5
MEM  minimal Eagle’s medium
MERS-CoV  Middle East Respiratory Syndrome coronavirus
mg  milligram
MHV-A59  mouse hepatitis virus, strain A59
min  minute
mL  milliliter
MOI  multiplicity of infection
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>N</td>
<td>nucleocapsid protein</td>
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<tr>
<td>N7-MTase</td>
<td>N7 methyltransferase</td>
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<tr>
<td>NendoU</td>
<td>nidovirus endoribonuclease</td>
</tr>
<tr>
<td>NF H20</td>
<td>nuclease free water</td>
</tr>
<tr>
<td>NF-κb</td>
<td>nuclear factor-kappa-light-chain enhancer of B cells</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
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<td>Nsp</td>
<td>nonstructural protein</td>
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<td>nt</td>
<td>nucleotide</td>
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<td>NTD</td>
<td>amino terminal domain</td>
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<td>OAS1/2</td>
<td>2’5’-oligoadenylate synthase 1 and 2</td>
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<td>Oligo-dT</td>
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<td>ORF</td>
<td>open reading frame</td>
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<tr>
<td>PABP</td>
<td>polyA binding protein</td>
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<tr>
<td>PAMP</td>
<td>pattern-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDCoV</td>
<td>porcine deltacoronavirus</td>
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<tr>
<td>PEDV</td>
<td>porcine epidemic diarrhea virus</td>
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<tr>
<td>PFU</td>
<td>plaque-forming units</td>
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<td>protein kinase R</td>
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<td>PLP1</td>
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<tr>
<td>polyA</td>
<td>poly adenosine</td>
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<tr>
<td>polyI:C</td>
<td>polyinosinic:polycytidylic acid</td>
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</table>
polyU      poly uridine
pp        polyprotein
PRR      pattern recognition receptor
PRRSV   porcine reproductive and respiratory syndrome virus
qPCR    quantitative PCR
RBD      RNA binding domain
RdRp     RNA-dependent RNA polymerase
RFP      red fluorescent protein
RFS      ribosomal frameshift
RIG-I    retinoic acid-inducible gene I
RLR      retinoic acid-inducible gene I-like receptors
RNA      ribonucleic acid
RNase L  ribonuclease L
RTC      replicase-transcriptase complex
RTPase   RNA triphosphatase
S        spike protein
SARS-CoV severe acute respiratory syndrome coronavirus
sgRNA    subgenomic RNA
snoRNA   small nucleolar RNA
ss       single-stranded
STAT1/2  signal transducer and activator of transcription 1/2
TC       tissue culture
TP       transducing particle
TPB      tryptose phosphate broth
TRS      transcription regulatory sequence
TRS-B    transcription regulatory sequence-body
TRS-L    transcription regulatory sequence-leader
Tyk2     tyrosine kinase 2
V  volume
XendoU  *Xenopus laevis* endoribonuclease
WT  wild type; wild-type
α  alpha
β  beta
λ  lambda
μg  microgram
μL  microliter
μM  micromolar
ABSTRACT

Coronaviruses (CoVs) are positive-sense RNA viruses that can emerge from endemic reservoirs and infect zoonotically, causing significant morbidity and mortality. CoVs encode an endoribonuclease (EndoU) that cleaves RNA in biochemical assays, but the target and function of EndoU activity during viral replication was not known. My work focused on characterizing the functions of EndoU during infection. I report that EndoU is an innate immune antagonist. To function as an immune antagonist, EndoU cleaves the 5′-Poly-Uridines from Negative-sense viral RNA, termed PUN RNA, which is the product of polyA-templated RNA synthesis. Using a virus containing an EndoU catalytic-inactive mutation, I detected a higher abundance of PUN RNA in the cytoplasm of infected cells compared to wild type CoV-infected cells. Furthermore, we found that transfecting PUN RNA into cells stimulates a robust, MDA5-dependent interferon response, and that removal of the polyU-extension on the RNA dampens the response. Overall, the results of this study reveal the PUN RNA to be a novel CoV MDA5-dependent pathogen-associated molecular pattern (PAMP). We also establish a mechanism for EndoU activity to cleave and limit the accumulation of this novel PAMP. Since EndoU activity is highly conserved in all coronaviruses, inhibiting this activity may serve as an approach for therapeutic interventions against existing and emerging CoV infections.
CHAPTER ONE
INTRODUCTION
The Biology of Coronaviruses

Overview and Classification

Coronaviruses (CoVs) are enveloped, positive-sense ribonucleic acid (RNA) viruses in the taxonomic order Nidovirales. The Coronaviridae family is divided into two subfamilies: Letovirinae, which contains the Alphacoronavirus genus, and Orthocoronavirinae, which contains four main genera: Alphacoronavirus, Betacoronavirus, Deltacoronavirus, and Gammacoronavirus (Lefkowitz et al., 2018). CoVs infect a wide range of avian and mammalian species with most infections resulting in respiratory or gastrointestinal disease.

Viruses in the Nidovirales order are organized based on the phylogenetic similarity of the genome structure. All nidoviruses have large positive-sense RNA genomes, with CoVs containing genomes of 27 to 32 kilobases (kb) in length. The nidoviruses were aptly named due to their ability to produce nested sets of RNA during infection. Nido means “nest” in Latin. CoVs produce 7-9 nested, subgenomic RNAs (sgRNAs), which amplify the 3’ end of the genome (Masters, 2006). Another common feature among nidoviruses is that the genomic organization is conserved with a large replicase gene at the 5’ end of the genome, followed by structural and accessory proteins at the 3’ end of the genome. The major differences in the nidoviruses derive from the size, number, and type of structural proteins, which significantly alter the structure and morphology of the virions (Fehr and Perlman, 2015).
**Genome Organization**

CoVs carry non-segmented, single-copy, single-stranded, positive-sense RNA genomes, which are about 30 kb in length. The viral genome has a similar appearance to host messenger RNA (mRNA) since the genomic RNA (gRNA) is capped at the 5’ end with a guanine residue methylated at the N7 position, while the 3’ end of the genome contains a poly-adenosine (polyA) tail. Since the viral RNA mimics host mRNA, the viral genome can be translated by host ribosomes after insertion of the viral genome into the cytoplasm of cells (Fehr and Perlman, 2015; Masters, 2006).

The 5’ end of the genome contains an untranslated region (5’ UTR) with a small leader (L) sequence. The 5’ UTR is highly structured with seven stem-loops being experimentally determined to be critical for viral replication (Brown et al., 2007; Guan et al., 2011; Yang and Leibowitz, 2015). The leader sequence includes the first 70-100 nt of the genome and a 6-8 nt transcription-regulating sequence (TRS) (Baric et al., 1983; Lai et al., 1984, 1983). Each sgRNA also contains identical TRS segments at the 5’ end of the RNA, which act as cis-acting regulatory elements and are critical for the discontinuous transcription that occurs during sgRNA replication (Budzilowicz et al., 1985; Sawicki et al., 2007; Sola et al., 2015). The 3’ end of the CoV genome contains an untranslated region (3’ UTR) between 270 to 500 nt in length that precedes the polyA tail. The 3’ UTR is also highly structured, containing an RNA pseudoknot and several cis-acting elements essential for RNA replication (Goebel et al., 2004b, 2004a; Yang and Leibowitz, 2015).

The remaining CoV genome can be divided into two major regions, with the first two thirds of the genome coding for the replicase polyproteins and the last third of the genome encoding structural and accessory proteins (Fig. 1B). Translation of the first two open reading
frames (ORF), ORF1a and ORF1b, produces two replicase polyproteins, polyprotein 1a (pp1a) and polyprotein 1ab (pp1ab) (Lai et al., 1994). While ORF1a is translated directly into pp1a, pp1ab is produced by a -1 nt ribosomal frameshift (RFS) that occurs at a pseudoknot-slippery sequence in ORF1. The slipping of the ribosome allows for continuation of translation through ORF1b, producing pp1ab (Bredenbeck et al., 1990; Brierley et al., 1987). Both pp1a and pp1ab are subsequently cleaved by viral proteases to generate 16 nonstructural proteins (nsp1-16), which have a wide-range of activities. These activities include altering the host cell endoplasmic reticulum (ER) membranes to form the replicase-transcriptase complex (RTC), replicating the viral genome, and altering host innate immune signaling. The remaining third of the genome contains multiple ORFs that encode for structural and accessory proteins. CoV structural proteins include the envelope (E), membrane (M), nucleocapsid (N), and spike (S) proteins (Fehr and Perlman, 2015; Masters, 2006). A subset of Betacoronaviruses also encode a hemagglutinin-esterase (HE) which is incorporated in the virion (Cornelissen et al., 1997; Kazi et al., 2005; Klausegger et al., 1999). ORFs for accessory proteins are spread among the structural protein ORFs. These accessory proteins differ for each CoV species and are generally dispensable for viral replication. Many of the accessory proteins provide a selective pathogenic advantage to the specific CoV species (Fehr and Perlman, 2015). This dissertation is focused on the murine CoV mouse hepatitis virus, strain A59 (MHV-A59), which is a Betacoronavirus. The genome of MHV-A59 contains 7 distinct viral mRNAs: 1 genomic full-length RNA and 6 sgRNAs. MHV-A59 encodes the replicase and structural proteins as well as four distinct accessory proteins: ns2, ns4a, ns4b, and ns5a (Masters, 2006). The functions of these proteins are discussed below.
**Figure 1. CoV Virion and Genome Structures.** (A) Cartoon representation of mature CoV virion. A host membrane that contains S, E, M, and HE proteins surround the viral (+) sense, genomic RNA. The genomic RNA is coated with N protein. (B) Cartoon representation of the CoV genome. The genome can be divided into two large segments, the replicase nonstructural proteins and the structural/accessory proteins. The first two/thirds of the genome encodes a large polyprotein that is cleaved into 16 nonstructural proteins (nsp1-16). The last third of the genome encodes for the structural proteins, S, E, M, N, and HE, as well as multiple accessory proteins. For MHV-A59, there are four known accessory proteins, ns2, ns4a, ns4b, and ns5a.

**Virion Structure**

**Coronavirus virion.** CoVs are spherical virions with diameters of approximately 125 nm (Bárcena et al., 2009; Neuman et al., 2006). CoVs have a distinct corona or crown of proteins extending from the virion like rays from a sun, hence the name “corona”-virus. This prominent feature of the enveloped virus are the large trimeric, club-like S proteins projecting out from the virion (Masters, 2006). The rest of the virion is contained within a host membrane-derived lipid envelope. The virus particle contains 4 main structural proteins: S, E, M, and N proteins (Fig. 1A).
**Spike.** The spike (S) protein is the main determinant host range and tissue tropism since the virus uses S protein for cell attachment and entry. The S protein is about 150 kilodaltons (kDa) in size and is heavily N-linked glycosylated. S protein utilizes an N-terminal sequence to associate with endoplasmic reticulum (ER) membranes and become incorporated into the virion (Neuman et al., 2006). For entry, the S protein is a class 1 fusion protein (Bosch et al., 2003; Collins et al., 1982; Walls et al., 2019). S protein forms a homotrimer that can engage with host cell surface proteins and/or sugars. For MHV-A59, the cellular protein receptor is carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1)(Holmes et al., 1990). The spike protein can be divided into two subunits, S1 which is involved in receptor binding and S2 which contains the stalk region of spike protein (Abraham et al., 1990; Luytjes et al., 1987). Some coronaviruses also require cleavage by host cell furin-like protease to separate S1 and S2 subunits of spike to mediate fusion and entry into cells.

**Envelope.** The envelope (E) protein is an 8-12 kDa transmembrane protein that is present in small quantities in the virion (Raamsman et al., 2000). The E protein encodes a predicted ion channel, but the function of the protein is still yet to be determined. For example, deleting the E protein is not always lethal to virus replication. In SARS-CoV, the ion channel function is not necessary for viral replication, but is necessary for the pathogenesis of the virus in vivo (Boscarino et al., 2008; Corse and Machamer, 2000; Fischer et al., 1998; Nieto-Torres et al., 2014; Verdiá-Báguena et al., 2012; Ye and Hogue, 2007).

**Membrane.** The membrane (M) protein is the most abundant structural protein in the virion. This small 25-30 kDa protein contains three transmembrane domains, with a small N-terminal glycosylated ectodomain and a larger endodomain. When present in the virion, the M protein promotes membrane curvature and ultimately drives the shape of the virion (Neuman et
al., 2011). The M protein is hypothesized to form a dimer, but likely utilizes multiple conformational states to function as a membrane altering protein (de Haan and Rottier, 2005; Fehr and Perlman, 2015; Masters, 2006).

**Nucleocapsid.** The nucleocapsid (N) protein binds to the viral RNA. The 43-50 kDa N protein contains two RNA binding domains, NTD and CTD (Chang et al., 2006; Hurst et al., 2009). The N protein can be highly phosphorylated, which is hypothesized to trigger structural changes and alter the affinity of RNA binding (Stohlman and Lai, 1979). The N protein has a high binding affinity for the TRSs and packaging signal in the viral RNA (Molenkamp and Spaan, 1997; Stohlman et al., 1988). N protein also interacts with nsp3 and the M protein, with the M protein interaction driving packaging of the viral RNA into the virion (Sturman et al., 1980). The N protein is found in the core of the virion, encompassing the genomic RNA.

**Hemagglutinin-esterase.** A subset of Betacoronaviruses also encode a hemagglutinin-esterase (HE) protein (King et al., 1985). The HE protein binds to sialic acids and contains acetyl esterase activity (Cornelissen et al., 1997). The HE protein is predicted to enhance the attachment of the S protein to host protein receptors and promote the spread of the virus through mucosa. Interestingly, the HE protein is selected against during passage of the virus in cell culture (Lissenberg et al., 2005).

**Replication Cycle**

**Overview.** CoV replication begins with the S protein binding to the cellular receptor. The virion membrane can fuse with the cell surface or be incorporated into intracellular vesicles through receptor-mediated endocytosis (Gallagher and Buchmeier, 2001; Millet and Whittaker, 2015). After a membrane fusion event, the single-stranded, positive-sense genome is released into the cytoplasm of cells. This positive-sense RNA resembles host mRNA so the viral mRNA
is translated by host ribosomes to produce the replicase polyproteins, pp1a and pp1ab. Pp1a and pp1ab are then cleaved by viral proteases to produce sixteen nonstructural proteins (nsps) involved in viral replication (Baker et al., 1993; Kanjanahaluethai et al., 2003; Masters, 2006; Schiller et al., 1998). The functions of each nsp are diverse, but nsps are functionally conserved between every coronavirus (Gorbalenya et al., 1989). For example, nsp12 encodes an RNA-dependent, RNA polymerase (RdRp) for each coronavirus. Nsp3, Nsp4, and Nsp6 alter host membranes to form convoluted membranes (CMs) and double-membrane vesicles (DMVs) to generate the replicase-transcription complexes (RTCs) (Angelini et al., 2013). CoV RTCs have two main functions. First, RTCs replicate full-length genomic RNA to be package into newly synthesized virions to infect new cells. Second, RTCs produce sgRNAs through discontinuous transcription to enhance production of structural and accessory proteins. Structural proteins are translated, inserted into the ER membrane, trafficked to the ER-Golgi intermediate compartment (ERGIC), and finally assembled into complete virions. N protein binds to newly synthesized genomic RNA, which then buds into ERGIC membranes containing the remaining structural proteins (Fehr and Perlman, 2015; Masters, 2006). These budded virions are then trafficked in vesicles to the cell surface, where the new infectious virions are released from the cell (Fig. 2).
Figure 2. Diagram of CoV Replication Cycle. The infectious virion binds to the cell surface receptor. The virion either fuses at the cell surface or is endocytosed and fused to the cell membrane in the endosome. Upon fusion, the genomic (+) RNA is released. The (+) RNA is translated into replicase polyproteins, which are proteolytically cleaved into individual nonstructural proteins (nsps). The replication proteins generate replicative membranes (convoluted membranes and DMVS) from the ER and replicate the viral (+) RNA into (-) gRNA and (-) sgRNAs. The (-) gRNA and (-) sgRNAs act as templates for (+) gRNA and (+) sgRNAs. These (+) RNAs then are translated into structural proteins in the ER. The gRNA interacts with structural proteins and virions bud into the ERGIC. The fully formed virions are then released from the cell by exocytosis.

Attachment and entry. The initial attachment of the virion to the host cell is initiated by the S protein binding to its receptors. Recent studies have shown not only does S protein
interact with a host protein receptor, but also contains a sialic acid binding domain that facilitates cell entry (Qing et al., 2020). The interaction between the S protein and host receptor is the primary determinant for what host species and tissues the virus can infect (Fehr and Perlman, 2015). Many Alphacoronaviruses utilize aminopeptidase N (APN) as their receptor, where Betacoronaviruses have a range of host protein receptors. SARS-CoV and HCoV-NL63 utilize angiotension-converting enzyme 2 (ACE2), whereas MERS-CoV uses dipeptidyl-peptidase 4 (DPP4) (Li et al., 2003; Raj et al., 2013; Wan et al., 2020). For MHV-A59, the cell receptor that S protein binds to is murine CEACAM-1, but MHV can infect cell lines from multiple species including monkey, feline, hamster, swine, and human cells (Schickli et al., 1997). Recent structural studies have shown that S protein may “breath.” The S1a domain of the S protein has two conformations, which open and close the receptor binding domain. Once the receptor binds to the S protein, then the S1a domain is locked into the open state (Tortorici and Veesler, 2019).

Many CoVs also utilize other host proteins for cell entry. Recently the role of tetraspanins and peptidases have been implicated in enhancing CoV entry into cells. Studies show that many CoVs utilize peptidases to cleave the S protein to expose the fusion peptide to the host membrane and facilitate fusion. The density and presence of host peptidases also alters the tropism for CoV and can facilitate zoonotic shifts (Belouzard et al., 2012, 2009; Millet and Whittaker, 2015). The cleavage is generally accomplished by acid-dependent proteolytic cleavage by cathepsin, transmembrane protease, serine 2 (TMPRSS2), or another protease, which then triggers cell fusion. S protein cleavage separates the receptor binding domain from the fusion domain, freeing the fusion peptide to insert into host membranes (Earnest et al., 2017; Glowacka et al., 2011; Park et al., 2016). Tetraspanins have been implicated in arranging host receptors and peptidases into lipid rafts. The tetraspanin, CD9 drives the formation of protein
rich domains on the cell surface, which is hypothesized to increase the interactions of the receptor-bound S protein with a host peptidase, thus enhancing viral entry (Earnest et al., 2017). Interestingly, there are interferon induced tetraspanins that can decrease cellular entry by CoVs, suggesting a inhibitory role for some tetraspanins (Hantak et al., 2019).

Upon insertion of the fusion peptide into the host membrane, the S protein undergoes a conformation shift indicative of class 1 fusion proteins. The S protein extends into the outer leaflet of the host membrane then folds into an anti-parallel six-helix bundle, pulling the cell and virion membranes into contact (Bosch et al., 2003). After formation of the bundle, the viral and cellular membranes begin mixing forming a hemi-fusion state. Ultimately, the membranes will completely fuse and release the viral genome into the cytoplasm of the cells (Fehr and Perlman, 2015).

**Replicase protein translation.** Upon release of the viral genomic mRNA into the cytoplasm of the cell, host ribosomes will begin translation of the replicase polyproteins. The replicase proteins are translated as two major polyproteins, pp1a and pp1ab. Pp1ab is generated through a pseudoknot and slippery sequence causing a frameshift to occur during ribosome translation. The RNA pseudoknot causes the ribosome to stall and shift -1 nt along the slippery sequence (5’-UUUAAC-3’). This frameshift causes the reading of the ORF to change and skip the pp1a stop codon, allowing for the remaining pplab to be translated. A majority of the time, the ribosome will unravel the pseudoknot and translate only pp1a, but as high as 25% of the time, the frameshift will allow for all of pp1ab to be translated (Lai et al., 1994). This frameshifting is present in all nidoviruses and is hypothesized to control the replicase protein expression in a precise ratio. Another hypothesis is that the frameshift delays the production of the 1b proteins until a suitable replication membrane structure has been formed.
After translation, pp1a and pp1ab are cleaved by viral proteases into individual nonstructural proteins. Pp1a is cleaved into nsp1-11 while pp1ab is cleaved into nsp1-16. CoVs contain either two or three proteases for polyprotein cleavage. While most coronaviruses encode two papain-like proteases (PLP1 and PLP2) within nsp3, *Betacoronaviruses*, SARS-CoV, and MERS-CoV only contain one domain designated PLpro. All coronavirus encode a 3C-like protease (3CLpro) in nsp5. The PLpros cleave between nsp1/2, nsp2/3, and nsp3/4 at a conserved LXGG/X motif (Baker et al., 1993; Kanjanahaluethai et al., 2003). 3CLpro cleaves the remaining 11 cleavage sites at a LQ/S motif to produce the individual nsps (Baker et al., 1989; Gorbalenya et al., 1989). The known and predicted functions of all of the nsps are listed in Table 1.
Table 1. Predicted Functions of Nonstructural Proteins. Listed are the functions that are associated with each nonstructural protein (Nsp) and the studies that determined these functions. Adapted from Fehr and Perlman 2015.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nsp1</td>
<td>Inhibit host translation; Degrade host mRNA; Block Innate Immune Signaling</td>
<td>(Huang et al., 2011; Kamitani et al., 2009, 2006; Tanaka et al., 2012)</td>
</tr>
<tr>
<td>Nsp2</td>
<td>Binds prohibitin proteins</td>
<td>(Cornillez-Ty et al., 2009; Graham et al., 2005)</td>
</tr>
<tr>
<td>Nsp3</td>
<td>Multi-Domain: DMV formation (Macrodomain): IFN antagonist (PLPros): LXGG-specific protease; Deubiquitinase; DelISGylase (DPUP, UbI2, NAB, G2M, SUD, Y): Unknown Functions</td>
<td>(Angelini et al., 2013; Baker et al., 1993; Chatterjee et al., 2009; Fehr et al., 2015; Frieman et al., 2009; Mielech et al., 2014; Niemeyer et al., 2018)</td>
</tr>
<tr>
<td>Nsp4</td>
<td>DMV Formation</td>
<td>(Clementz et al., 2008; Gadlage et al., 2010)</td>
</tr>
<tr>
<td>Nsp5</td>
<td>3CL-protease</td>
<td>(Lu et al., 1995)</td>
</tr>
<tr>
<td>Nsp6</td>
<td>DMV Formation</td>
<td>(Oostra et al., 2008)</td>
</tr>
<tr>
<td>Nsp7</td>
<td>Hexadecameric complex with nsp8 Helicase for RdRp (nsp12)</td>
<td>(Zhai et al., 2005)</td>
</tr>
<tr>
<td>Nsp8</td>
<td>Hexadecameric complex with nsp7; Helicase for RdRp (nsp12); Potential Primase; Adenyltransferase</td>
<td>(Imbert et al., 2006; Tvarogova et al., 2019; Zhai et al., 2005)</td>
</tr>
<tr>
<td>Nsp9</td>
<td>RNA binding protein</td>
<td>(Egloff et al., 2004)</td>
</tr>
<tr>
<td>Nsp10</td>
<td>Cofactor for nsp14 and nsp16 Promotes 2’-O-MT and ExoN activities</td>
<td>(Bouvet et al., 2014; Decroly et al., 2011)</td>
</tr>
<tr>
<td>Nsp11</td>
<td>Unknown function</td>
<td>(de Haan and Rottier, 2005)</td>
</tr>
<tr>
<td>Nsp12</td>
<td>RNA-dependent RNA polymerase</td>
<td>(Brockway et al., 2003)</td>
</tr>
<tr>
<td>Nsp13</td>
<td>DNA/RNA helicase</td>
<td>(Konstantin A Ivanov et al., 2004; Ivanov and Ziebuhr, 2004)</td>
</tr>
<tr>
<td>Nsp14</td>
<td>N7 Methyltransferase Exoribonuclease: RNA proofreading activity</td>
<td>(Denison et al., 2011; Eckerle et al., 2007; Minskaia et al., 2006)</td>
</tr>
<tr>
<td>Nsp15</td>
<td>Endoribonuclease of Uridines; IFN antagonist</td>
<td>(Bhardwaj et al., 2004, 2006; Deng et al., 2017, 2019; K. A. Ivanov et al., 2004; Kindler et al., 2017)</td>
</tr>
<tr>
<td>Nsp16</td>
<td>2’-O-Methyltransferase (2’-O-MT); IFN Antagonist</td>
<td>(Chen et al., 2011; Decroly et al., 2008)</td>
</tr>
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</table>

**Replication complex structure.** During replication, CoVs utilize host membrane to derive replicative membrane structures. The two major structures generated during most CoV replication are double membrane vesicles (DMVs) and convoluted membranes (CMs)(Gosert et al., 2002; Knoops et al., 2008; Prentice et al., 2004; Shi et al., 1999; van der Meer et al., 1999). In the nidovirus family, certain replicative structures are conserved. For example, coronaviruses
and arteriviruses all form DMVs in some capacity. Interestingly, some nidoviruses do not contain convoluted membranes, but instead alter membranes to produce paired membranes or spherules from the ER (van der Hoeven et al., 2016). Since all nidoviruses alter host membranes, the process is hypothesized to be crucial for viral replication and pathogenesis. Studies have shown that DMVs contain newly synthesized RNA during MHV infection, again implicating the necessity of the replication structures (Gosert et al., 2002).

Interestingly, nidoviruses contain a conserved region of the replicase polyprotein that drives the rearrangement of host membranes. For CoVs, the proteins necessary for membrane rearrangement is nsp3, nsp4, nsp5, and nsp6. Studies have shown that nsp3, 4, and 6 can rearrange membranes during expression in cells, absent of viral RNA synthesis and replication (Angelini et al., 2013; Clementz et al., 2008). Nsp5 is the main protease necessary for cleavage of nsp4 and nsp6 from the polyprotein. Nsp3 contains many functional domains and upon expression in cells, nsp3 will drive formation of perinuclear clusters of large multilamellar vesicles and membrane bundles. Nsp4 and nsp6 do not have any known enzymatic activity, but alter membrane rearrangement. Nsp4 shows reticular localization, but does not induce membrane rearrangements on its own. When nsp3 and nsp4 are paired, maze-like bodies of tubular membranes are formed inside the cells. When nsp6 is expressed, single-membrane vesicles accumulate around microtubule organization centers. It is hypothesized that the membrane pairing activity of nsp3 and nsp4 and the vesicle induction by nsp6 ultimately leads to the formation of DMVs and CMs in cells. (Gosert et al., 2002; Knoops et al., 2008; Neuman et al., 2014).

**RNA replication and transcription.** Following the translation, cleavage, and assembly of the replication complex proteins and structures, viral RNA synthesis occurs. RNA synthesis
generates new gRNA as well as sgRNA. SgRNA serves as mRNA for structural and accessory proteins. All positive-sense (+) sgRNAs are produced 3’ co-terminal with the sgRNAs being nested within each other. To generate more gRNA and sgRNA, the positive-sense RNA is first replicated into negative-sense (-) gRNAs and sgRNAs, which then acts as a template for positive-sense RNA synthesis. The negative-sense RNAs are about a 0.1% as abundant as positive-sense RNA and contain a poly-uridylate and anti-leader sequences (Masters, 2006; Sola et al., 2015).

Viral RNA replication begins with synthesis of negative-sense gRNA and sgRNA. The transcription is completed by nsp12, the RNA-dependent, RNA polymerase (RdRp). Also nsp7 and nsp8 are hypothesized to be necessary for RNA synthesis. Nsp7 is predicted to form a helicase for RNA binding, while nsp8 is a predicted primase. In fact, the structure of the nsp7-nsp8-nsp12 complex has been solved by cryo-EM (Kirchdoerfer and Ward, 2019). The current model for RNA synthesis is through two steps, continuous replication of genomic RNA and discontinuous transcription of sgRNA. RNA structure and sequence at the 3’ UTR of the (+) RNA recruits the replication proteins to the end of the genome. Nsp8 will generate a short RNA primer, which then binds in the polyA tail of the positive sense, forming the beginning of the (-) RNA. Nsp12 then synthesizes the remaining genomic (+) RNA is a continual transcription process (Masters, 2006; Sola et al., 2015).

Interestingly, each (+) sgRNA contains a short leader sequence (about 100 nt) at the 5’ end of the RNA similar to the full-length genomic RNA. The “attachment” of the leader sequence is hypothesized to occur during (-) sgRNA synthesis though a mechanism of discontinuous transcription (Sawicki et al., 2007; Sola et al., 2015)(Fig. 3). CoVs contain multiple transcription regulatory sequences (TRS) in the beginning of each sgRNA. The TRS
within the leader sequence (TRS-L) binds to TRSs in the body (TRS-B) which precede each sgRNA. The TRS has a conserved core sequence which is 6-7 nt in length. Since the core sequence is identical between the TRS-L and TRS-B sites, the TRS-L can basepair with a nascent (-) sense TRS-B. This base pairing drives the template to switch from the nascent RNA to the leader sequence and transcription continues through the leader to the end of the RNA. This process generates (-) sgRNA that contains the TRS sequence with an anti-leader sequence attached. The attachment of leader sequences implies a premature termination of synthesis and a template switch to the leader sequence, which would require long-distance RNA-RNA interactions. After the replication of the (-) gRNA and sgRNAs, these (-) sense RNAs act as templates for (+) mRNA synthesis. RNA secondary structure and sequences in the anti-leader sequence recruits the replication machinery. The replication machinery then begins transcription of the leader sequence and ORFs. Interestingly, longer sgRNAs can produce the smaller, nested sgRNAs as well.
Figure 3. Model of Discontinuous RNA Transcription during CoV Replication. This diagram represents the hypothesis that the (-) sense, sgRNAs obtain leader sequences through discontinuous transcription. Upon infection, the (+) sense, genomic RNA is released into the cytoplasm and replication complex proteins are translated. 1) Viral proteins will bind to the genomic RNA and form an RNA/Protein complex. Proteins will interact with the transcription regulatory sequences (TRS) at the leader region (TRS-L) or body (TRS-B) of the genomic RNA. The proteins will then facilitate long distance RNA interactions between the TRSs. 2) The RdRp will begin transcription of the (-) sense RNA starting in the polyA tail. 3) Upon transcription, the RdRp will continue to transcribe new (-) sense RNA. 4) After reaching a TRS, occasionally the RdRp will switch templates from the TRS-B to the TRS-L. This results in the RdRp to now transcribe the leader sequence to the end of a shortened (-) sense RNA, generating the (-) sense sgRNA. 5) Upon reaching the terminal end of the leader sequence, the newly transcribed (-) sense, sgRNA is released.
After transcription of the (+) gRNA and sgRNAs, the RNA is processed by numerous viral proteins to prevent host RNA sensor recognition. To pass as mRNA, viral RNAs are capped at the 5’end by a canonical 4-step capping mechanism of: 1) RTPase activity, encoded by nsp13 helicase, which hydrolyzes the gamma-phosphate of the mRNA; 2) guanylyltransferase activity to add GMP to the 5’-diphosphate RNA, which has yet to be assigned to an nsp; 3) N7-MTase activity, encoded by nsp14, which methylates the guanosine, leading to a cap-0 structure; and 4) 2’-O-methyltransferase activity, encoded by nsp16, carrying out further methylations and producing to cap-1 and cap-2 structures. Thus CoV RNA appears like cellular mRNAs, which allows them to escape host detection (Chen and Guo, 2016; Sawicki et al., 2007; Sola et al., 2015). The 3’ end of the genome also contains a long polyA tail, similar to host mRNA. This tail has recently been proposed to be attached through a non-canonical cytoplasmic adenylating signal that is encoded within the 3’UTR. Briefly host cleavage and polyadenylation specificity factor (CPSF) binds to an AGUAAA signal which recruits host proteins such as polyA binding protein (PABP). These host proteins will cleave at a cytidine residue and synthesize a new polyA tail on the RNA (Peng et al., 2016). Also, a study has determined that nsp8 contains 3’-terminal adenylyltransferase activity, which could add a polyA tail onto the end of the RNA (Tvarogova et al., 2019).

Since coronaviruses have a long RNA genome, the viruses also encode a proofreading system to maintain sequence fidelity. Nsp14 encodes an exonuclease (ExoN) as well as the N7-MTase, which upon ExoN mutation leads to a 15- to 20-fold increase in mutation accumulation (Eckerle et al., 2007; Minskaia et al., 2006). The removal of ExoN activity also increases the susceptibility of CoVs to mutagens such as ribavirin and 5-fluorouracil (Denison et al., 2011). Interestingly nsp10 has been linked to ExoN and nsp16 methyltransferase activities (Bouvet et
al., 2014; Decroly et al., 2011). Nsp14 and Nsp16 bind to nsp10 in overlapping sites on nsp10 which suggests a molecular switch, mediating interactions between RNA for proofreading and mRNA capping machineries.

**Virion assembly and release.** Following viral mRNA transcription, viral structural proteins are translated and the S, E, and M proteins are inserted into the endoplasmic reticulum. The proteins are trafficked to the ER-Golgi intermediate compartment (ERGIC). The genomic mRNA is encapsidated by N protein, which then binds to M protein in the ERGIC and buds into a new virion (Krijnse-Locker et al., 1994; Tooze et al., 1984). While M protein directs the protein-protein interactions for virion formation, E protein is also necessary for viral particles to form. S protein is involved in this step but is not required for packaging. The interaction site between N protein and M protein has been mapped to the C-terminus of the endodomain of M protein and the CTD of the N protein, however it is unclear how the nucleocapsid traffics to the ERGIC with viral RNA in tow. It is also unknown how only (+) sense RNA is packaged. MHV contains a packaging signal in the nsp15 coding sequence but mutating this signal does not affect virus production. Also, most coronaviruses do not contain a similar packaging signal, so this may be MHV specific (Fehr and Perlman, 2015; Masters, 2006).

After mature virions bud from the ERGIC, the virions are trafficked to the cell surface in vesicles and released by exocytosis. Additionally, S proteins that are not packaged into virions are transported to the cell surface where they function as cell-cell fusion proteins. Briefly, S proteins will interact with receptors on neighboring cells, causing fusion of cell membranes and formation of multinucleated syncytia. This allows the virus to spread cell to cell without exposure to extracellular factors like neutralizing antibodies.
Innate Immune Responses to RNA Virus Infection

Overview

The host immune system is constantly encountering and combatting pathogen invasions. Due to the presence of pathogens, innate and adaptive immune systems have evolved to detect and suppress the growth of invading pathogens. For viral infections, the innate immune system provides the initial immune response. The innate immune system senses viral particles, viral proteins, and viral nucleic acids and signals for an appropriate immune response to impair the growth and disease caused by the virus. The sensing of the viral infection is accomplished by pattern recognition receptors (PRRs). PRRs will recognize pathogen-associated molecular patterns (PAMPs), which will activate the PRR to signal for an anti-viral state in cells. One major antiviral signal produced during innate immune activation is Type I Interferon (IFNα and IFNβ). IFNs are cytokines that activate multiple interferon-stimulated genes (ISGs), which in turn have multiple antiviral functions to inhibit viral replication. Through autocrine and paracrine signaling, IFN binds to IFN receptors on the cell surface to signal the upregulation of hundreds of ISGs, which then induce an anti-viral state (Müller et al., 1994; Trinchieri, 2010).

Pathogen-Associated Molecular Patterns (PAMPs)

In order to stop viral replication, cells must first recognize that it has been infected with the virus. Cells initiate the innate immune response by detecting fragments or characteristics of the virus replication cycle. PAMPs are pathogen-derived molecular signals that are recognized as “non-host” by host sensors. PAMPs can be proteins, lipids, nucleic acids, or small molecules, but are not naturally produced by host cells absent of infection. PRRs will recognize and bind to a unique PAMP, which leads to activation of specific cellular pathways. For this document,
specific RNA PAMPs and PRRs will be discussed, specifically RNA PAMPs during CoV infection and RNA-specific PRRs (Kato et al., 2006).

**RNA PAMPs.** RNA PAMPs are ribonucleic acids that are recognized as non-cellular RNA. Usually RNA PAMPs contain unique secondary structures or are “missing” cellular RNA modifications. To understand how viral RNAs can differ from host RNA, it is first important to know what cellular RNAs look like.

Generally cellular RNA is generated in the nucleus or mitochondria of cells, then is transported to the cytoplasm, where the RNA is most active. Host RNA polymerases (Pol-I, Pol-II, and Pol-III) transcribe different types of RNAs. Pol-I transcribes ribosomal RNAs, Pol-II transcribes mRNA, and Pol-III transcribes tRNA. These polymerases generate a precursor transcript with a 5’ triphosphate on the RNA. Before translocation out of the nucleus or mitochondria, the RNA is then modified by host proteins to remove the 5’ triphosphate. Ribosomal RNA is cleaved by a phosphatase to remove the triphosphate. mRNA is capped through N7-guanosine methylation. Host tRNAs are cleaved to remove the triphosphate or methylated to prevent detection. The 3’-end of mRNA also contains a poly-adenosine (polyA) tail that is attached through host polyadenylation factors. Host RNAs also contain internal modifications. ADAR1 deaminates adenosine residues to inosines, which destabilize RNA stem loop structures. This is used to prevent Alu elements in certain mRNAs from forming dsRNA stem loops that are immunostimulatory. Host tRNAs are modified by ribose 2’O methylation of internal guanosines, which dampens immune stimulation by the tRNAs (Bouvet et al., 2014; Brocard et al., 2017; Ramanathan et al., 2016). If viral RNAs do not mimic host RNA structures and modifications, then they may activate innate immune sensors.
Two predominant viral RNA signals that activate host sensors are a 5’ triphosphate on RNA and dsRNA. Since most viruses use relatively simple replication machinery, the viral RNAs resemble premature cellular RNAs and contain 5’ triphosphate ends. CoV (+) sense RNA actually lacks the 5’ triphosphate due to the viral proteins that cap the RNA (Kindler and Thiel, 2014). The RNA that activates innate immunity during CoV infection was thought to be dsRNA (Deng et al., 2017; Kindler et al., 2017; Roth-Cross et al., 2008). Since CoVs replicate through long strands of complement RNA, there are dsRNA species formed from the gRNA and each sgRNA that are thousands of basepairs long (Lai et al., 1982; Sethna et al., 1991). Long complemented dsRNA species do not normally exist in host cells, so these long dsRNA are detected as PAMPS by host sensors. Previous studies utilize synthetic polyI:C as a long dsRNA PAMP to determine the pathways that detect dsRNA. Researchers hypothesized that CoV dsRNA formed by complementary strands of RNA thousands of basepairs in length will form a PAMP and activate host sensors.

**Development of dsRNA antibodies.** To study dsRNA in cells, researchers generated monoclonal antibodies directed against dsRNA epitopes (Schonborn et al., 1991). To develop the dsRNA antibodies, mice were immunized with yeast L-dsRNA (Sweeney et al., 1976), and murine plasmablasts were isolated and screened for antibody specificity. The anti-dsRNA monoclonal antibodies (mAbs) were screened for their binding to ssRNA, ssDNA, dsDNA, and dsRNA. The researchers selected antibodies that only bound to dsRNA with high affinity with no additional binding to ssRNA, ssDNA, or dsDNA. The developed antibodies are now available for purchase from Scicons Inc., and designated as mAb K1 and J2 (www.scicons.eu). The anti-dsRNA mAbs have mainly been used to detect viral dsRNA during infection of cells. One of the techniques for viral dsRNA detection is immunofluorescence. For CoV infection, the dsRNA
signal is detected by immunofluorescence and the dsRNA signal co-localizes with CoV replication complexes (Weber et al., 2006). Using immuno-gold EM staining with a dsRNA antibody, the dsRNA signal is localized within DMVs during SARS-CoV infection (Knoops et al., 2008). This dsRNA result led to the hypothesis that CoV dsRNA is sequestered within DMVs, which prevents the dsRNA from being detected by host PRRs.

Although these antibodies have been integral in detecting viral dsRNA, the exact epitopes that the antibodies bind to on the viral RNA are not fully understood. A previous study found that the J2 anti-dsRNA mAb antibody bound to a dsRNA signal that was at least 40 nucleotides (nt) long (Bonin et al., 2000). The dsRNA antibody also preferentially bound to polyA:U or mixed based pairs compared to polyI:C. It remains to be determined if the epitope that these antibodies recognize are RNA sequence or RNA structure dependent.

**Interferon Signaling**

Once the viral PAMPs are produced, the cell must recognize and react to the viral infection. One characterized innate immune pathway in response to viral infections is the interferon signaling pathway. Interferons were discovered in the 1950’s as supernatant cytokines that would inhibit influenza virus replication ( Isaacs et al., 1957; Isaacs and Lindenmann, 1957). Upon detection of viral PAMPS, PRRs will signal for cells to express interferon, which is then secreted by the cells. The secreted interferon will interact with interferon receptors, which leads to an upregulation of genes involved in forming an antiviral response (Müller et al., 1994). The interferon response is crucial for cells to control viral replication and to limit damage to the host during a viral infection.

**Type I interferons.** Interferon (IFN) is a family of cytokines that are recognized as key component for defense against viral infections. There are three major types of IFN, type I (IFNα
and IFNβ), type II (IFNγ), and type III (IFNλ). Each type of IFN has its own specific cellular receptor that the IFN signals through. Type I signals through IFNα receptor (IFNAR), Type II signals through the IFNγ receptor (IFNGR), and Type III signals through the IFNλ Receptor (IFNLR) (Sadler and Williams, 2008). For this dissertation, I will address type I IFN expression and signaling pathways.

Type I IFNs have many subsets of cytokines with 13 IFNα’s, IFNβ, IFNκ, IFNε, IFNο, IFNτ, and IFNδ all signaling through the IFNAR. While the function of the overall class of type I IFNs has been well characterized for inhibiting viral infections, what the differences are between the subsets of type I IFN is not well known. Studies using IFNAR−/− mice have shown that mice lacking type I IFN signaling have increased susceptibility to numerous virus and microbial infections (Müller et al., 1994). These studies show that a type I IFN response is necessary to control viral pathogenesis in vivo and limit the ability of viruses to replicate inside the host cell.

**IFN expression stimulated by RIG-I-like receptors.** Type I IFN can be produced by almost any cell type (de Weerd and Nguyen, 2012). Transmembrane or cytosolic receptors, such as RIG-I-like receptors (RLRs), will recognize a PAMP and activate the IFN signaling pathway. These receptors will bind to PAMPs and activate a host signaling cascade to induce the cells to express IFN. The RLR class of receptors consist of retinoic acid-inducible gene I (RIG-I), melanoma differentiation associated protein 5 (MDA5), and Laboratory of Genetics and Physiology protein 2 (LGP2). RLRs belong to the Asp-Glu-Ala-Asp (DEAD) box family and contain an RNA helicase domain with ATPase activity and a C-terminal Domain (CTD), which mediates the specificity of RNAs detected by the sensor. RIG-I and MDA5 also contain two caspase activation and recruitment domains (CARDs), which allow for signaling by CARD dimerization with the mitochondrial antiviral signaling protein (MAVS) (Yoneyama et al., 2004).
Deletion studies have shown that RIG-I and MDA5 recognize different types of viral infections, indicating that each sensor recognizes a specific RNA target (Kato et al., 2006). In contrast to RIG-I and MDA5, LGP2 is not well understood. LGP2 can act as a negative regulator for RLR signaling but can also activate IFN (Saito and Gale Jr, 2008). Recent studies have shown that LGP2 acts as a positive regulator for MDA5 signaling (Bruns et al., 2014; Uchikawa et al., 2016). An important aspect of RLRs is that they recognize and bind to dsRNA, although not all types of dsRNAs will activate the sensors.

The host pattern recognition receptor that is central to the studies in this dissertation is MDA5. Previous studies showed that MDA5 binds to and recognizes long dsRNA signals (>1,000 bp)(Kato et al., 2008), but MDA5 has also been shown to be activated by higher-order structured RNA (Pichlmair et al., 2009) and RNAs lacking 2-O-methylation (Züst et al., 2011). Upon multiple MDA5 proteins binding to a long dsRNA strand, the CARD domains of MDA5 will bind and induce oligomerization of MDA5 proteins. The oligomerized MDA5 proteins then act as a scaffold for binding and oligomerization of downstream signaling proteins like mitochondrial antiviral-signaling protein (MAVS). The binding of MDA5 to MAVS leads to a nucleation of MAVS proteins, which then form MAVS helical filaments on the mitochondrial surface (Reikine et al., 2014) The MAVS filaments serve as a platform for further signaling events. MAVS signals a kinase cascade to phosphorylate TBK1 and IKKi, which then activates IRF3 to translocate to the nucleus of the cell. IRF3 interacts with IRF7 and binds to promoter sites upstream of IFN to begin transcription of IFN mRNAs (Trinchieri, 2010). The IFN mRNA is subsequently translated and the IFN protein is secreted from the cell for autocrine and paracrine signaling. The secreted IFN will then bind to host receptors, with type I IFN interacting with IFNAR on surface of the cell (Fig. 4).
**Activating interferon stimulated genes.** For IFN to induce an antiviral state in a cell, the IFN cytokine must signal for the induction of IFN-stimulated genes (ISGs). Upon binding of type I IFN to the IFNAR, the IFNAR activates a signaling cascade inside the cell. IFNAR is present on all cell types, so this signaling pathway could occur in any cell of the body (de Weerd and Nguyen, 2012). IFNAR signals through two Janus family kinases, Jak1 and Tyk2, which results in the recruitment of STAT1 to STAT2 and the formation of a STAT1-STAT2 heterodimer. This STAT1-STAT2 heterodimer migrates to the nucleus of cells and interacts with transcription factors, such as IFN regulatory factor (IRF) 9 and IFN-stimulated gene Factor 3 (ISGF3). The activated transcription factors then bind to upstream IFN-stimulated response elements (ISRE), which promotes transcription of IFN-stimulated genes (ISGs) (Trinchieri, 2010). The IFN-induced transcription is regulated by the post-translational modification of STATs, chromatin remodeling, the epigenetic landscape, and other transcription factors, co-activators, and co-repressors (Ivashkiv and Donlin, 2014). The binding of IFNs to the IFNAR leads to upregulation of over 300 genes, most of which have been shown to have antiviral functions (Der Zhou 1998). These interferon-stimulated genes (ISGs) will produce an antiviral state not only in the virally infected, IFN-producing cells, but also in uninfected neighboring cells, limiting the spread of the viral infection (Fig. 4).
Figure 4. IFN Signaling Pathways. MDA5 senses dsRNA in the cytoplasm of the cell and oligomerizes on the dsRNA. The MDA5 activates MAVS which leads to a phosphorylation of TBK1 and IKKi. The activated TBK1 phosphorylates IRF3 and IRF7, which translocate to the nucleus and signal for production of IFN mRNA. IFN protein is produced and secreted from the cell. Type I IFN binds to and activates IFNAR at the cell surface. IFNAR activation phosphorylates Jak1 and Tyk2, which then phosphorylate STAT1 and STAT2, causing dimerization. Dimerized STAT1 and STAT2 bind with IRF9 and ISGF3, translocate to the nucleus, and bind to an ISRE to signal for production of ISGs.

Interferon-Stimulated Genes (ISGs)

Upon IFN signaling, cells will induce multiple proteins that have a range of anti-viral functions. These functions can be RNases, proteases, ubiquitinating proteins, proteins that alter membrane rigidity or permeabilization, etc. Some of these proteins are ADAR1, ISG20, IFIT1, IFIT2, ISG15, ISG54, Mx1, PKR, and OAS. Many of the host PRRs that regulate IFN signaling are also upregulated by IFN, forming an amplification signaling loop. In addition to the
production of ISG proteins, IFN-induced microRNAs that can also alter expression of cellular proteins (Pedersen et al., 2007). Two of the ISG proteins, PKR and OAS, are discussed below.

**Protein Kinase R.** Protein Kinase R (PKR) is a host dsRNA receptor. PKR is an ISG that can be induced by type I or type III IFN signaling. The upregulation of PKR during antiviral states leads to the alteration of numerous cellular pathways (Gal-Ben-Ari et al., 2019; Kuhen and Samuel, 1997). Upon PKR binding to dsRNA, PKR forms a homodimer and autophosphorylates itself (Taylor et al., 2001). The phosphorylated PKR is then an active kinase which will phosphorylate numerous host proteins. One protein PKR phosphorylates is eIF2α, which prevents eIF2α from initiating translation by host ribosomes thus inhibiting translation of cellular proteins (Hoang et al., 2018). PKR activation also leads to the activation of the FADD apoptotic pathway, eventually leading to cell death (García et al., 2007). Regardless of whether eIF2α phosphorylation will inhibit the protein translation, the apoptosis of the infected cells leads to antiviral response and inhibition of viral replication. (Gal-Ben-Ari et al., 2019; Krahling et al., 2009).

**2’5’ Oligoadenylate Synthase.** 2’5’ oligoadenylate synthetases 1 and 2 (OAS1/2) are proteins from a family of interferon-induced PRRs that bind and recognize dsRNA. OAS binding to dsRNA activates OAS enzymatic activity, which polymerizes ATP into 2’-5’-linked oligomers (Rebouillat et al., 1998). The 2’5’ oligoadenylates then bind to RNAse L and activate the RNAse activity of the enzyme (Dong and Silverman, 1995; Ogden et al., 2015). This process is regulated by a 2’5’ phosphodiesterase, which degrades the 2’5’ oligoadenylate and prevents RNAse L activation (Silverman and Weiss, 2014). RNAse L will cleave any host and viral RNA. The RNAse L activity will eliminate the viral genome, prevent viral protein production, and damage
host machinery that are necessary for viral replication. Eventual loss of the host RNA leads to apoptosis of the cell, further preventing viral replication (Silverman, 2007).

Coronavirus Immune Antagonists

Overview

Viral infection can induce the expression of IFNs just hours after initial viral infection. Since IFN signaling and ISGs induce an antiviral state, many viruses encode proteins that will inhibit or antagonize the innate immune signaling pathways. CoVs are notorious for delaying IFN responses (Channappanavar and Perlman, 2017; Menachery et al., 2014; Roth-Cross et al., 2008; Totura and Baric, 2012). CoV-infected cells will eventually express IFN and stimulate the production of ISGs, but the delayed IFN response leads to a dysregulation of the overall innate immune response. The expression of IFN during CoV infection is stimulated through a MDA5-mediated pathway, suggesting that viral dsRNA is detected during infection (Roth-Cross et al., 2008).

The delayed IFN signaling during CoV infection leads to detrimental dysregulation of innate immune signaling during in vivo infection. There are two pathogenic types of human CoVs, low pathogenic and highly pathogenic CoVs. Low pathogenic CoVs cause a mild respiratory illness, whereas high pathogenic CoVs, like SARS-CoV and MERS-CoV, will infect lower airways and can cause fatal pneumonia. The CoV-induced pneumonia is associated with massive inflammatory cell infiltration and elevated pro-inflammatory cytokine/chemokine profiles, which results in acute lung injury and acute respiratory distress syndrome. For both SARS-CoV and MERS-CoV, viral infection leads to extensive infiltration of neutrophils and macrophages in the lungs of infected patients. The lung histology showed lung consolidation and edema, which led to loss of lung function. The increased inflammatory cell infiltration is driven
by significant upregulation of pro-inflammatory chemokines, such as CCL3, CCL5, CCL2, and CXCL10. Similarly, infected patients have enhanced expression of pro-inflammatory cytokines, such as IFN\(\gamma\), IL-1, IL-6, and IL-12 (Channappanavar and Perlman, 2020, 2017). Interestingly, treating mice with IFNs early during SARS-CoV and MERS-CoV infection significantly reduced the pro-inflammatory response and reduced immunopathogenic disease (Channappanavar et al., 2019, 2016). These studies highlight that appropriate timing of IFN signaling is crucial for controlling the pathogenesis of CoV infections.

One cell type that is important in the regulation of the innate immune response to CoVs is the macrophage. For MHV, macrophages play a crucial role in recognizing and inducing the type I IFN signal (Cervantes-Barragán et al., 2009; Roth-Cross et al., 2008). Priming macrophages with IFN, leads to an enhancement of an antiviral state and decrease in the immunopathogenesis caused by CoV infection (Zhou et al., 2010). Using virus infection of macrophages, we can identify and evaluate the viral factors involved in antagonizing innate immune pathways. We and others found that removing the activity of viral innate immune antagonists resulted in reduced levels of virus replication in cell culture and attenuation of disease in vivo (Deng et al., 2017; Kindler et al., 2017). Many groups hypothesize that mutating the CoV immune antagonists, which allows for early detection of the virus replication and an early IFN response, will produce a live-attenuated vaccine strain (Deng et al., 2017, 2019; Menachery et al., 2018). Many studies have therefore sought to determine the mechanisms of which CoV proteins will inhibit IFN signaling and innate immunity.

**Hiding the Viral RNA/Mimicking Host mRNA**

One strategy for CoVs to inhibit innate immune signaling has been to mimic host mRNA and hide the viral PAMPs from detection by host sensors. The genomic positive-sense RNA has
characteristics of host mRNA, so will appear as “native” RNA to host sensors. Viral proteins are involved in capping viral RNAs. RIG-I is able to detect RNA containing 5’-triphosphates, which normally are not present on host RNA. CoVs encode an RNA-triphosphatase (nsp13), a N7-methyltransferase (nsp14), and a 2’-O-methyltransferase (nsp16). These viral proteins mimic host machinery to cap the viral RNA, thus preventing immune activation (Kindler and Thiel, 2014). CoV mRNA also contains a polyA tail, again mimicking host mRNA. The addition of the polyA tail is possible performed by nsp8 (Tvarogova et al., 2019).

In addition to mimicking host mRNA, viral dsRNA signals are proposed to be sequestered by viral replication complexes. Immuno-gold electron microscopy staining showed that viral dsRNA signal was localized within DMVs (Knoops et al., 2008). The DMVs are not open to the cytoplasm of cells, so cytoplasmic sensors cannot physically interact with RNA inside of DMVs. While the dsRNA signal is localized to DMVs early in infection, the dsRNA will disperse into more cytoplasmic staining late in infection (Becares et al., 2016). This dispersal of the dsRNA signal may be the signal that induces late IFN signaling during CoV infection. While dsRNA may be sequestered away from host sensors and other viral RNAs mimic host mRNA, further studies are necessary to determine what the RNA signal is what activates host innate immune pathways late during CoV infections.

**Overexpression of CoV Innate Immune Antagonists**

Many studies have sought to identify the viral protein(s) that antagonize host signaling pathways. To determine which viral proteins are important, researchers overexpressed CoV proteins in the context of IFN stimulation to determine if the viral proteins would inhibit the IFN signal. For example, if SARS-CoV nsp1, PLpro, nsp7, nsp15, or ORF6 proteins are expressed during the infection of an IFN-stimulating virus, then the IFN stimulation is reduced, showing
potential roles of IFN antagonism for these proteins (Frieman et al., 2009). Overexpression studies have provided evidence that indicates that viral proteins can inhibit innate immune pathways. For CoV, many accessory proteins can inhibit innate immune signaling. SARS-CoV ORF3b and ORF6 will prevent the translocation of IRF3 to the nucleus after MDA5 activation (Freundt et al., 2009; Kopecky-Bromberg et al., 2007). Additionally ORF6 can block nuclear import, thus inhibiting the signaling pathway of IFN (Hussain and Gallagher, 2010). The overexpression of SARS-CoV ORF3a induces ER stress in cells and downregulates the IFNAR1 protein expression, which will dampen IFN responses (Minakshi et al., 2009). For MERS-CoV, overexpression of ORF4a will inhibit MDA5 activation though interactions with dsRNA, as well as prevent PKR activation in cells (Niemeyer et al., 2013; Rabouw et al., 2016).

Overexpression of replicase proteins can also inhibit innate immune signaling. SARS-CoV PLPro, which contains protease, deubiquitinating activity, and deISGylating activity, can inhibit IFN induction (Clementz et al., 2010), can block IRF3 and NF-κB signaling (Frieman et al., 2009), and inhibit STING signaling (Sun et al., 2012). MHV PLP2 also functions as protease, deubiquitinase, and deISGylatilase, which can prevent IFN induction in cells (Mielech et al., 2014; Yang et al., 2014). Overall, ectopic expression of CoV proteins have led to the identification of multiple potential IFN and innate immune antagonists.

**Generating Coronaviruses with Inactive Immune Antagonists**

To determine if CoV proteins functioned as immune antagonists, CoVs containing mutations in IFN antagonists were generated using reverse genetics. A list of all innate immune antagonists that have been determined during CoV infection is listed in Table 2.
Table 2. **Innate Immune antagonists during CoV Infection.** Listed are the studies that generated viruses that contained mutations in innate immune antagonists.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Viral Protein</th>
<th>Target; Enzymatic Activity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SARS</td>
<td>ORF6</td>
<td>Inhibits Nuclear Import</td>
<td>(Frieman et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Nsp1</td>
<td>Block Phosphorylation of STAT1</td>
<td>(Wathelet et al., 2007)</td>
</tr>
<tr>
<td>MERS</td>
<td>Ns4a</td>
<td>Inhibits PKR Phosphorylation</td>
<td>(Comar et al., 2019)</td>
</tr>
<tr>
<td></td>
<td>Ns4b</td>
<td>Inhibits activation of RNase L</td>
<td>(Comar et al., 2019)</td>
</tr>
<tr>
<td></td>
<td>Nsp16</td>
<td>Necessary for IFN resistance and pathogenesis; 2’-O-Methyltransferase</td>
<td>(Menachery et al., 2017, 2018)</td>
</tr>
<tr>
<td>MHV</td>
<td>Ns2</td>
<td>Inhibits RNaseL activation; 2’-phosphodiesterase</td>
<td>(Zhao et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Nsp15</td>
<td>Inhibits production of dsRNA PAMP; Endoribonuclease</td>
<td>(Athmer et al., 2018; Deng et al., 2017; Kindler et al., 2017)</td>
</tr>
<tr>
<td></td>
<td>ORF5a</td>
<td>Inhibits IFN expression</td>
<td>(Koetzner et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Nsp3/PLP2</td>
<td>Inhibits IFN expression; Deubiquitinase, Protease, DeiSGylase</td>
<td>(Mielech et al., 2015)</td>
</tr>
<tr>
<td></td>
<td>Nsp3/Macromdomain</td>
<td>Inhibit IFN expression;</td>
<td>(Deng et al., 2019)</td>
</tr>
<tr>
<td></td>
<td>Nsp14</td>
<td>Necessary for IFN resistance; Exonuclease</td>
<td>(Case et al., 2017)</td>
</tr>
<tr>
<td>HCoV-229E</td>
<td>Nsp15</td>
<td>Inhibits activation of dsRNA sensors; Endoribonuclease</td>
<td>(Kindler et al., 2017)</td>
</tr>
<tr>
<td>FIPV</td>
<td>ORF7</td>
<td>Necessary for IFN resistance</td>
<td>(Dedeurwaerder et al., 2014)</td>
</tr>
<tr>
<td>TGEV</td>
<td>ORF7</td>
<td>Prevents activation of dsRNA sensors/IFN expression</td>
<td>(Cruz et al., 2013, 2011)</td>
</tr>
<tr>
<td></td>
<td>Nsp14</td>
<td>Inhibits dsRNA pathways; Exonuclease</td>
<td>(Becares et al., 2016)</td>
</tr>
<tr>
<td>PEDV</td>
<td>Nsp15</td>
<td>Inhibits IFN expression; Endoribonuclease</td>
<td>(Deng et al., 2019)</td>
</tr>
<tr>
<td></td>
<td>Nsp16</td>
<td>Necessary for IFN resistance; 2’-O-Methyltransferase</td>
<td>(Hou et al., 2019)</td>
</tr>
</tbody>
</table>
The generation of IFN-stimulating or IFN-sensitive CoVs has been driven by deleting or mutating the sequences that encode putative interferon antagonists. Since overexpression studies implicated accessory proteins as IFN antagonists, researchers hypothesized that deletion or mutation of accessory proteins would result in attenuated viruses. For example, catalytic mutations in MERS-CoV ns4a and ns4b have shown that ns4a prevents the phosphorylation of PKR and induction of IFN, whereas ns4b inhibits activation of RNAse L (Comar et al., 2019).

Interestingly, studies using mutant CoVs have also implicated numerous replicase proteins as IFN antagonists. SARS-CoV Nsp1 had been determined to decrease phosphorylation of STAT1, thus inhibiting the signaling for multiple ISG production (Wathelet et al., 2007). Mutating nsp16 decreases the IFN resistance and pathogenesis of MERS-CoV (Menachery et al., 2017, 2018). For MHV, there have been multiple viruses generated with mutated IFN antagonists. MHV lacking ns2 phosphodiesterase activity exhibit activation of RNAse L (Zhao et al., 2012). Multiple mutations in nsp15 lead to increased activation of dsRNA sensing pathways, which eventually leads to attenuation of the viral replication (Athmer et al., 2018; Deng et al., 2017; Kindler et al., 2017). Also mutations in nsp3 have led to attenuation of viral replication and activation of IFN signaling (Deng et al., 2019; Knoops et al., 2008; Mielech et al., 2015). Overall, CoVs contain multiple proteins that will interact and inhibit many host innate immune pathways. For this dissertation, I will discuss the mechanism for CoV nsp15/EndoU in inhibiting the detection of dsRNA by host sensors.

**Nonstructural protein 15/EndoU**

**Overview**

For this dissertation, I will focus on one CoV protein that we have found to be an IFN antagonist. This protein contains an endoribonuclease domain that cleaves at uridine residues,
hence the name EndoU or NendoU (Snijder et al., 2003). EndoU is encoded by nsp15 and is translated as part of pp1ab before cleavage into a separate EndoU protein by the 3CLpro. Here I will discuss the background information on EndoU, which led us to study this protein in the context of innate immune antagonism.

**Discovery and Conservation of EndoU**

EndoU/nsp15 is a protein that is generated during proteolytic processing of the pp1ab polyprotein. The nsp15 protein generated during MHV infection is 35 kDa (Heusipp et al., 1997) and is localized to replication complexes in perinuclear regions of infected cells (Shi et al., 1999). EndoU was first characterized by using bioinformatics to compare the viral sequence with known sequences of other viral and host proteins. A previous study sought to describe the unknown functions of many of the cleavage products of the CoV polyproteins and found that SARS-CoV EndoU had a domain that was conserved with a known endoribonuclease called XendoU (Snijder et al., 2003). XendoU is a host endoribonuclease found in *Xenopus laevis* that cleaves polyU-specific RNA, produces 2’-3’-cyclic phosphate termini, and processes small nucleolar RNA (snoRNA) (Laneve et al., 2003).

EndoU has been found to be conserved across the nidovirus family. All known coronaviruses encode EndoU within nsp15. Arteriviruses contain an EndoU domain in nsp11. In CoVs, EndoU contains less than 50% amino acid conservation, but the catalytic histidines and endoribonuclease domain folds are fully conserved (Deng and Baker, 2018). Due to this conservation, EndoU was first named NendoU for being a nidovirus-specific EndoU (Snijder et al., 2003). Interestingly, *Mesoniviridae* and roniviruses, which infect invertebrates, were found to belong to the nidovirus family, but lack the EndoU domain (Lauber et al., 2012; Nga et al.,
These findings suggest that EndoU is only conserved among vertebrate-infecting nidoviruses, thus might play a role only during vertebrate infections.

**EndoU Biochemical Cleavage Assays**

With EndoU being conserved among CoVs, many studies were conducted to determine the structure and function of EndoU during CoV replication. One study expressed and purified EndoU from SARS-CoV, MHV-CoV, and IBV-CoV. They found that purified EndoU cleaves ssRNA and dsRNA, but not ssDNA or dsDNA (Bhardwaj et al., 2004). An additional study found that EndoU from SARS-CoV and hCoV-229E both cleaved ssRNA and dsRNA, but more effectively cleaved dsRNA. This study also showed that EndoU produced 2’-3’ cyclic phosphate ends similar to XendoU (K. A. Ivanov et al., 2004). Both studies determined that Mn$^{2+}$ was necessary for EndoU activity in the biochemical assays and that EndoU cleaves at uridine residues. A follow-up study found that EndoU only cleaves uridines at the 3’ of uridine residues (Bhardwaj et al., 2006). Interestingly, the RNA sequence recognized and cleaved by EndoU during the biochemical assays was not specific. One study tried to determine if only specific uridines of a viral RNA stem loop were cleaved to determine if structure of the RNA played a role in specificity (Bhardwaj et al., 2006). They found that not all uridines of the RNA stem loop were cleaved, but multiple uridines were cleaved with no specific epitope emerging. This result suggests that EndoU cleavage can be influenced by RNA structure, but an exact cleavage site during viral replication was still unknown.

For MHV-A59, biochemical studies show that EndoU will cleave ssRNA and dsRNA. Importantly, mutation of the two catalytic histidines, H262 and H277, to alanines resulted in loss of RNA cleavage activity (Kang et al., 2007). Further studies have confirmed EndoU activity is conserved in CoVs such as MERS-CoV (Zhang et al., 2018) and even arteriviruses (Cao et al.,
2008; Nedialkova et al., 2009) showing conservation of EndoU cleavage activity and specificity across the nidovirus family.

**EndoU Overexpression Studies**

To study EndoU in the cellular environment, many studies overexpressed EndoU and measured the effect on host gene expression pathways to determine which pathways were perturbed by EndoU activity. Many pathways were inhibited by the overexpression of EndoU. EndoU inhibits MAVS-mediated apoptosis (Lei et al., 2009), can bind to retinoblastoma tumor suppressor protein and block tumorigenesis (Bhardwaj et al., 2012), and can block IFN production (Frieman et al., 2009). Researchers reported that overexpression of EndoU prevents phosphorylation and nuclear translocation of the NF-κB p65 subunit. (Liu et al., 2019).

Additionally, overexpression of PRRSV EndoU/nsp11 blocked MAVS and RIG-I signaling, indicating a role of EndoU of modulating IFN signaling during arterivirus infection (Sun et al., 2016). Overall, overexpression of EndoU in cell culture can inhibit multiple pathways. The main pathways that were reported were MAVS-related IFN signaling pathways. The expression of EndoU can inhibit these pathways in the absence of viral replication and infection, indicating a possible role for EndoU as an IFN antagonist.

One characteristic of overexpression is that EndoU is no longer co-localized to the replication complex, but has cytoplasmic localization (Liu et al., 2019; Sun et al., 2016). This cytoplasmic localization may alter the protein and RNA interactions of EndoU. During viral replication, EndoU is confined to the viral replication complex and may only interact with proteins and RNAs near the replication complex. Also, we note that in our hands, EndoU activity during overexpression prevents the translation of multiple biosensors, such as luciferase and RFP (data not shown). While overexpression studies may highlight new pathways for EndoU to
interact with, we propose that studying EndoU in the context of viral infection will provide a more accurate method to determine the function EndoU.

**EndoU Structure**

Researchers have shown that EndoU can be expressed and purified for biochemical assays, and the structure of EndoU has been determined for several CoVs. The first study did not resolve the crystal structure of EndoU, but using cryo-EM, determined that SARS-CoV EndoU can form either monomeric or hexameric oligomers. Interestingly, mutations that disrupt hexameric formation also disrupt the RNA cleavage activity of EndoU. This suggests that the hexamer formation by EndoU is necessary for activity during viral replication (Guarino et al., 2005). The hexameric, X-Ray crystal structures of EndoU for SARS-CoV (Ricagno et al., 2006), MHV-A59 (Xu et al., 2006), and MERS-CoV (Zhang et al., 2018) have been solved (Fig. 5). X-Ray crystal structure for the arterivirus PRRSV EndoU/nsp11 and PDCoV EndoU show that functional EndoU forms a dimer, showing divergence from the other CoVs in oligomerization.

Further studies sought to determine how RNA was bound and recognized by EndoU. One study crystalized polyU RNA with EndoU and found that the RNA bound in a channel formed during the oligomerization of EndoU (Bhardwaj et al., 2006). Two strands of RNA ran along the EndoU complex into the enzymatic active site. Another study removed the domain that drove hexamer formation from EndoU and found that the active pocket changed between trimmed monomeric EndoU and full-length hexameric EndoU (Joseph et al., 2007). Researchers tried to determine how RNA bound into the catalytic mutant of SARS-CoV EndoU (Bhardwaj et al., 2008). This study determined that the RNA binding pocket had unreported flexibility and that EndoU binding pocket structure is altered by the hexamer formation of the EndoU subunits. Overall, purified EndoU oligomerizes to form an active RNAse complex.
Figure 5. X-Ray Crystal Structure of MHV-A59 EndoU. The catalytic histidines are highlighted in red. This structure was solved by Xu et al. (Xu et al., 2006) and is available at the RCSB Protein Data Bank (PDB# 2GTH).

Reverse Genetics EndoU-Mutant Viruses

In order to study EndoU in the context of viral replication, CoVs with mutations in EndoU were generated. The first attempt was using hCoV-229E, but a replicative virus could not be recovered, suggesting that EndoU was necessary for viral replication (K. A. Ivanov et al., 2004). Similarly, catalytic mutations in EndoU for the arterivirus EAV could not be recovered. Mutations in other conserved, non-catalytic sites showed reduced plaque size and a decrease in the yield of infectious virus produced compared to wild-type infection (Posthuma et al., 2006).

In MHV-A59, viruses containing EndoU catalytic mutations, H262A and H277A, could be recovered. These viruses generated similar plaque sizes in DBT cells, but had about a 10-fold reduction in viral titers upon infection, suggesting that EndoU activity was necessary for efficient viral replication. When studying viral RNA production, the study found that EndoU-mutant
viruses produced less positive and negative-sense sgRNAs, which led to a decrease in the production of the spike protein compared to wild-type virus. Researchers found that if EndoU was transiently transfected into infected cells, then viral RNA synthesis was partially restored (Kang et al., 2007).

More recently studies have utilized different cell types to determine the role of EndoU during viral infection. One study in situ tagged EndoU to study interaction of EndoU with different replication complex proteins. The researchers found that EndoU localized with replication complexes during infection and that during immunoprecipitation, EndoU was precipitated with nsp12 and nsp8. These results indicate that EndoU complexes with the RNA polymerase during replication (Athmer et al., 2017). Interestingly, since the in situ tag was added to the packaging signal of MHV, this tagged virus had increased packaging of negative-sense RNA in the virions and induced increased IFN production upon infection (Athmer et al., 2018).

One of our studies was published at the same time of another study that focused on EndoU (Kindler et al., 2017). The findings in this study support our study with a virus with an EndoU catalytic mutation (H277A) that can replicate as efficiently as wild-type virus in the absence of innate immune signaling. Kindler and colleagues found that an EndoU-mutant virus induced IFN in an MDA5-dependent manner and activated multiple host sensors such as PKR and OAS. The activation of the innate immune sensors led to an attenuation of the viral replication in macrophages and during in vivo infections. Our studies utilizing an EndoU-catalytic mutant (H262A) virus are discussed in this dissertation.

Currently, work in the Baker Lab has produced another CoV that contains an EndoU catalytic mutation. Deng et al. have produced a EndoU-mutant porcine epidemic diarrhea virus (PEDV), that can efficiently replicate in Vero cells. In IFN-responsive PK1 cells, the EndoU-
mutant virus has increased type I and type III IFN signaling. The EndoU-mutant virus also contained a mild attenuation during infection of piglets compared to the wild-type infectious clone (Deng et al., 2019). Overall, EndoU-mutant viruses can be generated with efficient replication if they are propagated in the absence of host innate immune sensing. Prior to our work, it was uncertain if EndoU played a role in inhibiting innate immunity during viral replication.

**Objective of Dissertation Research**

The goal of my project is to determine the role of EndoU during viral infection. While EndoU has been shown to cleave ssRNA or dsRNA in biochemical assays, the target and function of EndoU during viral replication was still unknown. Previous studies had mixed results on whether EndoU was necessary for viral RNA synthesis. Since overexpression of EndoU in cell inhibits IFN signaling, we also sought to determine if EndoU inhibited innate immune signaling during viral infection. To test if EndoU was an IFN antagonist or played a role in viral replication, we generated an EndoU-mutant (EndoUmuts) MHV-A59. We predicted that in the absence of IFN signaling, the EndoUmuts virus would grow similar to wild-type MHV. With IFN signaling intact, we hypothesized the EndoUmuts virus would be attenuated and stimulate a heightened IFN response compared to wild-type MHV. Since EndoU was proposed to cleave dsRNA during infection, we hypothesized that EndoUmuts viruses would have an increased activation of dsRNA sensors due to an increased abundance of a dsRNA epitope. We predicted that one role of EndoU would be to reduce the dsRNA in infected cells, reducing the signal to activate host sensors. Here I describe my efforts to i) characterize the IFN antagonism of EndoU during mutant infection; ii) determine the mechanism for EndoU to inhibit the activation of dsRNA sensors; iii) and finally evaluate EndoU-mutant viruses as potential vaccine candidates.
Overall, the contribution of this dissertation is to provide mechanistic details for the role of EndoU activity during viral infection and provide a target for the development of antivirals and for the generation of live-attenuated vaccine strains for CoVs.
CHAPTER 2
MATERIALS AND METHODS

Viruses and Cells

Viruses

An infectious clone of Mouse hepatitis virus strain A59 (MHV-A59) (genbank accession no. AY910861) was previously generated by Dr. Anna Mielech in Dr. Susan Baker’s Lab at Loyola University Chicago. EndoU mutant viruses containing a catalytic site mutation (H262A) or a protein destabilization mutation (T98M) were previously generated by Dr. Anna Mielech and Dr. Xufang Deng in the Baker Lab (Deng et al., 2017). Viruses were propagated in DBT cells and stored at -80°C. Briefly, T-75 flasks of DBT cells were infected with virus at a multiplicity of infection of 1 for 1 hr in 2 mL of serum-free media at 37°C with 5% CO₂. After 24 hrs, supernatants were collected and cleared of cell debris by centrifugation at 1,000xg for 10 min at 4°C. Virus-containing cell-free supernatants were aliquoted and stored at -80°C. Titers of viral stocks were determined by plaque assay on DBT cells. Infectious clones of wild-type PEDV or EndoUmut PEDV (H226A) were previously generated by reverse genetics and full-genome-sequenced by Dr. Xufang Deng in the Baker Lab (Deng et al., 2019).

HEK 293T Cells

Human embryonic kidney 293T cells (CRL-11268, ATCC) were cultured at 37°C with 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM) (10-017-CV, Corning) containing 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1% HEPES, 1% L-glutamine, 1% sodium pyruvate, and 1% nonessential amino acids, 1% HEPES, 1% L-glutamine, 1% sodium
pyruvate, and 1% nonessential amino acids, 1% HEPES, 1% L-glutamine, 1% sodium pyruvate, and 1% penicillin/streptomycin. When confluent in a T-75 flask, the monolayer was washed with phosphate-buffered saline (PBS), then cells were removed by addition of 1 mL of 0.25% trypsin-versene solution for 2 min at 37°C. For routine passaging, cells were split 1:10 every 3-4 days.

**DBT Cells**

Delayed brain tumor (DBT) cells were cultured at 37°C with 5% CO₂ in MEM (M0268, Sigma) containing 5% FBS, 10% tryptone phosphate broth (TPB), 1% L-glutamine, and 1% penicillin/streptomycin. When confluent in a T-75 flask, the monolayer was washed with PBS, then cells were removed by addition of 1 mL of 0.25% trypsin-versene solution for 2 min at 37°C. For routine passaging, cells were split 1:10 every 3-4 days.

**AML12 Cells**

AML12 hepatocytes (CRL-2254, ATCC) were cultured at 37°C with 5% CO₂ in DMEM/F-12 (12400-024, Invitrogen) supplemented with 1% penicillin/streptomycin, 10% FBS, Insulin, Transferrin, and Selenium (41400045, Life Technologies) and Dexamethasone (40ng/mL)(D4902, Sigma). When confluent in a T-75 flask, the monolayer was washed with PBS, then cells were removed by addition of 1 mL of 0.25% trypsin-versene solution for 20 min at 37°C. For routine passaging, cells were split 1:5 every 3-4 days.

**L929 Cells**

L929 cell line was gifted from Dr. Francis Alonzo (Loyola University Chicago). These cells secrete macrophage colony stimulating factor (M-CSF), which is used to promote macrophage differentiation from bone marrow (Weischenfeldt and Porse, 2008). The cells are maintained at 37°C with 5% CO₂ in DMEM (10-017-CV, Corning) supplemented with 10% FBS, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, and 1%
penicillin/streptomycin. When confluent in a T-75 flask, the monolayer was washed with PBS, then cells were removed by addition of 1 mL of 0.25% trypsin-versene solution for 5 min at 37°C. For routine passaging, cells were split 1:10 every 3-4 days. For supernatant collection, cells were passaged 3.75 x 10⁵ cells into 75 mL media into a T-125 flask. Cells were incubated at 37°C with 5% CO₂ for 6 days, then supernatant was filtered with a 0.2µm filter (73520-994, VWR) and stored at -20°C.

**Generation of Bone Marrow-Derived Macrophages (BMDMs)**

Bone marrow was collected from femurs of C57BL/6 (#000664, The Jackson Laboratory) or IFNα1 receptor knockout (IFNAR⁻/⁻) mice (breeder pair provided by Deborah Lenschow, Washington University in St. Louis, St. Louis, MO). Differentiated BMDMs were maintained in BMDM media containing DMEM (10-017-CV, Corning) supplemented with 30% L929 cell supernatant, 20% FBS, 1% L-glutamine, 1% sodium pyruvate, and 1% penicillin/streptomycin. For generation, 5 x 10⁶ bone marrow cells were plated in 100 x 26-mm petri dishes (25387-030, VWR) with 15 mL BMDM media with 50 µM β-mercaptoethanol (2-ME). After 3 days of incubation at 37°C with 5% CO₂, 10 mL of BMDM media with β-mercaptoethanol was added. Following another 3 days of differentiation, BMDMs were either plated for experiments or harvested and stored in liquid nitrogen until use (1 x 10⁷ cells/tube). To remove BMDMs from plate, cells were washed with 10 mL of cold PBS, incubated for 30 min at 4°C in 10 mL PBS, and then gently rinsed from the plates by manual pipetting. For liquid nitrogen storage, 1 x 10⁷ cells/mL were suspended in BMM media with 10% DMSO and stored in liquid nitrogen until use. For viral infections, BMDMs were thawed and plated in 100 × 26-mm Petri dishes in BMM media without β-mercaptoethanol. After 3 days of incubation at 37°C with 5% CO₂, cells were
plated onto tissue culture dishes (6 x 10^5 cells/mL plated dilution). The plated BMDMs were allowed to adhere and rest for at least 24 hrs before subsequent infection experiments began.

**Experimental Methods**

**Immunofluorescence Studies**

BMDMs (1.5 x 10^5 cells per well) AML12 hepatocytes (1.5 x 10^5 cells per well) were plated on a glass coverslip in a 24-well plate. Glass coverslips were autoclaved and baked dry before use. After incubating at 37°C with 5% CO₂ for 24 hrs, the cells were infected with wild-type or mutant MHV at an MOI of 0.1 in serum-free media. Briefly, cell supernatant was removed by vacuum and cells were washed with 2 mL of PBS. 300 µl of infectious media was added to the cells and the cells were incubated at 37°C with 5% CO₂ for 1 hour with tilting the plate every 15 min. After incubation, infectious media was removed, 2 mL of complete media with FBS was added, and cells were incubated for indicated times at 37°C with 5% CO₂.

At the indicated timepoints, cells were washed with 500 µL of PBS, then fixed with 500 µL of 3.7% formaldehyde in PBS by incubating for at least 20 min at room temperature. Cells were then washed 2 times with 500 µL PBS and permeabilized with 500 µL of 0.1% Triton X-100 (T8787, Sigma) in PBS by incubating at room temperature for at least 30 min. Cells were again washed 2 times with 500 µL PBS, then 500 µL of 5% normal goat serum (NGS) was added to block non-specific interactions. Depending on the antibodies used for staining, 10% FBS in PBS was also used for blocking. Fixed cells treated with blocking buffer were incubated overnight at 4°C. Blocking buffer was removed, and the coverslips were stained with antibodies in 0.5% NGS in PBS. Primary antibodies were used as follows: anti-nsp2/3 (1:1,500, Schiller *et al* (Schiller et al., 1998)), anti-dsRNA (1:500, Scicons, K1), and anti-nsp15 (1:500, Schiller et al). Secondary antibodies used are: donkey anti-rabbit IgG alexafluor 488 (1:1,000; A-21441,
Invitrogen) and goat anti-mouse IgG Alexa Fluor 568 (1:1,000; A11004, Thermofisher). Nuclei were visualized with Hoescht 33342 (1:2,500; H1339, Life Technologies). Briefly, 300 µL of primary antibody solution was added to the fixed cells and were incubated at room temperature for 2 hours. The primary antibody solution was removed, and the cells were washed 2 times with 500 µL of PBS. After addition of 300 µL of secondary antibody solution with Hoescht 33342 stain, the cells were incubated at room temperature for 1 hour while covered with aluminum foil. After incubation, the cells were washed 2 times with 500 µL of PBS. To prepare the samples for viewing, I added 10 µL drop of Fluorogel Mounting Medium (50-247-04, Fisher Scientific) on microscope slides, carefully added the coverslip, and sealed the edges of the coverslip to the slide with clear coat nail polish. Cells were imaged by collecting z-stack images with a Deltavision wide-field fluorescent microscope (Applied Precision, GE) equipped with a digital camera (CoolSNAP HQ, Photometrics). Images were taken with either a 20x, a 60x, or a 100x lens. Samples were excited with light generated by an Insight SSI solid-state illumination module (Applied Precision, GE) and deconvolved with SoftWoRx deconvolution software (Applied Precision, GE). All images were collected under identical acquisition conditions and processed using Imaris 7.6.4 (Bitplane).

**dsRNA Immunoprecipitation, RNA-Sequencing, and RT-qPCRs**

IFNAR<sup>-/-</sup> BMDMs, C57Bl/6 BMDMs, or AML12 cells were plated with 2 mL of cell dilution into 6 well plates. The cell concentration for BMDMs is 6 x 10<sup>5</sup> cells per mL. AML12 cell concentration is 3.5 x 10<sup>5</sup> cells per mL. Cells were infected with wild-type or EndoUmut MHV at a MOI of 1. Briefly, cell supernatant was removed by vacuum and cells were washed with 2 mL of PBS. 400 µL of infectious media was added to the cells and the cells were incubated at 37°C with 5% CO<sub>2</sub> for 1 hour with tilting the plate every 15 min. After incubation,
infectious media was removed, 2 mL of complete media with FBS was added, and cells were
incubated for indicated times at 37°C with 5% CO2.

At indicated times post infection, cell supernatants were removed and cells were washed
1 time with 2 mL of PBS. To obtain total RNA, I added 350 µL of RLT buffer to lyse the cells
and collected the samples into 1.5 mL tubes. Cellular RNA was isolated with RNeasy kit (74104,
Qiagen) according to the manufacturer’s instructions. After RNA isolation, RNA concentrations
were measured by nanodrop.

To immunoprecipitate the dsRNA, I mixed 1 µg of cellular RNA in a 1.5 mL tube with 2
µL anti-dsRNA antibody (K1, Scicons) or mouse anti-β-actin (A00702-40, Genscript) and 500
µL of RNA binding buffer (50mM Tris pH 8.0, 150mM NaCl, 1% NP-40, and 20 U/mL
Ribolock (EO0381, Thermofisher). The RNA immunoprecipitation mixture was incubated
overnight at 4°C, with mixing on tube rotator. After overnight incubation, 50 µL of protein G
beads (LSKMAGG02, Millipore) were added and the mixture incubated rotating for 4 hours at
4°C. Protein G beads were precipitated with magnets then washed 5 times with 500 µL cold
binding buffer. Washing was performed by addition of binding buffer then slowly spinning the
tubes for 10 min at 4°C. The last wash was removed and 350 µL of RLT buffer was added to the
precipitated beads. After incubating the beads in RLT buffer for 10 min spinning at room
temperature, the RLT buffer mixture was added to the RNeasy Column and RNA was purified
with RNeasy Kit. Purified RNA was either reverse transcribed into cDNA with RT2 First Strand
kit (330411, Qiagen) or sent for RNA sequencing to the Genomics Facility at the University of
Chicago. For cDNA synthesis, 8 µL of purified RNA per sample was reverse transcribed into
cDNA, since RNA concentration was not measurable by nanodrop. qPCR of cDNA was
performed as described below. For qPCR, viral N gene was measured using primers listed in Table 4.

For RNA-sequencing, RNA was processed by University of Chicago Genomics Facility. cDNA sequencing libraries were generated with TruSeq Stranded Total RNA with Ribo-zero subtraction (Illumina), then sequenced on Illumina HiSeq4000. RNA-sequencing reads were analyzed with Galaxy’s online platform (www.usegalaxy.org). Reads were groomed, clipped, and mapped with Hisat2 to the wild-type MHV strain A59 (genbank accession no. AY910861) or host genome (GRCm38 Ensembl build of the C57BL/6J). The number of reads at individual viral nucleotides was calculated by plotCoverage. RNA seq data has been deposited and is available in the NCBI GEO database (Accession number: GSE144886).

**Quantitative Polymerase Chain Reaction (qPCR)**

At indicated timepoints, 350 µL of RLT buffer was added to lyse the cells and cellular lysate was collected into 1.5 mL tubes. Cellular RNA was isolated with RNeasy kit (74104, Qiagen) according to the manufacturer’s instructions. Briefly, 350 µL of 70% EtOH was added to the cell lysate, mixed, and added to the RNeasy column. The columns were centrifuged at 14,000 rpm for 1 min, then the flowthrough is discarded. The columns were washed with 700 µL of RWI buffer, then 2 times with 500 µL of RPE buffer. After last wash, the column was placed into a new collection tube and centrifuged at max speed for 2 min to dry out column. Then, the column was transferred to a 1.5 mL tube, 35 µL of nuclease-free (NF) H2O was added to column, and incubated at room temperature for 1 min. The RNA was eluted from the column by centrifuging the column and tube at max speed for 1.5 min. Purified RNA was measured by nanodrop.
To generate cDNA, I reverse transcribed 500 ng of purified RNA into cDNA with RT2 First Strand kit (330411, Qiagen). For cDNA synthesis, 8 µL of purified RNA/H₂O was mixed with 6 µL of GE2 buffer in a 0.2 mL PCR tube and incubated for 5 min at 37°C. The mix was cooled on ice and 6 µL of BC4 buffer was added. The reverse transcription reaction was incubated at 42°C for 15 min, then 95°C for 5 min to inactivate the reverse transcriptase, and finally a 4°C hold. cDNA was then diluted with 91 µL of nuclease-free NF H₂O.

qPCR reactions were performed using RT2 Kit (330502, Qiagen). The measurements of qPCR mixture per well is shown in Table 3. Each sample was quantified from triplicate wells. qPCR cycles were 1) 95°C for 10 min, 2) 95°C for 15 sec, 3) 60°C for 1 min, and repeated steps 2-3 for 40 cycles. A melt curve was performed after the qPCR reaction to determine melting temperatures of PCR products. Primer mixtures used are IFNβ1 (PPM03594C, Qiagen), β-actin (PPM02945B, Qiagen), and 18s rRNA (PPM57735E, Qiagen). Other primer sets are listed in Table 4.
Table 3. PCR Reaction Mixtures.

<table>
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<tr>
<th>Component</th>
<th>/Rxn (µL)</th>
<th>Component</th>
<th>/Rxn (µL)</th>
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<td>Primer Fwd (10 µM)</td>
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<td>Primer Rev (10 µM)</td>
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Table 4. List of MHV Primers Used in these Studies.

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<th>Primer set</th>
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<td>IFNλ2/3</td>
<td>F 5’-AGTGGAAGCAAGAAGGATG-3’</td>
</tr>
<tr>
<td>IFNλ2/3</td>
<td>R 5’-GAGATGAGGTTGAAGAG-3’</td>
</tr>
<tr>
<td>N Gene</td>
<td>F 5’-AGCAGACTGCAACTACTCAACCCACTG-3’</td>
</tr>
<tr>
<td>N Gene</td>
<td>R 5’-GCAATAGGCATCTCCTGTCTTCTGCA-3’</td>
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<tr>
<td>Negative-Sense cDNA</td>
<td>5’-GAATTCTGGTGTGCGATGAAC-3’</td>
</tr>
<tr>
<td>PolyU qPCR Set 1</td>
<td>R 5’-TGTGTGAGAAGTGTAGCAAGG-3’</td>
</tr>
<tr>
<td>PolyU qPCR Set 2</td>
<td>R 5’-TGTGTGAGAAGTGTAGCAAGG-3’</td>
</tr>
<tr>
<td>PolyU Length Set 1</td>
<td>R 5’-CATATGTGAGCAGCTCAAGATG-3’</td>
</tr>
<tr>
<td>PolyU Length Set 2</td>
<td>R 5’-CATATGTGAGCAGCTCAAGATG-3’</td>
</tr>
<tr>
<td>Probe</td>
<td>F 5’-6-FAM-TAACCATAA/ZEN/GAAGGCAATGCAAGG-3’</td>
</tr>
<tr>
<td></td>
<td>F 5’-GATAGGGATTCGCCTGCTG-3’</td>
</tr>
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</table>
Table 5. List of PEDV Primers Used in these Studies.

<table>
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<tr>
<th>Primer Set</th>
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<td>Negative-sense cDNA</td>
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<tr>
<td>PolyU qPCR Set 1</td>
<td>R 5’-CACTGTCATGAGGGGAACG-3’</td>
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<td></td>
<td>F 5’-GTCCATTGAGTCAATACCTTG-3’</td>
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<tr>
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<td>F 5’-GGGGATCCGCGGTGTTTTTTTTTTT-3’</td>
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<tr>
<td>PolyU Length Set 1</td>
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<td></td>
<td>F 5’-GGGGATCCGCGGTGTTTTTTTTTTT-3’</td>
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<tr>
<td>PolyU Length Set 2</td>
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<tr>
<td></td>
<td>F 5’-GATAGGGGATCCGCCTG-3’</td>
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<tr>
<td>Probe</td>
<td>5’-6-FAM-CCTTTTCACGAGTAATCAGAGATCCGC-Iowa Black FQ-3’</td>
</tr>
</tbody>
</table>

**PolyU Extension PCRs**

IFNAR−/− BMDMs (6 x 10⁵ cells/mL) or AML12 cells (3.5 x 10⁵ cells/mL) were plated into 6 well plates. Cells were infected with wild-type or EndoUmut MHV at a MOI of 1. For PEDV, Dr. Xufang Deng infected PK1 or Vero cells with wild-type or EndoUmut PEDV at an MOI of 0.1. Briefly, cell supernatant was removed by vacuum and cells were washed with 2 mL of PBS. 400 µL of infectious media was added to the cells and the cells were incubated at 37°C with 5% CO₂ for 1 hour with tilting the plate every 15 min. After incubation, infectious media was removed, 2 mL of complete media with FBS was added, and cells were incubated for indicated times at 37°C with 5% CO₂. At indicated times post-infection, RNA was isolated by RNeasy Kit. For strand-specific cDNA, 500 ng of RNA was reverse transcribed with Omniscript (205113, Qiagen). Briefly, 15 µL of RNA (500 ng) in NF H₂O was mixed with the primer and incubated at 95°C for 5 min to denature the RNA. The RNA/primer mix was immediately chilled on ice. Next, 2 µL 10x Buffer, 2 µL dNTPs (5 µM), and 1 µL Omniscript was added to the sample mixture and cDNA was synthesized at 50°C, the temperature shown to reduce self-primed cDNA synthesis (Haddad et al., 2007). For negative-sense RNA, the cDNA primer was
5'-GAATTCTGGTGGTGTGGTGATGAAC-3' for MHV and 5'-GCAGCATTGCTCTTTGGTG-3' for PEDV. For total RNA, the cDNA primers were random hexamers and positive-sense RNA primer was oligo-dT. The cDNA was diluted by addition of 91 µL of NF H2O. qPCR was performed with SsoAdvanced Universal Probes Supermix (1725281, Bio-rad). qPCR reaction was performed with an annealing temperature of 60°C with either primer set PolyU qPCR Set 1 or PolyU qPCR set 2. Primers are listed in Tables 4 and 5.

PolyU length nested PCRs were performed with PFU Ultra Polymerase (600380, Agilent) with an annealing temperature of 60°C. cDNA was generated as described above. For negative-sense RNA, the cDNA primer was 5'-GAATTCTGGTGGTGTGGTGATGAAC-3' for MHV and 5'-GCAGCATTGCTCTTTGGTG-3' for PEDV. For positive-sense RNA, the cDNA primer was 5'-GGGGATCCGCGGTTTTTTTTTT-3'. A PCR was performed with Set 1 primers for PolyU Length. Then the product was diluted 1:1000 and utilized in a PCR with Set 2 primers for PolyU length. PCR products were separated on a 10% polyacrylamide gel with TBE running buffer by loading PCR products in 6x Loading Dye (NEB) and running the gel at 180V for 45 min. The gel was then transferred to a dish and stained with SYBR Green II dye (S7564, Thermofisher) in 50 mL of TBE. Primers for cDNA synthesis and PCRs are listed in Tables 4 and 5. For polyU length sequencing, sequencing libraries were generated with custom amplicon primers with nextera XT indexes and the amplicons were sequenced on an Illumina Miseq V2 500 with paired-end 250bp reads. Reads were groomed, clipped, and mapped with Hisat2 to the wild-type MHV strain A59 (genbank accession no. AY910861).

RNA Transfections

Gene blocks (gBlocks) containing a T7 promoter, a segment of the viral genome segment, and a HindIII cleavage site were synthesized by Integrated DNA technologies (IDT).
The viral sequences are listed in Table 6. To clone these geneblocks into plasmid DNAs, the pCAGGs vector was digested with EcoRI-HF (R3101S, NEB)and HindIII (R3104S, NEB) restriction enzymes, treated with Antarctic Phosphatase (M0289S, NEB), and gel purified from an 1% agarose gel with Wizard SV Gel and PCR Purification Kit (A9281, Promega). Viral genome segments were cloned into the digested pCAGGs vector by Gibson Assembly. Briefly, 50ng of digested vector was mixed with ~25 ng of a gBlock and 2x NEBuilder Hifi DNA Assembly Mix (E2621S, NEB). The mixture was incubated at 50°C for 1 hour, then transformed into competent *E. coli* cells. Colonies containing pCAGGs-viral gene plasmids were selected using ampicillin (100 ng/mL). The DNA plasmids were maxiprepped with PureYield Plasmid Maxiprep System and sequenced to ensure the correct viral sequence was present.

RNAs of the viral sequences were generated through T7 transcription. Briefly, plasmids were digested with HindIII-HF, then purified with Wizard Gel and PCR Purification Kit. RNA was *in vitro* transcribed using 1 µg of digested plasmid. The plasmid was mixed with 4 µL of 5x Transcription Buffer, 2 µL of rNTPs (N0466S, NEB), 1 µL Ribolock, and 2 µL of T7 RNA polymerase (EP0111, Thermofisher) in a 20 µL total volume reaction. The mixture was incubated at 37°C for 5 hours. The RNA was then purified by adding 1:3 volume of 7.5M LiCl precipitation solution and 1:3 volume of NF H₂O. The RNA/LiCl solution was incubated overnight at -20°C, then centrifuged at maximum speed at 4°C for 30 min. After removal of the LiCl, the pellet was washed with 300 µL of ice-cold 75% EtOH. After removal of the EtOH wash, the RNA pellet was dried then resuspended in 35 µL of NF H₂O. RNA concentrations were determined by nanodrop and RNA was stored at -80°C until use.

For RNA transfections, AML12 cells (3.5 x 10⁵ cells/mL) were plated into 24 well plates and incubated for 24 hours at 37°C with 5% CO₂. Poly I:C (P1530, Sigma) or 5 pmol of *in vitro-
transcribed RNA was transfected into AML12 cells using Lipofectamine 2000 (11668027, Thermofisher). Briefly, RNA was diluted into 50 µl of OptiMEM (11058021, Thermofisher) and 3 µl of Lipofectamine 2000 was added. The RNA mixture was incubated at room temperature for 20 min, then added dropwise into AML12 wells. The plates were gently mixed by tilting then incubated for 8 hours at 37°C with 5% CO₂. After incubation, RNA was isolated using RNeasy kit as described above and qPCR for IFNβ1 (PPM03594C, Qiagen) and 18s rRNA (PPM57735E, Qiagen) was performed as described above.
### Table 6. List of RNA Constructs for In Vitro Transcription.

<table>
<thead>
<tr>
<th>RNA construct</th>
<th>Gene Block Sequence</th>
</tr>
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<tr>
<td>N5</td>
<td>UUUUUUUUUGUGAUUCUCAACUAAUGCCGAUAUCAACUCAUUUACUAGGGCAUUGCAGGAAUACGUAAUGAGACGGAUAGAGACCUAUGACCAUGCAACAGAAGACAUCCACAUUCUGAUAGAGAGUGUCACUUCUGCCACAAGUUAGGAUGUGGCAUCACCAACUACUUCUGAUAGAGAGUGUCCUAUCCGACUUUCUCGCGAGGGGUUACCACCGAGCGCCGACAUAGGAAUAUCAUUCUUUACAUAUAGAUGAUAGGCAUCACCAACUCAUCUAC</td>
</tr>
<tr>
<td>P3</td>
<td>GUAGUGCCGCAUGGGUUAGAAGAUGACUCUAUAUGGAAGAAUGAUAUCUUGUGCCGCCCUUGCGAGAAGAGCGAAGACACUCUCUAUCGAAGAUGAGCUUGGCUGUCACUACUGUUAUGAAGAAGAGAUGUCACUACUGUUAUGACAGCAAGACAUCCACAUUCUGAUAGAGAGUGUCCUAUCCGACUUUCUCGCGAGGGGUUACCACCGAGCGCCGACAUAGGAAUAUCAUUCUUUACAUAUAGAUGAUAGGCAUCACCAACUCAUCUAC</td>
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<td>N3</td>
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<td>P5</td>
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<td>N5.100</td>
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<td>N5.8U</td>
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</tr>
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</table>

### Generation of MDA5-Knockdown Cells

A modified CRISPR/Cas9 protocol, based on the GeCKO system (Shalem et al., 2014) was used to knock-down the expression of MDA5 or RIG-I in AML12 cells. Single-guide RNAs
were identified with Benchling (Benchling, Inc) to target the *Ifih1* (MDA5) or *Ddx58* (RIG-I) genes. The sequence used for the clonal MDA5-knockdown (MDA5-KD) AML12 cells discussed in Figure 23 targeting *Ifih1* was 5’-ATGGACGCAGATGTTCGTGG-3.’ The remaining guide RNA sequences are listed in Table 7. Complementary DNA versions of guide RNA were annealed and inserted into a pLentiCRISPRv2-puro (Addgene #52961) cassette between flanking BsmBI sites. Briefly, the complementary DNA oligos were annealed by mixing the oligos with 1x T4 DNA ligase buffer containing ATP, and T4 PNK ligase (M0201S, NEB). Then the reaction was incubated at 37°C for 30 min, 95°C for 5 min, and ramping from 95-25°C for at 5°C intervals for 5 min holding at each interval. The annealed oligos were then mixed with BsmBI-linearized pLentiCRISPRv2-puro plasmid and ligated with T4 DNA ligase (M0202S, NEB) at 16°C overnight. The ligated plasmid was then transformed into competent *E. coli* and miniprepped with PureYield Miniprep Kit (A1222, Promega).

Transducing particles (TPs) were generated by transfecting HEK-293T/17 cells with pLentiCRISPRv2-puro, pPax2 (lentiviral packaging plasmid), and pHEF-VSV-G (vesicular stomatitis virus glycoprotein) and collecting supernatant. Briefly, 5 x 10⁶ HEK-293T/17 cells were plated into a 10 cm dish and incubated at 37°C 5% CO₂. After overnight incubation, the plasmids were mixed with OptiMEM and Trans-LTI (Mirus), incubated for 20 min at room temperature, then added dropwise to the 293T cells. Supernatants were collected at 24- and 48-hours post transfection. TPs were isolated by centrifuged the cell supernatant at 1000xg for 10 min at 4°C then filtering the supernatant through a 0.45uM filter (Millipore Sigma). TPs were stored at -80°C until use.

AML12 cells (2x 10⁵ cells/mL) were plated into 6-well plates and incubated overnight at 37°C 5% CO₂. The AML12 cells were then transduced with the TPs by removing the AML12
supernatant, washing the cells with 2 mL of PBS, then adding 300 µL of Purified TP supernatant. The cells were then incubated for 1 hrs at 37°C 5% CO₂ with tilting to mix the plate every 15 min. After the 1 hour incubation, 2 mL of complete media was added and the cells were incubated for 24 hours at 37°C 5% CO₂. Transduced AML12 cells were then selected by replacing the media with complete media containing 1µg/µl puromycin (InvivoGen) for 96 hrs. The polyclonal populations of selected cells were then grown to confluency in puromycin-selection. To obtain monoclonal knock-down cell populations, the KD AML12 cells were diluted to a 1 cell/100 µl dilution and 100 µl of solution was plated into a 96-well flat-bottom plate. Wells containing 1 colony were then selected and grown to confluency. To reach confluency, the cells were grown about 3 weeks with fresh complete media added to the cells every 3 days. Knockdown of MDA5 was determined by western blot using rabbit anti-MDA5 (SAB3500356, Sigma) and mouse anti-actin (A00702-40, Genscript).

Table 7. List of Guide RNA Sequences for CRISPR/Cas9 Knockdowns.

<table>
<thead>
<tr>
<th>Single Guide RNAs</th>
<th>Sequence</th>
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<tr>
<td>Ifih1-1</td>
<td>CAAGTGGAGACACTCGTCAT</td>
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<td>Ifih1-2</td>
<td>ATGGACGCAGATGTTCGTGG</td>
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<td>Ifih1-3</td>
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<tr>
<td>Ddx58-1</td>
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</tr>
<tr>
<td>Ddx58-3</td>
<td>GATATCATTTGGATCAACTG</td>
</tr>
</tbody>
</table>

RNA Cleavage Assay

Cleavage of RNA substrates were performed according to Kang et al (Kang et al., 2007). Purified, wild-type EndoU was kindly gifted by Dr. C. Kao (Formerly of Indiana University,
currently at Aligos Therapeutics). Briefly, 1 µM RNA was mixed with EndoU in Cleavage Buffer (50 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT) with or without 5 mM MnCl₂. Reactions were incubated at 30°C for the indicated time and reactions were stopped by addition of RNA Gel Loading Buffer (B0363S, NEB), followed by incubation at 95°C for 5 min. Reaction products were immediately loaded into a 10% poly-acrylamide gel with TBE buffer or a 1% agarose gel with TAE buffer and bands were separated by electrophoresis. Gels were moved into a staining box then stained with SYBR Green II dye in 50 mL TBE buffer. The gels were visualized with a ChemiDoc XRS+ imager (Bio-rad) and processed with Image Lab software (Bio-rad).

For RNA transfection, 500 ng of in vitro transcribed RNA was mixed with EndoU in Cleavage Buffer (50 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT) with or without 5 mM MnCl₂. The mixture was incubated at 30°C for 45 min, then the mix was heated to 95°C for 5 min, then mixed with 40 µL OptiMEM and 3 µL Lipofectamine 2000. The RNA mixture was incubated at room temperature for 20 min, then added dropwise into AML12 wells. The transfection mixture and cells were gently mixed by tilting the plates, then incubated for 8 hours at 37°C with 5% CO₂. After incubation, RNA was isolated using RNeasy kit as described above and RT-qPCR for IFNβ1 (PPM03594C, Qiagen) and 18s rRNA (PPM57735E, Qiagen) was performed as described above.

**Viral Titration by Plaque Assay**

IFNAR⁻/⁻ BMDMs (6 x 10⁵ cells/mL), C57Bl/6 BMDMs (6 x 10⁵ cells/mL), or AML12 cells (3.5 x 10⁵ cells/mL) were plated into 6 well plates. Cells were infected with wild-type or EndoUmut virus at a MOI of 0.1. Briefly, cell supernatant was removed by vacuum and cells were washed with 500 µL of PBS. 250 µL of infectious media was added to the cells and the cells were incubated at 37°C with 5% CO₂ for 1 hour with tilting the plate every 15 min. After
the 1 hour incubation, inoculum was replaced with 500 μL of fresh, complete medium. Cells were then incubated at 37°C with 5% CO2. At indicated time points, cell culture supernatants were collected and stored at -80°C.

Serial dilutions of supernatants were made in serum-free media and analyzed by plaque assay in DBT cells. Briefly, DBT cells (2.5 x 10^5 cells/mL) were plated into 6 well plate and incubated for 20 hours at 37°C with 5% CO2. After incubation, the DBT cells were subsequently infected with diluted supernatants. Cell supernatants were removed by vacuum and cells were washed with 500 μL of PBS. 3000 μL of infectious media was added to the cells and the cells were incubated at 37°C with 5% CO2 for 1 hour with tilting the plate every 15 min. After incubation, infectious media was removed and cells were overlaid with MEM containing 2% FBS and 0.4% Noble Agar. Cells were incubated for 48 hours at 37°C with 5% CO2. After 48 hours, the cells were fixed with 3.7% formaldehyde in PBS for 30 min. After fixation, the formaldehyde and agar mixtures were removed and the fixed cells were stained with 0.1% crystal violet solution for 1 hour at room temperature. The crystal violet stain was removed and the cells were washed with distilled H2O and dried. The number of plaques was quantified by manual counting. Titers were obtained from three independent assays for each sample.

**EM Imaging**

IFNAR^-^ BMDMs or C57Bl/6 BMDMs were plated at 3 x 10^5 cells/mL in a 12-well plate and grown overnight at 37°C with 5% CO2. The BMDMs were infected with wild-type or EndoUmut MHV at an MOI of 2. Cell supernatant was removed by vacuum and cells were washed with 1 mL of PBS. 300 μL of infectious media was added to the cells and the cells were incubated at 37°C with 5% CO2 for 1 hour with tilting the plate every 15 min. After the 1 hour incubation, inoculum was replaced with 1 mL of fresh, complete medium. Cells were then
incubated at 37°C with 5% CO₂. At indicated time points, the cell supernatant was removed and the cells were washed 2 times with PBS. The cells were fixed for 1 hr with 5% acrolein (A24001, Sigma Aldrich) in phosphate buffer (pH 7.2). Cells were washed twice with PBS, stained, and then embedded in epoxy resin. Staining and embedding was performed by Dr. David Rademacher of the Loyola University Chicago Imaging Core. Images were taken with a Philips CM120 120kV transmission electron microscope (TEM) at Loyola University Chicago Imaging Core.

**Flow Cytometry**

Triplicate wells of 6 x 10⁵ BMDMs were plated in a 12-well plate for 6 hpi experiments or six well plates for the 12 hpi experiments to lessen syncytia formation. After 24 hour incubation at 37°C with 5% CO₂, the BMDMs were infected with wild-type or EndoUmut MHV at an MOI of 0.1. After 6 hours or 12 hours, cells were removed from the wells by addition of cold PBS and incubation at 4°C for 30 min. Cells were manually pipetted off the wells and transferred into a 96-well, V-bottom plate. Cells were centrifuged at 2,000xg in the plate to pellet cells at the bottom, then resuspended in PBS containing fixable viability dye (65-0865-14, eBioscience) and stained for 30 min at room temperature. After washing the cells 2 times with PBS, the cells were fixed with 3.7% formaldehyde in PBS. After 2 washes with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS. The cells were then washed 2 times with PBS and blocked with PBS containing 5% normal goat serum (NGS). Cells were labeled with anti-dsRNA (5ug/mL, K1, Scicons), and goat anti-mouse Alexafluor 568 (1;1000). Cells were analyzed using an LSR Fortessa cell analyzer (BD Bioscience). Flow Cytometry data were analyzed using FlowJo software (Treestar).
**Evaluating Pathogenesis of MHV in Mice**

All experiments were completed using protocols reviewed and approved by the Loyola University Chicago IACUC. C57BL/6 mice were purchased from Jackson Laboratory and Ifnar<sup>-/-</sup> C57Bl/6 mice were gifted from Dr. Deborah Lenschow of Washington University in St. Louis and bred in the Loyola University Chicago Animal Facility. For intraperitoneal infections, 6-wk-old female mice were injected with 60,000 pfu of wild-type or EndoUmut viruses in 100 ul PBS. Mice were then monitored by percentage of initial body weight until they were euthanized. Mice were euthanized at indicated time points by inhalation of CO<sub>2</sub>, and organs were harvested for analysis. For viral titers, tissues were removed, weighed, and dissociated with 1 mm zirconia/silica beads (11079110z, BioSpec Products) for 40 seconds in a bead beater (MP Biomedical). Tissue extract was separated from the beads and stored at -80°C until use. Viral titers were measured by plaque assay in DBT cells as described above. H&E staining of liver slices was performed by the Tissue Processing Core Facility at Loyola University of Chicago. For intracranial infections, 6-wk-old female mice were inoculated with 600 pfu in 20 ul PBS. Infections were performed by Dr. Xufang Deng in the Baker lab. Infected mice were monitored for body weight daily and euthanized when weight loss was over 25% of original body weight.

**Western Blotting**

For western blotting experiments, 1 mL of IFNAR<sup>-/-</sup> BMDMs (6 x 10<sup>5</sup> cells/mL) or AML12 cells (3.5 x 10<sup>5</sup> cells/mL) was plated into 12 well plates. After 24 hour incubation at 37°C with 5% CO<sub>2</sub>, the cells were infected with wild-type or EndoUmut MHV at an MOI of 0.1. After incubation for indicated times at 37°C with 5% CO<sub>2</sub>, cell lysates were collected by lysing cells with 150 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) and moving cell lysate into 1.5 mL tubes. The lysates were then centrifuged at 14,000xg at 4°C to pellet nuclei. Supernatants were then transferred to a new 1.5 mL tube and 2x Sample Buffer (10% Glycerol, 5% β-mercaptoethanol, 3% SDS, Bromophenol Blue, 1.0 M Tris-HCl buffer pH 6.8) was added to denature proteins. Proteins were heated at 95°C, then cooled to room temperature before evaluation by SDS-PAGE.

Cell lysates were separated using 10% SDS-PAGE, then transferred to a PVDF membrane using a semi-dry transfer apparatus (Bio-rad). The conditions for the transfer were: 25V and 1.0A for 30 min. After transfer, membranes were blocked with 5% milk in TBS-T overnight at 4°C. Primary antibodies were diluted in 5% milk in TBS-T then either incubated at room temperature for 1 hour or overnight at 4°C. Membranes were then washed 3 times with TBS-T for 10 min, then incubated at 1 hour at room temperature with 1:2500 dilution of HRP-conjugated secondary antibodies. Membranes were again washed 3 times with TBS-T for 10 min before addition of Western Lightning Plus-ECL (PerkinElmer, Inc.) chemiluminescent substrate and visualization of the blots on a FluorChem E (Protein Simple). Band sizes were determined relative to PageRuler Plus Prestained Protein ladder (26619, Thermofisher). The antibodies used for western blotting are anti-nsp1 (anti-p28, (Schiller et al., 1998)), anti-nsp2/3 (D3, (Shi et al., 1999)), anti-N protein (J3.3, gift from Dr. John Fleming, University of Wisconsin), and anti-β-actin (A0702-40, Genscript). The secondary antibodies used were donkey anti-rabbit-Ig-HRP (6440-05, Southern Biotech) and goat anti-mouse-Ig-HRP (1010-05, Southern Biotech).

**Generation of Figures, Graphics, Tables, and Reference Management**

The graphic figures and illustrations were generated using Adobe Illustrator. All figures that incorporate experimental data and statistical analysis were generated using GraphPad Prism 6 software (Graphpad.com). Tables were created using Microsoft Word (Microsoft). All
reference materials were organized and incorporated using Mendeley software version 1.19.3 (Mendeley Ltd.)

Data Availability Statement

RNA-sequencing data has been deposited and is available in the NCBI GEO database (Accession number: GSE144886).
CHAPTER 3

EXPERIMENTAL RESULTS

In this chapter, I describe our work investigating the roles of EndoU during CoV infection. First, I describe the effects of active versus inactive forms of EndoU on viral replication and immune cell activation. Second, I describe experiments investigating the mechanism used by EndoU to antagonize host immune signaling. Lastly, I describe in vivo infection experiments that document a role of EndoU in MHV virulence. Overall, these results parse out how EndoU inhibits the sensing of viral RNA by host innate immune pathways and how inactivating EndoU may be a new approach for generating live-attenuated vaccines for CoVs.

Characterizing EndoU-mutant Viruses in Cell Culture

In this section, the experiments address the question of whether EndoU plays a role in viral replication and whether EndoU antagonizes innate immune signaling pathways during viral infection of macrophages. I hypothesize that since viruses that encode catalytic mutations in EndoU can be generated through reverse genetics and can grow to high titers in cell culture, that EndoU is not necessary for efficient viral replication. Instead, I predict EndoU inhibits the signaling of host innate immune pathways. Since the viral dsRNA signal can activate MDA5, PKR, and OAS (Deng et al., 2017), I predict EndoU can block the dsRNA signal from being detected by these host sensors. The following experiments are designed to determine if MHV with EndoU mutations activate multiple PRRs leading to attenuation of the mutant-MHV replication in macrophages.
Coronaviruses Encoding EndoU Mutations Exhibit Impaired Replication in Macrophages

To determine if EndoU played a role in viral replication or inhibiting innate immunity, we infected bone marrow-derived macrophages (BMDMs) with WT-MHV or two viruses containing EndoU mutations (EndoUmut)(Fig. 6). The first virus named N15m3 contained a catalytic histidine to alanine mutation (H262A) in the EndoU protein. The second virus contained an EndoU destabilizing mutation (T98M), but also contained a mutation in nsp3 (R971A). To control for the nsp3 mutation, a third mutant virus was generated (N3m), which contained the nsp3 mutation, but lacked the nsp15 mutation. Upon infection of wild-type C57Bl/6 BMDMs, we found that EndoUmut viruses had significantly decreased viral replication compared to wild-type virus (Fig. 7A). This defect in viral replication is noticeable as early as 12 hpi, and the difference becomes more predominant as time progresses. The N3m virus replicated similarly to wild-type MHV. Interestingly EndoUmut viruses do not contain a defect in viral replication during infection of IFNAR\(^{-/-}\) BMDMs, which lack type I interferon signaling (Fig. 7B). These experiments suggest that EndoUmut viruses are attenuated through IFN signaling in BMDMs. EndoU is not necessary for general viral replication, since there is no replication defect in IFNAR\(^{-/-}\) BMDMs. Instead, EndoU is necessary for efficient viral replication in IFN-responsive cells.
Figure 6. Diagrams of mutant MHV-A59 strains. Depicted are the viral genome of wild-type and each EndoUmut virus described in this study. N15m3 contains a H262A catalytic mutation in nsp15/EndoU. N15m1 contains a T98M destabilizing mutation in nsp15/EndoU and an R971A mutation in nsp3. N3m contains the R971A mutation in nsp3.

Figure 7. Evaluating the Growth Kinetics of EndoUmut Viruses in BMDMs. Cells were infected with WT- or EndoUmut-MHV (N15m1 and N15m3) at an MOI of 0.1. N3m is a control virus that contains an identical nsp3 mutation as N15m1, but lacks the EndoU mutation. At indicated timepoints, cell supernatants were collected and viral titers were measured by plaque assay in DBT cells. (A) C57Bl/6 BMDMs infected with viruses and viral titers measured from 4 to 24 hours post infection. (B) IFNAR−/− BMDMs infected with viruses and viral titers measured from 4 to 24 hours post infection. Values were analyzed using a nonlinear regression test. ***P < 0.001; n.s., not significant. Data are representative of two to three independent experiments and presented as the mean ± SD.
Another phenotype that was associated with EndoUmut virus attenuation was increased cell death during infection of wild-type BMDMs (Fig. 8). At 12 hpi during an multiplicity of infection (MOI) of 1 infection or 24 hpi during an MOI of 0.1 infection, EndoUmut virus induced increased cell death compared to wild-type virus. This was observed by a decrease in cell density left on the plate and an increase in cell floating or lifted up off the plate. In IFNAR−/− BMDMs, EndoUmut and wild-type viruses induce similar cell morphology. All viruses formed syncytia, which are multinucleated cells fused together by viral spike protein. The syncytia form at an identical rate beginning around 9 hpi and form to a similar extent in both EndoUmut and wild-type virus infections. The IFNAR−/− BMDMs begin to form syncytia early during replication, but do not detach from the plate like wild-type BMDMs. We hypothesize that the increased cell death is one factor that attenuates EndoUmut viruses during macrophage infection.

**EndoUmut CoVs Stimulate Early and Robust IFN Responses**

To determine EndoU is an IFN-antagonist, we quantitated the expression of a type I IFN gene by qPCR during EndoUmut virus infection. We found that in wild-type BMDMs, EndoUmut viruses induced significantly more IFNα11 expression at 8 hpi, 12 hpi, and 16 hpi compared to WT or the N3m control virus (Fig. 9A). With the increase in IFN expression, the amount of viral RNA replication was decreased as measured by expression of the N gene region of the viral genome (Fig. 9B). At 12 hpi and 16 hpi, EndoUmut viruses had attenuated viral RNA production, which ultimately leads to the decrease in viral titers found in Figure 7. These data show that EndoU is an IFN antagonist during viral replication, and viruses lacking this antagonist activity have attenuated viral RNA production during infection of macrophages.
Figure 8. Evaluation of Cell Morphology During EndoUmut Virus Infection. BMDMs were infected with WT- or EndoUmut-MHV at an MOI of 1 or 0.1. Cells were monitored over time and at 12 or 24 hpi, images were taken of the cells. We observed differences cell number and morphology during infection of IFN-competent BMDMs.

Figure 9. Evaluating IFN Stimulation by EndoUmut Viruses in C57Bl/6 BMDMs. Cells were infected with WT- or EndoUmut-MHV (N15m1 and N15m3). At indicated timepoints, cells were lysed and RNA was collected. After cDNA synthesis, qPCR was used to quantify the levels of (A) Ifna11 mRNA and (B) viral N gene RNA. Values were analyzed using a two-way ANOVA test by time in A–C or an unpaired t test in D. *P < 0.05; n.s., not significant. **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are representative of two to three independent experiments and presented as the mean ± SD. These experiments were performed by Dr. Robert Mettelman and Dr. Xufang Deng in Deng, Hackbart, et al. 2017.
To determine if the increased expression of IFN mRNA led to increased production of interferon-stimulated genes (ISGs), we tested if an EndoUmut virus increases the amount of ISG54 protein expressed in the cytoplasm of BMDMs. We found that EndoUmut virus induced increased expression of ISG54 protein, not only in infected cells, but also neighboring cells (Fig. 10). While WT had some low levels of ISG54 seen in infected cells, the EndoUmut cells were substantially brighter. These results indicate that ISG protein production is enhanced during EndoUmut virus. One important note is that the IFN is working in both an autocrine and paracrine fashion, so an anti-viral response is being mounted in uninfected cells as well as infected cells.

Figure 10. Evaluating Expression of ISG54. C57Bl/6 BMDMs were infected with WT- or EndoUmut-MHV at an MOI of 1. At 12 hpi, the cells were fixed, stained with anti-ISG54, anti-N protein, and Hoescht 33342 stain for nuclei. Cells were then imaged and evaluated for expression of ISG54 during infection.

To determine if the EndoU antagonist activity is detected in a stable cell line, we also infected AML12 hepatocytes and measured viral replication and IFN stimulation. We found that EndoUmut and wild-type MHV replicated to similar titers in AML12 cells (Fig. 11A) and had a similar production of intracellular viral RNA (Fig. 11B). Mirroring the BMDMs, EndoUmut
virus induced a type I IFN response in AML12 cells compared to wild-type virus (Fig. 11C). EndoUmut virus also induced a type III IFN response in the hepatocytes as measured by IFNλ expression (Fig. 11D). Interestingly, while EndoUmut infection produces a heightened IFN response in AML12 cells, the viral replication of EndoUmut virus did not differ compared to wild-type virus. One hypothesis for why replication is similar is that the IFN response is too slow to effectively induce ISGs and halt the viral replication. It appears that peak viral titers have been reached by 16 hpi, similar to peak expression of IFN mRNA production. The expression of IFN may be too little and too late then to signal for production of ISGs to prevent the production of infectious viral particles. Since this experiment was performed at a high MOI (MOI of 1), it would be interesting to determine if the kinetics of viral replication is affected when cells are infected lower MOIs. A low MOI may allow the IFN to signal before high titers of infectious virus would enter the culture supernatant, thus allowing for an antiviral response to be mounted against the EndoUmut virus.
Figure 11. Growth Kinetics of Wild-type and EndoUmut MHV-A59 in AML12 Hepatocytes. AML12 cells were infected with wild-type (WT) or EndoUmut MHV-A59 at an MOI of 1. At indicated timepoints, supernatant and intracellular RNA was collected. (A) Growth kinetics of WT- and EndoUmut-viruses as measured by plaque assay in DBT cells. (B) Viral gene expression measured by qPCR of N gene transcripts. Gene expression is normalized to 18s rRNA and set relative to WT at 4hpi. (C) IFNβ1 gene expression as measured qPCR. (D) IFNλ2/3 gene expression as measured by qPCR. Gene expression is normalized to 18s rRNA and set relative to mock. Values were analyzed by student T-tests. Data are representative of three independent experiments and presented as mean ± standard deviation.

EndoUmut CoVs Activate Multiple dsRNA Sensors, Leading to Apoptosis in Macrophages

Upon seeing enhanced cell death and increased IFN production during EndoUmut infection of BMDMs, we sought to determine if other host dsRNA sensors were also activated during EndoUmut virus replication. We evaluated PKR activation by analyzing phosphorylation of eIF2α. Upon PKR binding to dsRNA, PKR will oligomerize and phosphorylate eIF2α, which
blocks the translation of cellular proteins. PKR will also activate FADD which leads to apoptosis in cells (Gal-Ben-Ari et al., 2019). We hypothesize that EndoUmut viruses will have increased activation of dsRNA sensors, including PKR activity. We observed that EndoUmut viruses have increased p-eIF2α, without an elevation in eIF2α (Fig. 12A). We also determined activation of OAS1/2, which produces 2’5’ oligoadenylate (2’-5’A). The 2’-5’A binds to and activates RNAse L, which then indiscriminately degrades cellular RNAs (Zhao et al., 2012). For OAS activation, we measured cellular RNA degradation by a bioanalyzer assay (Fig. 12B). We observed that EndoUmut viruses have increased RNA degradation in BMDMs, which decreases the RNA integrity number (RIN), compared to wild-type MHV infections. The activation of PKR and OAS, which leads to host translational shutoff and RNA degradation, ultimately causes apoptosis in the BMDMs. We determined that the BMDMs underwent caspase-dependent apoptosis over necroptosis and pyroptosis. While necrostatin-1 (Nec-1), a necroptosis inhibitor, or caspase-1 inhibitors (VX-765), a pyroptosis inhibitor, did not prevent the virus-induced cell death, a pan-caspase inhibitor (zVAD) prevented the EndoUmut induced cell death (Fig. 12C). These data suggest that EndoUmut viruses activated multiple host dsRNA sensors and the BMDMs underwent apoptosis. In conjunction with the viral replication data, we hypothesize that the macrophage apoptosis blocks and prevents infectious virus production.
Figure 12. Evaluation of PKR and OAS Activation by EndoUmut Viruses. C57Bl/6 BMDMs were infected with wild-type or EndoUmut viruses at and MOI of 0.1. (A) At 8 hpi, cell lysates were collected and evaluated for p-eIF2α, eIF2α, N protein, and β-actin by Western Blotting. (B) RNA was extracted from cells at indicated timepoints. 200 ng of total RNA was analyzed by bioanalyzer for RNA degradation patterns. RNA integrity numbers (RIN) and the positions of 28S and 18S ribosomal RNA are shown. (C) Infected cells were subsequently treated with either DMSO, zVAD (20 µM), Nec-1 (25 µM), or Vx-765(20 µM). Cell viability was measured by CellTiter Glo Assay (Promega). Results were analyzed by two-way ANOVA. These experiments were performed by Dr. Xufang Deng in Deng, Hackbart, et al. 2017.

The Mechanism of EndoU for Antagonizing IFN Signaling

In this section, I describe experiments aimed at investigating the mechanisms EndoU uses to ensure that viral RNA is not recognized by host PRRs.

**EndoU Activity Prevents the Dispersal of dsRNA from Replication Complexes**

Since we observed a difference in host dsRNA sensor activation in EndoUmut virus-infected cells as compared to wild-type virus-infected cells, we sought to identify differences in
the dsRNA signal during virus infections. We began the investigation of viral dsRNA by performing immunofluorescence with the K1 anti-dsRNA antibody. We hypothesized that the dsRNA is escaping from replication complexes during EndoUmut virus infection. The goal of these experiments was to if EndoUmut virus had increased abundance and dispersal of dsRNA signal away from viral replication complexes compared to wild-type MHV infection. We found that indeed EndoUmut viruses had an increase in dispersed dsRNA away from replication complexes. During infection of IFNAR−/− BMDMs, wild-type- and EndoUmut-MHV had similar production of replication complexes as measured by nsp2/3 foci (Fig. 13A and B). In line with our hypothesis, EndoUmut virus had increased dsRNA foci detected in the BMDMs, and the number of “free” dsRNA away from replication complexes was also increased compared to wild-type virus. While observing if the dsRNA signal now lacks colocalization with nsp15, we performed immunofluorescence with an anti-nsp15 antibody and the K1 antibody. We again found that the viral dsRNA signal is dispersed from nsp15 during EndoUmut virus infection, which leads to a decrease in co-localized foci of nsp15 and dsRNA (Fig. 13C and D).
Figure 13. Mutation of Nsp15 Affects dsRNA Distribution in Virus-Infected BMDMs.
IFNAR−/−BMDMs were infected with WT or N15m3 at an MOI of 0.1. Cells were fixed at 6 hpi and stained with (A) anti-nsp2/3, anti-dsRNA, and Hoescht 33342 or (B) anti-nsp15, anti-dsRNA, and Hoescht 33342. Surfaces for puncta were created based on dsRNA and nsp2/3 fluorescence, and fluorescence was measured within each surface. The foci from 25 images were counted using IMARIS software program. (A) Images of subcellular localization of dsRNA and nsp2/3 (Upper) and quantification of foci (Lower). (B) Images of subcellular localization of dsRNA and nsp15 (Upper) and quantification of foci (Lower). Percent colocalization of nsp15 with dsRNA was calculated by dividing dsRNA+ nsp15+ foci by total dsRNA foci. Values were analyzed by an unpaired t test and error bars represent the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant. Data are representative of two to three independent experiments. (Scale bars, 5 μm.)

A previous study described that the CoV dsRNA signal disperses from replication complexes late during infection (Becares et al., 2016). We therefore predicted that wild-type MHV would have dispersed dsRNA similar to the EndoUmut at a later timepoint. We found that the dispersal difference observed during EndoUmut infection is detected early during MHV-infection. As wild-type infection continues, the dsRNA also disperses from the replication complexes (Fig. 14A). The dispersal of dsRNA at 12 hpi is similar between wild-type and
EndoUmut MHV, leading to a similar number of total and free dsRNA foci during viral infection (Fig. 14B). These data highlight that EndoU prevents an early dispersal of dsRNA from viral replication complexes. This suggests that timing of the dispersal and detection of the dsRNA is important in preventing immune activation and promoting viral replication.

**Figure 14. Evaluating dsRNA Foci at 12 HPI in BMDMs.** IFNAR\(^{-/-}\) BMDMs were infected with WT- or EndoUmut-MHV at an MOI of 0.1. Cells were fixed at 12 hpi and stained with anti-nsp2/3, anti-dsRNA, and Hoescht 33342. Surfaces for puncta were created based on dsRNA and nsp2/3 fluorescence, and fluorescence was measured within each surface. The foci from 25 images were counted using IMARIS software program. (A) Images of subcellular localization of dsRNA and nsp2/3. (B) Quantification of fluorescent total dsRNA, free dsRNA, and total nsp2/3 foci. Values were analyzed by an unpaired t test and error bars represent the mean, *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant. Data are representative of two to three independent experiments. (Scale bars, 5 μm.)
EndoU Activity Reduces the Accumulation of an Epitope Recognized by an Anti-dsRNA Antibody in CoV-Infected Hepatocytes

To determine if this phenotype is present in a stable cell-line, we infected IFN-responsive AML12 hepatocytes with wild-type or EndoUmut MHV and measured accumulation of replication complexes (anti-nsp2/3) and K1 dsRNA foci at 8 hours post-infection (hpi) (Fig. 15A). We quantified the number of nsp2/3 foci and dsRNA foci from 50 individual cells. We found that while the number of nsp2/3-labeled replication complexes were not significantly different (Fig. 15B, right), the total number of dsRNA foci per cell was elevated in EndoUmut-infected cells (Fig. 15B, left). Median fluorescent intensity of the individual dsRNA foci was also brighter in EndoUmut-infected cells (Fig. 15C). These results again indicate that EndoUmut infection results in increased abundance of an epitope recognized by the K1 anti-dsRNA antibody in multiple cell types.
Figure 15. Evaluating the Accumulation of an Epitope Recognized by K1 antibody in Virus-Infected AML12 Hepatocytes. AML12 hepatocytes were infected with WT- or EndoUmut-MHV at an MOI of 0.1. Cells were fixed at 8 hpi and stained with K1 anti-dsRNA antibody, anti-nsp2/3, and Hoescht 33342 nuclei stain. (A) Subcellular localization of dsRNA and nsp2/3 foci was visualized. (B) Foci for dsRNA and nsp2/3 were quantified using Imaris software from 50 individual cells. (C) The median fluorescent intensity was calculated for each individual dsRNA foci and compared between WT- and EndoUmut-infections. Values were analyzed by student T-tests. Data are representative of three independent experiments and presented as individual cell points with mean ± standard deviation. n.s.: not significant

EndoU does not Alter Viral Replication Complex Structure or Formation

Since we observed a dispersal of dsRNA away from replication complexes, one possibility for EndoU activity was that EndoU alters the formation of the viral DMVs which are believed to sequester viral dsRNA during infection. To test this hypothesis, we performed electron microscopy (EM) imaging on virally infected BMDMs. In both IFNAR−/− BMDMs (Fig. 16A) and C57Bl/6 BMDMs (Fig. 16B), we found that wild-type and EndoUmut viruses formed DMVs and convoluted membranes. The DMVs during EndoUmut infection did not appear to be deformed or decreased number, so we concluded that the viral replication complexes were not altered by EndoU activity. These results are in line with a previous study that showed that only
nsp3, nsp4, and nsp6 are necessary to form DMVs and convoluted membranes (Oudshoorn et al., 2017).

Figure 16. Evaluating Replication Complexes using Electron Microscopy. BMDMs were infected with WT- or EndoUmut-MHV at an MOI of 1. At 9 hpi, the cells were fixed, stained, and imaged by electron microscopy. (A) IFNAR−/− BMDMs were infected and imaged by EM. (B) C57Bl/6 BMDMs were infected and imaged by EM.

EndoUmut- and Wild-type Viruses have Similar Expression of Viral Proteins

Since viral replication is not altered by EndoU activity, we hypothesized that viral protein production does not change between wild-type and EndoUmut infections. We found that EndoUmut virus infection of both AML12 cells (Fig. 17A) and IFNAR−/− BMDMs (Fig. 17B) produced similar levels of nsp1 and N protein compared to wild-type infection. The timing of protein production also does not differ with N protein and Nsp1 becoming detectable at 6 hpi. This data suggests that the formation of the dsRNA signal in the absence of EndoU does not alter viral protein production, thus the viral replication does not differ between wild-type and EndoUmut virus in the cells.
Figure 17. Analysis of Viral Protein Production During EndoUmut Infection. (A) AML12 cells or (B) IFNAR−/− BMDMs were infected with WT- or EndoUmut-MHV at an MOI of 0.1. Cellular protein lysate was collected at indicated timepoints and separated on a 10% SDS-PAGE gel. After transfer, blots were stained with antibodies against N protein (J3.3), Nsp1 (anti-p28), or host β-actin.

The Viral RNA Recognized by the K1 Antibody During CoV Infection is Negative-Sense RNA

Since the dsRNA recognized by the K1 antibody accumulates in the absence of EndoU activity, we sought to identify the dsRNA epitope. To this end, we sequenced the RNA precipitated with the K1 anti-dsRNA antibody. We obtained ~30 million reads for each total RNA sample and ~10 million reads for immunoprecipitated samples. Upon mapping the reads to the mouse genome, we found similar read counts to host genes from both wild-type- and EndoUmut-infected cells (data available at NCBI GEO database, GSE144886). We then mapped the reads to the MHV-A59 genome (Genbank accession no. AY910861), and separated the viral reads by strand specificity, expecting to identify complementary sequences from positive- and
negative-sense RNA. Surprisingly, we found that the majority of reads from the immunoprecipitated RNA sample mapped to negative-sense RNA (Fig. 18A). We discovered that 99.8% of the reads from the input RNA sample mapped to positive-sense RNA. In contrast, 99.8% of the reads from the immunoprecipitated RNA mapped to negative-sense RNA. We found that the reads from the input RNA sample mapped across the entire MHV genome, as expected (Fig. 18B and D). Similarly, we found that the reads from the immunoprecipitated RNA sample also mapped across the entire genome (Fig. 18C and E). We concluded that the K1 antibody immunoprecipitated full-length, negative-sense RNAs. When comparing the read counts between wild-type virus- and EndoUmut virus-infected samples, we found an 8-fold increase (6 x 10^5 read counts versus 4 x 10^6 read counts) in the abundance of the reads from the EndoUmut virus-infected samples (Fig. 18A). These results are consistent with the increase in dsRNA foci observed in EndoUmut-infected cells by immunofluorescence staining (Fig. 13).
Figure 18. RNA-Seq Analysis of Input Viral RNA and RNA Immunoprecipitated with Anti-dsRNA Antibody K1. IFNAR<sup>−/−</sup> BMDMs were infected with WT- or EndoUmut-virus at an MOI of 1. At 6 hpi, RNA was purified, mixed with anti-dsRNA antibody, precipitated with protein G beads, and purified off the beads. Input RNA and immunoprecipitated RNA samples were evaluated by RNA-sequencing. (A) Summary of RNA reads mapped to MHV-A59 genome. Values in tables are the means of three biological triplicates. (B-E) Total reads mapped to the viral genome. Reads were mapped to the positive-sense RNA from input RNA (B) and immunoprecipitated RNA (C). Reads were mapped to the negative-sense RNA from input RNA (D) and immunoprecipitated RNA (E). Read counts were quantified for each nucleotide of the genome, then averaged into bins of 200 nucleotides for three biological triplicates. The black (WT) and red (EndoUmut) lines represent the mean of each bin and shaded regions are the standard deviation from the mean. Data are representative of two independent experiments.
To determine the abundance of the dsRNA signal in other cell types, we infected IFNAR$^{-/-}$ BMDMs, C57Bl/6 BMDMs, and AML12 cells with either wild-type or EndoUmut virus, performed the anti-dsRNA immunoprecipitation, generated cDNA with random hexamers, and evaluated the abundance of the captured viral RNA by qPCR. We consistently detected elevated levels of viral RNA immunoprecipitated by the dsRNA antibody from EndoUmut virus-infected cells as compared to the levels detected in wild-type virus-infected cells (Fig. 19A). The total input viral RNA was similar between wild-type- and EndoUmut-infected cells (Fig. 19B). Overall, our sequencing and qPCR results suggest that EndoU reduces the accumulation of a negative-sense viral RNA epitope that can be recognized by the anti-dsRNA antibody.

![Quantifying Viral RNA Immunoprecipitated with Antibody K1](image)

**Figure 19. Quantifying Viral RNA Immunoprecipitated with Antibody K1.** IFNAR$^{-/-}$ BMDMs, C57Bl/6 BMDMs, and AML12 cells were infected with WT- or EndoUmut-virus (EUmut) at an MOI of 1. At 6 hpi, RNA was collected and processed for dsRNA immunoprecipitation (IP) with anti-dsRNA antibody or an isotype control. (A) CoV RNA immunoprecipitated using K1 Ab was quantified using primers to the nucleocapsid (N) gene by qPCR. (B) CoV RNA from input RNA was quantified by measuring the N gene expression. Viral RNA was normalized to 18s rRNA and set relative to WT. Values were analyzed by student T-tests. Data are representative of three independent experiments and presented as mean ± standard deviation. n.s.: not significant
EndoU Activity Limits Abundance and Length of PUN RNAs

Previous studies showed that the 5’-end of the CoV negative-sense RNA contains polyU extensions (Hofmann and Brian, 1991), and that EndoU cleaves at uridine residues (Bhardwaj et al., 2008, 2006, 2004; Guarino et al., 2005; Ivanov et al., 2004; Kang et al., 2007). Therefore, we considered the PolyU-containing, Negative-sense RNA, which we termed PUN RNA, as a potential target for EndoU activity. We hypothesized that PUN RNAs accumulate in the absence of EndoU activity. To quantitate the PUN RNAs, we generated cDNA from the negative-sense RNA using a strand specific primer and performed a series of qPCRs with primers shown in Figure 20A. Primer Set 1 flanks a taqman probe and provides a measurement of total negative-sense RNA. Primer Set 2 measures the PUN RNA. By normalizing Set 2 to Set 1, we can compare relative proportions of the negative-sense RNA that contain polyU sequences. To control for potential “self-priming” of the viral RNA during cDNA synthesis, we performed cDNA synthesis in the presence or absence of the (-) sense cDNA primer and quantified RNA expression by qPCR (Fig. 20B). For both Set 1 and Set 2 qPCRs, we detected a significantly higher signal with the (-) sense primer compared to no primer. When comparing wild-type- and EndoUmut-infected cells, we detected a 10-fold increase in PUN RNAs from EndoUmut-infected cells as compared to wild-type virus-infected AML12 cells (Fig. 20C, left) and detected a 60-fold increase in IFNAR+ BMDMs (Fig. 20D, left). To determine if the polyA tail on the positive-sense RNA was similarly reduced by EndoU activity, we used either random hexamers or oligo-dT primers for reverse transcription and determined that the abundance of polyA tails on positive-sense RNA does not differ between wild-type and EndoUmut infections (Fig. 20C and D, middle and right). We concluded that EndoU activity reduces the abundance of negative-sense RNA that contains polyU extensions.
Figure 20. Quantifying PUN RNAs from Virus-Infected Cells. IFNAR−/− BMDMs and AML12 cells were infected with WT- or EndoUmut-virus at an MOI of 1 and RNA was purified from cell lysates. (A) Schematic of cDNA and qPCR design. cDNA was generated using cDNA primers specific to the negative-sense RNA, random hexamers for total RNA, or oligo-dT primers for positive-sense RNA. qPCR was performed with either primer set 1 or primer set 2 for each polyU qPCR. Nucleotide number where (-) sense cDNA primer and probe bind to viral RNA are labeled. (B) qPCR of cDNA synthesized with no primers or (-) sense cDNA primers. (C) PolyU qPCR of negative-sense RNA (left) or PolyA qPCR primed with random hexamers (middle) or oligo-dT primers (right) from AML12 cells at 8 hpi. (C) PolyU qPCR of negative-sense RNA (left) or PolyA qPCR primed with random hexamers (middle) or oligo-dT primers (right) from IFNAR−/− BMDMs at 6 hpi. Set 2 is normalized to Set 1 and are presented as mean ± standard deviation. Values were analyzed by student T-tests. Data are representative of three independent experiments. n.s.: not significant. nd: not detected.
To determine if EndoU reduces the lengths of the polyU-extensions on the PUN RNA, we completed a nested PCR reaction to obtain polyU-containing PCR products with a minimum predicted size of ~ 100 bp (Fig. 21A). We detected PCR species of ~100 bp from both wild-type- and EndoUmut-infected cells, and detected a smear of larger PCR species unique to EndoUmut virus-infected cells (Fig. 21B). To determine if the length of polyA tails on the positive-sense RNA was affected by EndoU activity, we generated cDNA with oligo-dT primers to select for polyA-containing RNAs and performed the nested PCR reactions. We found that the products generated from positive-sense RNA were similar between wild-type and EndoUmut viruses, consistent with our previous results indicating that the polyA tail is not cleaved by EndoU activity (Fig. 21C). To determine if the smear of PCR amplicons represents extended polyU sequences, we sequenced the amplicons with next generation sequencing and found that EndoUmut PCR amplicons had an increase in the number of reads and proportion of products with extended polyU sequences (Fig. 21D). The most striking feature of the sequencing results is the bimodal distribution of the polyU extensions present in the EndoUmut-infected cell samples. We found that the majority (65%) of the reads from wild-type virus infection contained 10 uridine residues. In contrast, only 35% of the reads from the EndoUmut virus-infected sample contained 10 uridine residues. This was not due to a difference in the number of reads with 10 uridines, but an increase in longer polyU extensions detected in EndoUmut-infected cells. We detected variability in the polyU extensions in the EndoUmut virus-infected sample, with 65% of the reads containing from 11-17 uridine residues. We note that while PUN RNAs in EndoUmut-infected cells are only a few uridines longer than the PUN RNAs from wild-type virus-infected cells, the PUN RNAs are 10-fold more abundant in EndoUmut virus-infected cells (Fig. 20). Overall these experiments revealed that EndoU activity reduces the abundance and length of
polyU extensions on PUN RNAs, consistent with our hypothesis that EndoU cleaves the PUN RNAs during virus replication.

Figure 21. Evaluating the Length of PolyU Extensions on PUN RNA. AML12 cells were infected with WT or EndoUmut virus at an MOI of 1. At 8 hpi, RNA was purified from cell lysates and polyU nested PCR was performed. (A) Schematic of nested PCR. Briefly, cDNA was generated with a strand-specific primer for negative-sense RNA or an oligo-dT-anchor primer for positive-sense RNA, then nested PCR was performed. (B) PolyU or (C) PolyA pcr products separated on a 10% polyacrylamide gel and stained with SYBR Green II. (D) PolyU PCR products were purified from a polyacrylamide gel (B) and sequenced with MiSeq Next-Gen Sequencing. (Left) Graph of read counts that contain a specific length of polyU extensions. (Right) Graph of proportion of reads that contain a specific length of polyU extensions. Data are representative of three independent experiments.
Since EndoU is conserved among coronaviruses, we sought to determine if EndoU reduces the abundance and length of the PUN RNAs in the alpha-coronavirus porcine epidemic diarrhea virus (PEDV). Although the EndoU domains of MHV and PEDV exhibit only about 50% overall amino acid similarity, the catalytic histidines are 100% conserved (Deng and Baker, 2018). We showed that inactivation of EndoU in PEDV results in an increased type I and type III IFN response during infection (Deng et al., 2019). To determine if EndoU limits the accumulation of PUN RNAs during PEDV infection, we infected cells with either wild-type or EndoUmut PEDV, isolated RNA and evaluated the levels of PUN RNAs. We found that, relative to wild-type virus-infected cells, EndoUmut virus-infected cells contained abundant PUN RNAs in PK1 (Fig. 22A) and Vero cells (Fig. 22B). Sequences of PCR products templated by PUN RNA revealed that the length of the polyU extensions on the PUN RNAs was increased during EndoUmut virus infection (Fig. 22C and E), with a similar bimodal distribution of polyU extensions shown in Fig 22D. During PEDV infection, we did not observe a difference in polyA-tail length (Fig. 22D). Taken together, these results indicate that PUN RNAs are generated during alpha- and beta-CoV replication, and that the highly conserved EndoU activity targets the polyU extensions in the PUN RNA.
Figure 22. Evaluating the Abundance and Length of PUN RNA during PEDV Infection. PK1 or Vero cells were infected with WT- or EndoUmut- PEDV at an MOI of 0.1. RNA was purified at 24hpi. (A) PolyU qPCR quantified in PK1 cells. (B) PolyU qPCR quantified in Vero cells. Set 2 is normalized to Set 1 and are presented as mean ± standard deviation. (C) PolyU or (D) PolyA nested PCR products from PK1 Cells. (E) PolyU PCR products from PK1 cells were purified from a polyacrylamide gel (C) and sequenced with MiSeq Next-Gen Sequencing. (Left) Graph of read counts that contain a specific length of polyU extensions. (Right) Graph of proportion of reads that contain a specific length of polyU extensions. Values were analyzed by a student T-test. Data are representative of two independent experiments.

PUN RNA is a PAMP

Since EndoU both reduces PUN RNA abundance and suppresses host MDA5 activation, we hypothesized that CoV PUN RNA is a PAMP. To directly test this hypothesis, we measured
IFN stimulation following introduction of PUN RNAs into AML12 cells. PUN RNA was synthesized by T7 in vitro transcription of digested plasmids that contained sequences representing the 5′-end or 3′-end of the viral genome (Fig. 23A). The PUN RNA, designated N5, and other coronavirus positive- and negative-sense RNA termini (P3, P5, N3) were transfected into AML12 cells. Total cellular RNA was harvested at 8 hours post-transfection (hpt) and subjected to qPCR for IFNβ1 mRNA expression. We found that the presence of PUN RNAs increased IFNβ1 expression by 2,000-fold (Fig. 23B), which was 4-fold higher than any other in vitro transcribed viral RNA, indicated that PUN RNA is a PAMP.

To determine if the polyU sequence contributed to the robust IFN stimulation of the PUN RNA, we transcribed PUN RNA either containing 12 uridines (N5) or no uridines (N5.NoU) at the 5′ end. We found that removing the 12 uridines from the PUN RNA significantly decreased the ability of that RNA to induce IFNβ1 expression (Fig. 23C). Also, removing sections of the 3′ end of the PUN viral sequence (N5.180 and N5.100) resulted in a decrease in IFNβ1 expression, suggesting the polyU sequence alone is not sufficient to induce the IFN response (Fig. 23C). Shortening the polyU extension to 8 uridines (N5.8U) or 4 uridines (N5.4U) also diminished the IFN activation by the PUN RNA (Fig. 23D). These results suggest that a polyU sequence of 12 U’s can enhance the IFN response to PUN RNA.

Previous studies documented that MHV-A59 infection induces IFN through MDA5 signaling (Deng et al., 2017; Roth-Cross et al., 2008). To determine if PUN RNA activates MDA5, we generated MDA5 knock-down (MDA5-KD) AML12 cells by CRISPR-Cas9 transduction (Fig. 23E) and measured IFN activation by virus infection or RNA transfection. Both viral infection and the transfection of the PUN RNA induces IFNβ1 expression in an MDA5-dependent manner (Fig. 23F and G). During viral infection of MDA5-KD cells, both
wild-type and EndoUmut virus infections had a significant reduction of IFNβ1 expression (Fig. 23F). IFNβ1 induction by *in vitro* transcribed PUN RNA also was significantly reduced in MDA5-KD cells (Fig. 23G). Importantly, we found that a single-stranded, *in vitro*-transcribed RNA activated MDA5, which was previously known to be activated by long complementary dsRNA. Taken together, these data suggest that the PUN RNA can act as an MDA5-dependent, viral PAMP.

**EndoU Cleaves PUN RNA and Dampens Activation of IFN**

To determine if EndoU activity can cleave the PUN RNA PAMP, we performed a series of *in vitro* cleavage assays (Kang et al., 2007). We incubated EndoU with 5’-negative-sense RNA containing a 12 U extension (RNA 1) or without the 12 U extension (RNA 2)(Fig 24A). When either RNA 1 or RNA 2 is mixed with EndoU in the presence of MnCl₂, the RNA is degraded over time (Fig. 24B). This degradation is most likely due to the presence of multiple uridines throughout RNA 1 and RNA 2, which is consistent with previous studies (Bhardwaj et al., 2006). To determine if the polyU extension is cleaved, we substituted the viral sequence uridines with adenosines and generated RNA 3 and RNA 4 (Fig. 24A). When mixed with EndoU and MnCl₂, the polyU extension of RNA 3 is cleaved, producing a cleavage product the size of RNA 4 (Fig. 24C). RNA 4 was not cleaved, consistent with the requirement of uridine residues for EndoU recognition and cleavage.
Figure 23. Determining if PUN RNA is a MDA5-dependent PAMP. RNA was in vitro transcribed from DNA constructs and transfected into AML12 cells. At 8 h post transfection (hpt), RNA was purified from cell lysates and IFNβ1 gene expression was measured by qPCR. (A) Schematic diagram of RNA products from in vitro transcription. (B) IFNβ1 gene expression induced by coronaviral RNA termini. (C) IFNβ1 gene expression by PUN RNA constructs. (D) IFNβ1 gene expression by PUN RNA constructs with varying polyU lengths. MDA5-knockdown AML12 cells (MDA5-KD) were generated by CRISPR/Cas9 transduction. (E) Western blot of WT and MDA5-KD AML12 cells for MDA5 and Actin. (F) WT and MDA5-KD AML12 cells were infected with WT- or EndoUmut-MHV at an MOI of 1. IFNβ1 expression was measured at 16 hpi. (G) In vitro transcribed PUN RNA was transfected into WT- or MDA5-KD AML12 cells. IFNβ1 expression was measured at 8 hpt. IFNβ1 gene expression is normalized to 18s rRNA and set relative to mock. Values were analyzed by student T-tests. Data are representative of three independent experiments and presented as mean ± standard deviation.
To determine if EndoU cleavage can decrease the ability of PUN RNA to stimulate IFN, we cleaved the PUN RNA with EndoU (Fig. 24D). In the presence of EndoU and MnCl$_2$, the PUN RNA was degraded into smaller RNA fragments. After EndoU treatment, we transfected the PUN RNAs into AML12 cells and measured IFN stimulation (Fig. 24E). We found transfecting the RNA treated with EndoU decreased the IFN stimulation activity. We note that the PUN RNA with MnCl$_2$ migrated faster in the agarose gel, possibly due to a change in RNA structure, but we do not observe a difference in IFN stimulation in the presence of MnCl$_2$ alone. Overall, EndoU is capable of cleaving and degrading PUN RNA, which then reduces the ability of PUN RNA to stimulate IFN.
Figure 24. Evaluating EndoU Activity on PUN RNA. RNA was cleaved by EndoU and separated by gel electrophoresis. (A) Sequences and length of RNAs 1-4. (B) EndoU cleavage of RNA 1 and RNA 2 performed for stated times and separated on a 10% polyacrylamide gel. (C) EndoU cleavage of RNA 3 and RNA 4 for 30 min and separated on a 10% polyacrylamide gel. (D) EndoU cleavage of in vitro transcribed PUN RNA (N5) for 45 min and separated on a 1% agarose gel. (E) RNA treated by EndoU cleavage was transfected in AML12 cells and at 8 hpt, RNA was purified from cell lysates and IFNβ1 gene expression was measured by qPCR. IFNβ1 gene expression is normalized to 18s rRNA and set relative to mock. Values were analyzed by student T-tests. Data are representative of three independent experiments and presented as mean ± standard deviation.

Evaluating the Pathogenesis of EndoU-mutant Viruses in Mice

In this section, I discuss the role of EndoU as a virulence factor during MHV infection in mice. Since EndoUmut viruses are attenuated in wild-type macrophages, I predict that
EndoUmut viruses will be attenuated *in vivo*. I hypothesize that the IFN response generated during the EndoUmut virus infection will stimulate an adaptive immune response and a memory response that will protect the mice from challenge with wild-type virus. The experiments that follow address the potential of using EndoUmut viruses as live-attenuated vaccine candidates.

**EndoUmut Viruses are Attenuated *In Vivo***

Because EndoUmut viruses induces multiple host innate immune sensors, we hypothesized that EndoUmut viruses will be attenuated compared to wild-type virus infection *in vivo*. To test this, we first infected C57Bl/6 mice intraperitoneally (IP) with $6 \times 10^4$ PFUs of wild-type or EndoUmut viruses. After 3- or 5-days post infection (dpi), we collected liver and spleen tissue for viral pathogenesis analysis. Interestingly upon IP infection, we did not detect infectious virus in the spleen or liver of EndoUmut virus infected mice at either 3- or 5-dpi, while we detected wild-type MHV titers as high as $10^5$ PFU/g of tissue (Figure 25A). We also performed H&E staining of liver slices to evaluate the pathogenesis of the infections. Wild-type infection induces plaque formation in the liver caused by immune cells infiltrating into the tissue to control the viral infection. In contrast, we detected no evidence of disease or immune cell infiltration in the liver during EndoUmut virus infections (Figure 25B).

A lethal challenge model of intracranial (IC) infection by MHV-A59 was also used to test EndoUmut virus attenuation. Briefly, 600 PFU of wild-type or EndoUmut viruses were injected into the mouse cranium and viral pathogenesis was measured by loss of body weight. Wild-type MHV infection leads to weight loss, requiring euthanasia of the mice by 7-9 dpi. Comparatively, all EndoUmut infected mice had transient weight loss and all mice recovered from the viral infection, leading to 100% survival (Figure 25C and D). The attenuation of EndoUmut viruses is
type I IFN-dependent, since infection of IFNAR-/- C57Bl/6 mice shows similar pathogenesis and mortality in both wild-type and EndoUmut MHV infections (Figure 25E).

Figure 25. Evaluating the Pathogenesis of EndoUmut Infection in Mice. Six-week-old C57BL/6 mice were intraperitoneally inoculated with 6.0 × 10⁴ pfu of WT or EndoUmut viruses (N15m1 and N15m3). (A) Liver and spleen were harvested at 3 and 5 dpi (DPI) and tested for viral titer by plaque assay. Red dashed line indicates the limit of detection. (B) At 5 dpi, mouse livers from A were harvested for pathology evaluation by H&E staining. Typical lesions of MHV infection in liver were indicated by arrowheads. (Magnification, 40×.) (C and D) Mice were inoculated by intracranial injection with 600 pfu of WT or mutant viruses. Viral pathogenicity was evaluated by (C) bodyweight loss and (D) survival rate. Data are a pool of two independent experiments. The P values of survival rate were calculated using a log-rank test. (E) Twelve- to 14-wk-old IFNAR-/- mice were intraperitoneally inoculated with 50 pfu of virus and monitored for mortality. The P values of survival rate were calculated using a log-rank test. WT vs. N15m1, P = 0.0047; WT vs. N15m3, P = 0.0719; N15m1 vs. N15m3, P = 0.0145. Mouse numbers (n) are indicated in parentheses. Data are a pool of two independent experiments. Error bars in A, B, and D represent the mean ± SEM. These experiments were performed with Dr. Xufang Deng and Dr. Robert Mettelman in the Baker Lab.
**EndoUmut Viruses Elicit a Protective Immune Response In Vivo**

Because the EndoU mutant viruses are highly attenuated for virulence in vivo, we wanted to determine if these viruses could elicit protective immunity against subsequent challenge with wild-type virus. C57Bl/6 mice were infected intraperitoneally with $6 \times 10^4$ pfu of EndoUmut viruses. Four weeks later, the same mice, as well as naïve, age-matched mice, were challenged with $6 \times 10^4$ pfu of WT virus and viral burden and liver pathology were assessed at 5 dpi. In contrast to naïve mice, mice that had been inoculated with EndoUmut viruses before challenge with wild-type MHV produced undetectable viral titers in tested organs (Fig. 26A) and no observable liver pathology (Fig. 26B). Even more striking were the results from intracranial infection. Here we challenged mice that had been previously inoculated 7 wk prior with EndoUmut or naïve mice with a 10-fold lethal dose of MHV-WT. We found that the immunized mice experienced only minor weight loss and fully recovered from infection, whereas the age-matched naïve mice succumbed to infection (Fig. 26C and D). This finding demonstrates that immunization with EndoUmut viruses protects mice from a subsequent lethal challenge, suggesting that EndoUmut viruses can elicit strong, protective immune memory in mice, which highlights their potential as vaccine candidates.
Figure 26. Evaluating if EndoUmut can Elicit a Protective Immune Response in Mice. Ten-week-old naïve C57BL/6 mice or mice immunized with mutant virus 4 wk prior (from Fig. 25A) were intraperitoneally inoculated with $6.0 \times 10^4$ pfu of WT virus. At 5 dpi, organs were harvested for (A) viral titration and (B) liver pathology. Red dashed line in A indicates limit of detection. Images of liver sections in B are representative of four mice per group. (Magnification, 40×.) Black arrowheads indicate the liver lesions caused by MHV infection. (C and D) Thirteen-week-old naïve mice and mice immunized seven weeks prior with N15m1 (from Fig. 7E) were challenged with $6.0 \times 10^3$ pfu WT virus by intracranial inoculation. Viral pathogenicity was evaluated by (C) body weight loss and (D) percent survival. Mouse numbers (n) are indicated in parentheses. The P values of survival rate were calculated using a log-rank test. Data are a pool of two independent experiments. Error bars in A and C represent the mean ± SEM. These experiments were performed with Dr. Xufang Deng and Dr. Robert Mettelman in the Baker Lab.
Our studies sought to determine the role that the CoV endoribonuclease plays during viral replication. Previous studies found that purified EndoU protein could cleave ssRNA and dsRNA at uridine residues, but how this activity functioned during viral replication was unknown. Previous researchers thought that EndoU activity was required for viral RNA synthesis, since several attempts at generating EndoU-mutant CoVs were unsuccessful. Also, previous studies found that overexpression of EndoU in cells can block multiple innate immune signaling pathways, thus indicating that EndoU may be an immune antagonist and virulence factor during viral infection. Our studies provided evidence for the role of EndoU activity in inhibiting innate immune signaling and provided mechanistic details for how EndoU inhibits the detection of a CoV pathogen-associated molecular pattern (PAMP).

We found that EndoU activity is not necessary for viral RNA production or viral replication, but is necessary to prevent the activation of multiple host innate immune sensors. We found that replication of EndoU mutant (EndoUmut)-MHV leads to an activation of multiple host sensors in wild-type macrophages and eventual attenuation of viral replication compared to wild-type virus infection. In IFN-signaling deficient macrophages (IFNAR−/− BMDMs), EndoUmut viral replication is identical to wild-type MHV. EndoUmut viruses have increased activation of the host PRRs that recognize viral dsRNA, including MDA5, PKR, and OAS/RNase L. MDA5 activation induces the production of IFN, PKR phosphorylates eIF2α to
inhibit protein translation, and OAS1/2 activates RNAse L to degrade intracellular RNA. The activation of multiple host sensors eventually leads to apoptosis in the macrophages and attenuation of EndoUmut virus replication. Overall, we report that EndoUmut viruses activate multiple innate immune sensors, driving macrophages to undergo apoptosis. This immune activation attenuates viral replication and reduces the production of infectious virions (Fig. 27).

**Figure 27. Diagram of Host dsRNA Sensor Activation by EndoUmut Viruses.** Viral dsRNA signals can activate host PRRS: MDA5, PKR, and OAS1/2. MDA5 activation leads to expression of IFNs. PKR activation leads to the shutdown of host translation machinery. OAS activation leads to RNAse L activation, which degrades host RNA. Overall, these responses lead to apoptosis in macrophage. We found that EndoU activity plays a critical role in preventing the activation of these sensors.

Since EndoU is an immune antagonist, we sought to determine if mutations in EndoU could be used for generation of potential live-attenuated vaccines. To test whether EndoUmut viruses are attenuated *in vivo*, we infected mice and determined if EndoUmut MHV caused severe pathogenesis. Upon IP or IC infections in wild-type mice, we found that EndoUmut viruses have attenuated viral replication and have decreased immunopathogenesis and mortality compared to wild-type MHV. These results indicated that the EndoUmut viruses stimulated an immune response and were effectively cleared by the mice without causing significant disease.
Even though the EndoUmut virus is substantially attenuated in mice, the mutant virus still elicited a protective immune response by protecting the EndoUmut-immunized mice from wild-type virus challenge. Therefore mutations in EndoU show potential as mutations for the generation of live-attenuated vaccines for CoVs.

Since EndoUmut viruses showed promise as a vaccine candidate, we sought to determine the mechanism for EndoU in inhibiting innate immune signaling. EndoU had previously been shown to degrade dsRNA, which is the PAMP for the EndoUmut-activated PRRs. We therefore hypothesized that EndoU would degrade the CoV-specific dsRNA PAMP during viral replication. We found that cells infected with EndoUmut viruses exhibited an increase in dsRNA signaling, but interestingly the dsRNA signal was formed by negative-sense viral RNA. Since EndoU cleaves at uridine residues, we hypothesized that EndoU cleaved a uridine motif in the negative-sense RNA. One feature that caught our attention was the polyuridine (polyU) head of the negative-sense RNA (Hofmann and Brian, 1991).

We hypothesized that during the synthesis of negative-sense RNA, the CoV RNA-dependent RNA polymerase generated negative-sense RNAs with variable lengths of polyU extensions. EndoU can recognize and cleave the polyU extensions, which limits the ability of the negative-sense RNA to form a viral PAMP. In the absence of EndoU activity, the polyU extension on the PUN RNA enhances the interactions of the PUN RNA with a complementary region of the viral genome to form an epitope recognized by MDA5 and other host sensors, signaling for activation of innate immunity.

Our studies reveal that CoV EndoU activity cleaves polyU-containing, negative-sense RNA (termed PUN RNA), which acts as a viral PAMP. EndoU cleaves the poly-uridine sequence on the PUN RNA, limiting the length and abundance of the polyU extension. We
determined that EndoUmut-infected cells had significantly more PUN RNAs compared to wild-type infection. The increased PUN RNAs were also longer in EndoUmut virus infected cells compared to wild-type MHV infected cells. To test if PUN RNA can activate host PRRs, we in vitro-transcribed PUN RNA and found in fact that PUN RNA stimulates MDA5 to signal for an IFN response in cells. Removal of the polyU extensions reduced the IFN stimulation by the PUN RNA, indicating that removal of the polyU sequence by EndoU could reduce the IFN stimulation by the PUN RNA. EndoU cleavage of in vitro-transcribed PUN RNA also reduced the immune stimulation by the PUN RNA. Overall, our studies revealed that the PUN RNA is a PAMP and that EndoU activity is essential for limiting the accumulation and length of PUN RNAs during CoV infection (Fig. 28).

Our studies have determined a role for EndoU inhibiting innate immunity during CoV replication. We have found that EndoU will prevent activation of dsRNA PRRs by cleaving the PUN RNA. We also discovered that PUN RNA is a CoV PAMP, which can activate MDA5. While we have determined the mechanism for EndoU to antagonize innate immunity, there are many interesting questions regarding innate immune signaling and CoV replication that are still unanswered. In the following sections, I will address these questions.
Figure 28. Model Depicting EndoU Cleavage of PUN RNAs. This model depicts how EndoU activity limits the generation of PUN RNA, which can act as a PAMP. We found that PUN RNAs with variable lengths of polyU sequences are generated in the absence of EndoU activity. We predict that these PUN RNAs can fold back and generate stem-loop structures by hybridizing with an A/G rich domain located within the PUN RNA or on adjacent RNAs. This stem-loop structure may be recognized as dsRNA by host PRRs, thus stimulating the host innate immune response. The function of EndoU during replication is to reduce the length of polyU sequences, thus limiting the potential for generating PAMPs.

What RNAs are Activating MDA5 during CoV Infections?

Identifying viral PAMPs and the host PRRs that are activated by the PAMPs is critical for developing strategies for treating viral infections. Previous studies implicated MDA5 as the PRR important for macrophages to produce an IFN response against coronavirus infection (Deng et al., 2017; Kindler et al., 2017; Roth-Cross et al., 2008). Consistent with these studies, we report that the PUN RNA acts as a novel PAMP, recognized by MDA5 (Fig. 23). We show that
PUN RNA can stimulate a type I IFN response and knocking down MDA5 by CRISPR-Cas9 limits the IFN stimulation by PUN RNA. The PUN RNA is then a novel PAMP that activates an IFN signal through MDA5. The PUN RNA is a unique RNA PAMP that is generated during CoV replication.

Canonically, MDA5 binds to long dsRNA species, such as poly I:C, to induce IFN signaling (Kato et al., 2006). For most viruses, the leading hypothesis is that complement dsRNA composed of positive- and negative-sense RNAs form the signal that activates host dsRNA sensors. Contrary to this hypothesis, one previous study showed that viral RNA that activated MDA5 formed a hybrid ssRNA/dsRNA structure (Pichlmair et al., 2009). In this study, the researchers immunoprecipitated RNA from encephalomyocarditis virus (EMCV)-infected cells with the K1 antibody and found that this RNA also bound to MDA5. Additionally, when this RNA was separated on an agarose gel, there was not only a dsRNA band, but also a large amount of RNA stuck in the well of the gel. When determining which of these RNAs activated MDA5, it was the larger well RNAs, not the dsRNA, that activated IFN signaling. This study suggested that the “normal” viral dsRNA was not the potent activator of IFN signaling, but instead a higher-ordered structure that is composed of ssRNA and dsRNA stimulated MDA5.

In line with this previous study, our study suggests that the negative-sense RNA may form a higher-order RNA structure that can bind and activate MDA5. While we have not yet investigated the double-strandedness of the negative-sense RNA, our immunoprecipitation with the dsRNA antibody yielded predominantly negative-sense RNA. This suggests the negative-sense RNA can form a dsRNA epitope recognized by an anti-dsRNA antibody without the presence of the complement positive-sense RNA. It would be interesting to determine if immunoprecipitated CoV RNA is similar to the immunoprecipitated EMCV RNA with the
formation of a higher-ordered RNA that possesses both ssRNA and dsRNA qualities. Future studies should investigate the nature of K1-immunoprecipitated RNA for being either ssRNA or dsRNA. Also, studies could focus on whether the polyU extensions and EndoU alter whether the viral negative-sense RNA is ssRNA or dsRNA.

To investigate CoV PAMPS that are detected by MDA5, we transfected various CoV RNA segments into cells and measured IFN stimulation. We found that PUN RNA stimulated high levels of IFN and the removal of the polyU extensions of the PUN RNA dampened the IFN stimulation. The PUN RNA IFN signal was mediated by MDA5. The looming question about this result is how does the polyU extension enhance MDA5 activation? One possibility is that the polyU’s stabilize an RNA secondary structure that is recognized by MDA5. The polyU sequence could bind in cis with the upstream viral RNA sequence, either generating or locking in place a unique RNA structure. Without the polyU extension, the negative-sense RNA could form an RNA structure that is not recognized by MDA5. Another possibility is that the polyU sequence binds in trans to other viral RNAs or potentially host RNAs that contain polyA sequences. The binding in trans may produce a short dsRNA sequence with the polyU extension but may also generate a higher-ordered, web-like RNA structure. This web-like structure of RNA would contain both ssRNA and dsRNA that would then be immunostimulatory, akin to the Pichlmair study (Pichlmair et al., 2009). Overall, future studies are needed to determine if the polyU extensions drives RNA structure changes in the PUN RNAs, which could ultimately alter the immune stimulation capabilities of the PUN RNA.

While PUN RNA from MHV-A59 is immunostimulatory, it has yet to be determined if the PUN RNA from other coronaviruses have a similar phenotype. Interestingly, the sequence of the 5’ UTR of the negative strand is not well conserved among coronaviruses. While SARS-CoV
and SARS-CoV-2 share the highest similarity (89%), the other beta-CoVs share about 45% RNA sequence similarity with MHV-A59 (Fig. 29). Future studies need to determine if other CoV PUN RNAs have a similar immunostimulatory effect. The main questions driving these studies would be to determine if polyU extensions enhance the immune stimulatory capabilities of all CoV PUN RNAs.

If all PUN RNAs stimulate MDA5, then the recognition of MDA5 may not be a sequence-dependent, but instead be RNA structure-dependent. This naturally leads back to the question of what RNA structures the PUN RNAs form compared to positive-sense RNA. Many studies have determined that the positive-sense RNA forms conserved RNA secondary structures, including pseudoknots, that are necessary for viral RNA replication (Yang and Leibowitz, 2015). Even though these structures are present in the positive-sense RNA, the positive-sense RNAs is not as immunostimulatory compared to the negative-sense RNA (Fig. 23). This result suggests that the complement RNAs may form different RNA structures. CoVs then may encode EndoU to combat the immune signaling stimulated by the PUN RNA.

One question is why did CoVs evolve to use EndoU to cleave the PUN RNA instead of mutating the viral sequence to prevent an MDA5-recognized structure from forming. With the positive-sense RNA structures being vital for RNA replication, any alterations to the positive-sense structure or sequences may be detrimental to viral replication and attenuate the virus. Therefore, the sequence of the complement negative-sense RNA cannot be altered, even though the negative-sense RNA can stimulate IFN. Since the viral sequence cannot be changed, CoVs may then use EndoU to combat the immune stimulation. EndoU will cleave the viral RNA thus antagonizing the cellular immune responses without forcing selection of the genomic RNA sequences.
Figure 29. RNA Sequence Alignment of (-) Sense, 5' UTRs of CoVs. Alignment of the first 500 nucleotides of the negative-sense 5' UTR of multiple CoVs. Coronaviruses that are aligned are MHV-A59 (AY910861), SARS-CoV (FJ882957.1), MERS-CoV (KJ614529.1), SARS-CoV-2 (MN985325.1), and PEDV-CoV (KF272920). Alignment was performed with Clustal Omega, then visualized with ESPript3.
Currently, many studies utilize nucleic acids as adjuvants to stimulate innate immune responses (Temizoz et al., 2018). The theory is that by producing RNAs that will stimulate an appropriate anti-viral response in conjunction with additional viral epitopes, then a robust immune response will be mounted to these additional viral epitopes. With the PUN RNA forming an MDA5-recognized structure, it would be interesting to determine if the PUN RNA could act as an MDA5 adjuvant to elicit robust IFN responses during immunizations. The PUN RNA could then be inserted into RNA vaccine constructs to act as an immune response boosting RNA structure. The PUN RNA would activate MDA5 within cells containing the RNA vaccine epitope, thus driving an appropriate anti-viral response to the viral epitopes.

**What are the RNA Epitopes that the dsRNA Antibodies Recognize?**

One of the surprising findings from our study was that antibody K1, which was developed as an anti-dsRNA antibody (Schonborn et al., 1991), recognizes CoV negative-sense RNA (Fig. 18). Our immunofluorescence studies showed that the epitope recognized by K1 accumulates in EndoUmut infected cells. Using RNA sequencing, we determined that the RNA bound by K1 was negative-sense RNA. We speculate that coronavirus negative-sense RNA forms a higher-order RNA structure recognized by the K1 antibody, and that this RNA is also recognized by host sensors. Supporting this idea, a previous study showed that the viral RNA immunoprecipitated by the K1 antibody during EMCV infection formed a higher-order RNA structure and could activate MDA5 (Pichlmair et al., 2009). Our approach using RNA-seq analysis of immunoprecipitated RNA could be widely used to determine if other unique dsRNA epitopes are generated during viral infections. Schonborn et al developed four anti-dsRNA antibodies: J2, J5, K1 and K2. These antibodies were generated against L-dsRNA from *S. cerevisiae* and each antibody has unique binding specificities to different dsRNA species. For
example, the K1 antibody was reported to be highly specific to poly I:C, whereas the J2 antibody is specific to L-dsRNA (Schonborn et al., 1991; Sweeney et al., 1976). The differing specificities suggest that each anti-dsRNA antibody recognizes unique dsRNA structures or sequences. Ultimately, structural studies are needed to fully elucidate the higher-order RNA structures that these dsRNA antibodies are recognizing during coronavirus infection.

Another interesting note about the dsRNA antibodies is that the antibodies do not detect RNA from every virus. The J2 antibody can detect a dsRNA signal from CoV, reoviruses, adenoviruses, herpes viruses, and vaccinia virus, so J2 can recognize RNA from both RNA and DNA viruses. Interestingly, the J2 antibody will not detect any signal from Influenza A virus or La Crosse fever viruses (Son et al., 2015; Weber et al., 2006). These studies bring up the idea that not all viruses will produce the dsRNA antibody signal, even though they produce dsRNA that can activate host dsRNA sensors (Kato et al., 2008; Pfaller et al., 2014). J2 recognizes antigens with dsRNA at least 40bps and has a 10 times high affinity to poly A:U or mixed based pairs than to poly I:C (Bonin et al., 2000). Although J2 will not recognize dsRNA during some viral infections, other dsRNA antibodies may bind and recognize viral RNA during these infections.

There are many unanswered questions about the binding specificities of these antibodies. First, is one antibody better at recognizing viral dsRNA? Both K1 and J2 can recognize dsRNA by immunofluorescence and immunoprecipitation, but they recognize different dsRNA species. While MDA5 can be activated by K1 immunoprecipitated dsRNA, whether J2 immunoprecipitated RNA activates MDA5 is unknown. It may be that J2 immunoprecipitated RNA will activate different RNA sensors, since it may recognize a different RNA epitope. The current results indicate that for MDA5-activating viruses, K1 may be the better antibody to
determine the dsRNA-activating epitopes. Further work is necessary to determine what the exact epitopes these antibodies are recognizing during viral infection. Additionally, the question of why certain viruses do not produce a dsRNA signal recognized by the antibodies needs to be addressed.

**Is the PUN RNA Cleavage Activity of EndoU Conserved Among Nidoviruses?**

Since EndoU is conserved among all coronaviruses and arteriviruses, the function of EndoU is hypothesized to be conserved among the viral family. We observed that EndoU from both alpha- and beta-coronaviruses can control the length and abundance of PUN RNAs. While the function of EndoU is conserved, the amino acid sequence conservation is only 40% between PEDV and MHV (Fig. 30). While other human beta-coronaviruses, such as SARS-CoV, MERS-CoV, and SARS-CoV-2, are more conserved to MHV than PEDV, the amino acid sequences are still less than 50% conserved. One interesting feature of the MHV genome is a 33 amino acid insert in the middle of EndoU. This inserted peptide actually forms a packaging signal for MHV for the genomic RNA to be bound and packaged into the virion selectively over sgRNAs (Athmer et al., 2017).

Although the overall amino acid sequence is not well conserved, the catalytic histidines of all the CoVs is conserved. The histidines are indicated by the green arrows in Figure 30. With conservation of catalytic histidines, mutations that remove EndoU activity should be able to be applied to any known or recently emerged coronavirus. The structure of these endoribonucleases also contain conserved secondary and tertiary protein structures (Nedialkova et al., 2009). This indicates that anti-viral therapeutics targeting the EndoU catalytic pocket might inhibit a broad spectrum of viruses in the nidovirus family. Interestingly, arteriviruses contain a nsp15 homolog, named nsp11, that also encodes an endoribonuclease. This suggests that anti-viral therapeutics
targeting EndoU activity may also be developed for arteriviruses, such as equine arteritis virus (EAV) or porcine reproductive and respiratory system virus (PRRSV). Also live-attenuated vaccines containing nsp11 catalytic mutations may also be developed. Further studies in arteriviruses would also strengthen the conserved activity of EndoU and provide further information for developing broad acting anti-virals or vaccines strategies. Overall, since the catalytic histidines of EndoU are conserved in the nidovirus family, EndoU is a promising candidate for the targeting of antiviral drugs and for generating live-attenuated vaccines.
Figure 30. Protein Sequence Alignment of Nsp15/EndoU. Alignment of the protein sequence of the endoribonuclease encoded by nsp15. Catalytic histidine residues are indicated by green triangles. Coronaviruses that are aligned are MHV-A59 (AY910861), SARS-CoV (FJ882957.1), MERS-CoV (KJ614529.1), SARS-2-CoV (MN985325.1), and PEDV-CoV (KF272920). Alignment was performed with Clustal Omega, then visualized with ESPript3.
Do Mutations in EndoU Reveal Unknown Mechanisms in CoV Replication?

Our study also raises interesting questions about CoV replication, particularly questions associated with the formation and usage of the polyU extension during CoV RNA synthesis. The first question is whether the polyU sequence is required for polyadenylation of the positive-sense RNA. Studies from influenza virus revealed that a short polyU sequence within a unique RNA stem loop mediates a stuttering mechanism used to polyadenylate the positive-sense RNA (Poon, 1999). In contrast, Peng et al implicate that CoVs utilize a non-canonical cytoplasmic polyadenylation site to synthesize the positive-sense polyA tail (Peng et al., 2016). They identified a conserved viral sequence on the positive sense RNA that could be bound by host proteins including cytoplasmic polyadenylation element binding protein 1 (CPEB1) (Richter, 2007), a protein that mediates polyadenylation. In addition, CoV nsp8 has been demonstrated to contain 3’ terminal adenylyltransferase (TAT) activity (Tvarogova et al., 2019). Nsp8 can synthesize the polyA tail on the positive-sense RNA and having a complement negative-sense RNA with a polyU extension greater than 5 U’s enhances the TAT activity. Since we observe EndoU controlling the abundance of longer polyU sequences which stimulate IFN, it would be interesting to determine if there is an “ideal length” of polyU sequences that lack immune stimulation by MDA5 but promote TAT activity during addition of the polyA tail.

Another question arises about how the polyU tail is primed and generated. Our studies reveals that the negative-sense RNA synthesis begins farther upstream within the polyA tail, thus generating longer polyU extensions that are eventually cleaved by EndoU activity. In vitro studies have shown that nsp8 and nsp7/8 complexes are capable of de novo initiation of RNA synthesis. The hypothesis predicts that nsp7/8 either starts the RNA synthesis then allows the nsp12 RdRp to finish the transcription or nsp7/8 generates RNA primers that are then utilized by
nsp12 for RNA synthesis (Imbert et al., 2006; te Velthuis et al., 2012). Our data suggests that if a primer is generated, it would most likely be a polyU sequence that would bind and interact with the positive-sense polyA tail. This interaction would then prime the RNA for negative-sense RNA synthesis. Another possibility is that a different primer or host miRNA is priming synthesis inside the polyA tail, and EndoU activity removes this additional sequence. Since our RNA-sequencing did not have sufficient reads over this region of the genome, it is unknown if there are additional sequences attached to the polyU sequence in the absence of EndoU activity.

One caveat of the in vitro studies discussed above are that the assays are performed in the absence of other viral replication complex proteins that may alter the binding, recognition, and activity of the RNA processing proteins. Studies have shown protein-protein interactions between nsp7, nsp8, nsp12, and nsp15, which may alter the activities of these proteins (Athmer et al., 2017; Kirchdoerfer and Ward, 2019). While EndoU fully degrades PUN RNAs in vitro, the cleavage activity may be more specific during viral infection due to interactions with other viral proteins in the membrane-associated replicase complex (Gosert et al., 2002; Knoops et al., 2008). While EndoU cleaves PUN RNA, there may also be other EndoU cleavage sites during CoV infection that were not detected in this study. Further studies are needed to fully elucidate the mechanisms CoVs use to alter and process viral RNAs.

**Do Other Viruses Contain PolyU Sequences and Mechanisms to Prevent PolyU Detection?**

Our studies raise the question of whether other viruses have mechanisms to limit polyU-containing RNA from activating host PRRs. The Hepatitis C Virus (HCV) genome encodes a long polyU stretch on the positive-sense RNA, but the polyU region is flanked by highly structured RNA, which may limit immune stimulation (Schnell et al., 2012). This study found that a 34 nucleotide polyU core sequence stimulates RIG-I signaling upon transfection of the
RNA. This core sequence is part of the positive-sense 3’UTR and is a section of a polyU/UC tract that is 100 nucleotides in length. The authors hypothesize that the polyU tract enhances RIG-I binding to the RNA, but RIG-I must also bind with the triphosphate at the 5’-end of the positive-sense RNA. This long-distance interaction may be possible through RNA folding and secondary structure. Although HCV contains a long polyU region, the polyU is not located at the terminal end of the RNA like the CoV polyU. Highly-structured RNA stem loops surround the HCV polyU region, which may alter the polyU interactions with other RNAs and prevent activation of dsRNA sensors by the polyU region (Adams et al., 2017). Furthermore, the HCV replication complexes may hide and sequester the viral RNA from recognition by host sensors (Neufeldt et al., 2016). So further studies are necessary to determine if the polyU region of the HCV genome is immunostimulatory during viral infection.

Polioviruses prime replication of the negative-sense RNA with a polyU sequence attached to VPg (Steil et al., 2010). The VPg protein is linked to a polyU sequence, which can then act as a primer binding to the polyA tail to start RNA replication. After RNA transcription and replication, the VPg linkage remains on the negative-sense RNA (Nomoto et al., 1977a), leaving the virion RNA to be VPg-capped. The VPg-linkage may prevent the exposure of a polyU structure to host sensors, thus preventing the polyU sequence from acting as a PAMP. The polyU sequence attached to VPg is relatively short (< 5 nt), so it would be interesting to determine if this polyU sequence is immunostimulatory. The positive-sense mRNA of PV also contains a short polyU head, but lacks the VPg linkage (Nomoto et al., 1977b). A cleavage event that removes the VPg linkage may be similar to EndoU cleavage of CoV polyU sequences and prevent stimulation by the viral RNA.
For influenza viruses, the polyU sequence on negative-sense RNA is essential for polyadenylation because it is part of a unique stem-loop structure (Poon et al., 1999). This study shows that the polyU sequence is necessary for influenza viruses to elongate the polyA tail on the positive-sense RNA. The polyU stretch is not located at the terminal end of the 5’ UTR but is located about 15 nucleotides from the 5’ end. The additional 15 nucleotides bind upstream of the polyU tail forming a stem-loop structure that allows for the polyU stretch to be unpaired to act as a ssRNA template. The additional RNA structure promoted by the 5’ terminal 15 nucleotides may prevent the polyU sequence from influencing RNA secondary structure, thus preventing immune activation by the RNA. Also it is worth noting that the negative-sense RNA is localized to the nucleus and would not be exposed to cytoplasmic dsRNA sensors. These examples illustrate that many viruses have polyU sequences that may potentially be PAMPs, and that each virus may have evolved unique mechanisms or structures that limit their detection. Future work would be necessary to determine if these polyU-containing sequences are in fact immunostimulatory, particularly if you remove the different viral mechanisms that may alter the polyU signal.

**Does EndoU Function like Other Viral Ribonucleases?**

Our study reveals that the coronavirus endoribonuclease activity is distinctly different from three other previously documented viral ribonucleases. Influenza PA-X is an endoribonuclease that selectively degrades host mRNA by hijacking host RNA splicing machinery (Khaperskyy and McCormick, 2015). PA-X inhibits the translation of host proteins to perturb the cell functions. PA-X selectively targets mRNA transcribed by RNA polymerase II in the nucleus of the cell. Host exonuclease Xrn1 then completes the degradation of the mRNA to prevent the mRNA from being translated into proteins. PA-X selectively targets specific host
RNAs to promote the shutoff of host machinery, while allowing the cells to survive to produce new virus particles.

Pestivirus RNAse E(rns) is an endoribonuclease that is secreted outside infected cells and degrades extracellular viral RNAs to block innate immune activation (Mätzener et al., 2009). This study determined that extracellular pestivirus dsRNA and ssRNA stimulates IFN signaling in cells. RNAse E(rns) is a viral envelope glycoprotein that is secreted to degrade extracellular RNAs, including the immunostimulatory pestivirus RNAs. Interestingly, viruses lacking RNAse E enzymatic activity activate 5’-triphosphate IFN signaling, such as RIG-I. This result suggests the RNA signal degraded by RNAse E differs from CoV EndoU as each virus activates a different PRR in the absence of their endoribonuclease activity.

Lassa virus encodes an nucleoprotein (NP) with exonuclease activity that will specifically degrade intracellular dsRNA (Hastie et al., 2011). The NP will block the activation of IRF3 signaling, which leads to IFN expression in the cells. The inhibition of IFN by NP has been shown in overexpression studies, so further work is needed to determine the mechanism and function of the NP exonuclease activity during viral infection.

While PA-X cleaves host RNAs to shut down the host cell functions, both RNAse E(rns) and Lassa virus NP cleave viral RNAs that are hypothesized to be PAMPs. Our study reveals an additional mechanism for a viral endoribonuclease to degrade a viral PAMP. Since the functions of the viral nucleases are distinct, the development or evolution of these nucleases probably occurred in distinct, separate events.

**Are there any Antivirals that Target EndoU?**

Although EndoU appears to be a promising target for inhibition of CoV replication, there are no approved anti-viral drugs developed. One study has developed drugs targeting the
enzymatic pocket of structurally similar endoribonucleases, XendoU and human PP11 endoribonucleases (Ragno et al., 2011). The small molecule inhibitors can block endoribonuclease activity during in vitro cleavage assays, but it has yet to be determined if these inhibitors are toxic in cell culture and could inhibit CoV EndoU activity. Ideally, these small molecule inhibitors would block EndoU activity without blocking host ribonucleases. Since Ragno et al. use structure-based drug screening, the specificity of the drugs to the XendoU family may prevent off target effects in cells. Another study sought to determine if RNAse A inhibitors would also inhibit EndoU activity in vitro and during viral infection (Ortiz-Alcantara et al., 2010). They ultimately determined that some of these compounds could inhibit infectious virus production, but also could have potential off-target effects due to the binding of these compounds to host nucleases. One potential avenue may be to find polyU analogs that could block EndoU activity. This could work as a competitive inhibitor for EndoU activity and therefore induce an antiviral IFN response during wild-type CoV infection. Overall, future studies are needed to investigate the efficacy of these XendoU drugs against CoV infections and specifically EndoU activity. Future work may also use these identified small molecule inhibitors as a base molecule in a drug development screen. The identified molecules would be modified to better fit the CoV EndoU catalytic pocket to increase specificity and decrease off-target side effects.

**Can EndoU-Mutant Viruses be Used as Live-Attenuated Vaccines?**

There are some studies that suggest that live-attenuated vaccines may be more efficacious than inactivated or subunit vaccines (Buchan et al., 2018; Kwong et al., 2015). While many of these vaccines are currently under development for CoVs, I hypothesize that a live-attenuated vaccine may be the most effective for CoVs. One benefit for live-attenuated vaccines is that most
do not require immune adjuvants. The viral replication stimulates a robust anti-viral immune response, which promotes a long-lasting memory response. Another benefit for live-attenuated vaccines is that active viral replication can produce viral quasi-species. Viral quasi-species are virus populations that contain multiple diverse and unique mutations in the viral genome that may alter protein sequences, particularly structural protein sequences (Toro et al., 2012; Viehweger et al., 2019). The mutations in viral structural proteins may provide additional epitopes for the stimulation of adaptive immune responses. While CoV quasi-species are viewed as a mechanism for CoVs to evolve and escape neutralizing antibodies, a vaccine strain that contained additional quasi-species epitopes would enhance the diversity of the host antibody repertoire, thus stimulating a more diverse vaccine response. One shortcoming of live-attenuated vaccines is that the vaccine must be maintained in a cold chain from production to delivery to allow for the vaccine strain to infect and immunize the patient. Also, mutating the virus to attenuate the virus in vivo may produce defects in viral replication. Replication defects would produce vaccine strains that would be difficult to mass produce and distribute. EndoUmut CoVs can replicate similar to wild-type virus in the absence of IFN signaling, therefore CoV vaccine strains containing EndoU mutations should be able to be mass produced.

One issue that might arise during vaccination is the potential for lack of long-term immunity generated by a CoV vaccine. Interestingly, wild-type CoV infections do not generate robust adaptive memory B-cell responses in humans (Tang et al., 2011). When SARS-CoV patients were analyzed 3 years post-infection, studies found that a majority of the patients lacked neutralizing antibodies specific for SARS-CoV (Liu et al., 2011). The reasons for why wild-type CoVs do not generate sustained immune memory responses are currently unknown. One possibility is that the subversion of the early innate immune system by CoVs alters the adaptive
immune responses. The early IFN induction seen during EndoU mutant virus infections could then promote an appropriate anti-viral immune response and stimulate long-term immunity after vaccination. Studies are needed to determine if CoV vaccines can sufficiently prime and stimulate an effective immune response to properly immunize a population.

While the EndoUmut virus used in these studies contains one catalytic site mutation (H262A), generating a live-attenuated mutant virus with only one mutation is not ideal. Reversion mutations either in the catalytic site or satellite sites may convert the attenuated mutant virus back into a virulent strain. In order to combat reversion, we can generate vaccine strains that contain multiple mutations in multiple immune antagonists. EndoU has two catalytic histidines, so mutating both catalytic histidine sites to alanine residues would decrease the likelihood of the virus regaining EndoU activity. Additionally, Menachery et al have shown that combining different IFN antagonist mutations can produce a reversion resistant CoV strain (Menachery et al., 2018). This study compared the pathogenesis of an nsp16-mutant SARS-CoV with a SAR-CoV mutant virus that contained a combination of mutations in nsp14 and nsp16. The SARS-CoV that contained multiple mutations in IFN antagonists had enhanced attenuation of viral replication and decreased pathogenesis in vivo compared to the nsp16 mutant virus. Ideally, the goal is to develop a vaccine strategy in which multiple conserved immune antagonists can be mutated to generate a vaccine strain. Since the mutations are conserved across the CoV family, so the strategy could be applied to any CoV. Future studies are needed to identify the constellation of IFN antagonists that should be targeted to generate the best vaccine. Our study emphasizes that EndoU catalytic mutations should be included in these vaccine strategies.
Another potential problem with live-attenuated vaccines is RNA recombination with another coronavirus that would allow a virus to regain virulence. Since CoVs are a relatively large RNA genome (~30kb), CoV strains recombine viral genomes with other CoV strains during in vivo co-infection. To make a stable live-attenuated vaccine, the virus should not be able to recombine with other viruses. One study described a vaccine platform approach that generated recombination-resistant coronaviruses (Graham et al., 2018). This platform takes advantage of the multiple TRS locations of CoVs. By mutating each TRS sequence of SARS-CoV to a new sequence, the mutant viruses were unable to recombine with wild-type SARS-CoV in vivo. Only viruses that contained an entire set of matching TRSs could replicate, which prevents the vaccine strain from gaining any genes from wild-type CoVs through recombination and producing a viable virus.

Overall, mutations in EndoU present ideal mutations to be included in a set of mutations for generating live-attenuated vaccines against CoVs. The EndoU sequence and function is conserved among CoVs, so this vaccine strategy could be applied to any known and recently emerged CoV. EndoUmut viruses can replicate similar to wild-type CoV in cell culture, but are attenuated in vivo and provide a protective immune response.

**Do CoVs have a Hierarchy of IFN Antagonists?**

One interesting aspect of these studies is that EndoUmut viruses are attenuated during infection of macrophages and in vivo, even though CoVs contain multiple IFN antagonists and innate immune antagonists that are still intact. One theory is that CoVs have a hierarchy of IFN antagonists. EndoU would be a vital IFN antagonist, since the virus lacking EndoU activity cannot grow in IFN-response cells. Our data suggests that EndoU prevents the initial dsRNA signal from forming and being detected by host sensors. Other IFN antagonists block pathways
downstream of the dsRNA detection. EndoU may dampen the initial dsRNA signal before other IFN antagonists have been effectively translated. This would grant the virus an advantage on blocking the immune signaling pathways and keep the initial dsRNA signal at a minimum.

Another possibility is that the other IFN antagonists block different pathways. While EndoU blocks one dsRNA signal that is detected by MDA5, other viral proteins will inhibit different signals or pathways that will ultimately signal for an IFN response in the cells. Other IFN antagonists may be necessary to completely shutoff IFN signaling early in infection. Even with EndoU, wild-type MHV produces a low level of PUN RNAs, which may be necessary for viral replication. EndoU may inhibit a significant portion of the signal but may not block all of the dsRNA signal that activates host sensors. Then additional viral proteins are needed to block the remaining dsRNA signaling or downstream IFN signaling that is stimulated in those cells.

Another hypothesis is that each IFN antagonist is necessary in specific cell types. We have studied EndoU in macrophages and hepatocytes, showing the importance of EndoU activity for IFN antagonism in these cells. In other cell types, such as lung or intestinal epithelial cells, EndoU may play a lesser role and other IFN antagonists may be more crucial for inhibiting the innate immune response. It would be interesting to determine if all EndoU inhibits IFN expression in all infectable cell types or if other viral antagonists have a more profound effect in other cell types, particularly the respiratory epithelium.

To study the potential hierarchy of IFN antagonists, studies should determine the function of each IFN antagonist in combination with EndoU catalytic mutations. One hypothesis is that EndoU inhibits the dsRNA signal that is necessary for activation of the immune pathways modulated by other viral proteins. With a robust activation of host dsRNA sensors during EndoUmut infections, we could study the effect of other CoV proteins on these pathways.
What are the Potential Consequences of CoV Outbreaks?

The current outbreak of SARS-CoV-2 reveals the constant threat of the emergence of CoVs from animal populations. Multiple studies have discovered many SARS-Like and MERS-Like CoVs that exist in bats and other mammalian species around the world (Corman et al., 2014; Hon et al., 2008; Lau et al., 2005; Menachery et al., 2015; Woo et al., 2018). These CoVs have the potential to bind to human receptors and infect human cells (Wan et al., 2020). SARS-CoV-2 is an example of one of these bat viruses emerging into humans. The current outbreak of SARS-CoV-2 has a mortality rate of ~2%, highlighting the deadliness of this virus (Huang et al., 2020; Munster et al., 2020; Zhou et al., 2020). The presence of similar CoVs that are lurking in animal reservoirs leads to the potential for CoV pandemics to occur regularly. CoVs also have the potential to spread quickly through respiratory transmission. The ever-looming threat of new strains of CoVs to infect humans emphasizes the need for broadly acting anti-virals and strategies to quickly generate vaccines.

The potential for CoV outbreaks is not limited to the human population. Multiple CoVs have emerged in pigs and generated billions of dollars of annual loss in the porcine industry (Langel et al., 2016). TGEV and PEDV emerged in the porcine population early in the 1990s, but recently porcine delta coronavirus (PDCoV) caused a pandemic outbreak in 2012 among pig farms in China and the USA (Zhang, 2016). More recently, researchers found that swine acute diarrhea syndrome coronavirus (SADS-CoV) emerged from bats to infect pigs in China, again causing significant loss in the pork industry (Cui et al., 2019). These outbreaks in the pig populations highlight that CoVs have potential for pandemics to occur in multiple species, easily jumping between host populations. Overall, the SARS-CoV-2 outbreak is the most recent CoV
pandemic, but unfortunately many other CoVs are poised to shift into the human population and cause significant disease.

Final Remarks

In summary, my work provides evidence for a novel mechanism used by the coronavirus EndoU to cleave a viral RNA PAMP, which would otherwise be recognized by MDA5. These studies identify a novel CoV PAMP, which we termed PUN RNA. EndoU cleaves the polyU extension of the PUN RNA, which can decrease the ability of MDA5 to activate and stimulate IFN signaling in cells. By removing EndoU activity, we can generate vaccine strains of CoVs that can replicate in the absence of IFN signaling but will be severely attenuated in vivo. EndoU therefore is a highly conserved virulence factor and a potential target for antiviral and vaccine strategies.
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VITA

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In August 2014, Matthew enrolled into the Interdisciplinary Program in Biomedical Sciences at Loyola University 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 determining the mechanisms for a coronavirus endoribonuclease to antagonize innate immune signaling. Matthew’s work was supported by an NIH RO1 awarded to SC Baker, Ph.D. and an NIH T32 Training Grant in Experimental Immunology awarded to KL Knight, Ph.D. Matthew will continue his research endeavors as a Postdoctoral Fellow in the laboratory of CB Lopez, Ph.D. at Washington University in St. Louis, Missouri. There Matthew will study the interactions of innate immune pathways and respiratory virus RNAs.