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Multifunctional Coronavirus Papain-Like Proteases as Targets for Antiviral Therapeutics and Vaccines

Anna Maria Mielech

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LOYOLA UNIVERSITY CHICAGO

MULTIFUNCTIONAL CORONAVIRUS PAPAIN-LIKE PROTEASES AS TARGETS FOR ANTIVIRAL THERAPEUTICS AND VACCINES

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

ANNA M. MIELECH

AUGUST 2014
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community. It not only gave me a strong foundation for the future career but also made me feel at home, which I truly appreciate.

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For Michal
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<td>3CLpro</td>
<td>picornavirus 3C-like protease</td>
</tr>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>BHK</td>
<td>baby hamster kidney cells</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>CCHFV</td>
<td>Crimean Congo hemorrhagic fever virus</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CoV</td>
<td>coronavirus</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>DBT</td>
<td>delayed brain tumor cells</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMVs</td>
<td>double membrane vesicles</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DUB</td>
<td>deubiquitinating activity</td>
</tr>
<tr>
<td>E</td>
<td>envelope protein</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<tr>
<td>EAV</td>
<td>equine arteritis virus</td>
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<tr>
<td>EC$_{50}$</td>
<td>half maximal effective concentration</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetertaacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol teretaacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>FIPV</td>
<td>Feline infectious peritonitis virus</td>
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</table>
g  g-force (m/s²)
G  guanine
HCoV  human coronavirus
HEK  human embryonic kidney
hpi  hours post infection
HPR  horseradish peroxidase
icMHV  infectious clone mouse hepatitis virus
IFN  interferon
ISG  interferon stimulated gene
kb  kilobase
LB  Luria-Bertani
Luc  luciferase
M  membrane protein
MEM  minimal essential medium
MERS  Middle East Respiratory Syndrome
μg  microgram
μl  microliter
ml  milliliter
μM  micromolar
mM  milimolar
MHV  mouse hepatitis virus
min  minutes
MOI  multiplicity of infection
Mpro  main protease
mRNA  messenger RNA
N  nucleocapsid
ng  nanogram
nm  nanometer
nM  nanomolar
nsps  non-structural proteins
nt  nucleotide
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>OTU</td>
<td>ovarian tumor domain</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PLP</td>
<td>papain-like protease</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>PRRSV</td>
<td>porcine respiratory and reproductive syndrome virus</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>S</td>
<td>spike glycoprotein</td>
</tr>
<tr>
<td>SARS</td>
<td>Severe Acute Respiratory Syndrome</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SV</td>
<td>Sendai virus</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>Ub</td>
<td>ubiquitin</td>
</tr>
<tr>
<td>UBL</td>
<td>ubiquitin like</td>
</tr>
<tr>
<td>USP</td>
<td>ubiquitin specific protease</td>
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</table>
ABSTRACT

Coronaviruses (CoV) are well known human pathogens. Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) and Middle East Respiratory Syndrome coronavirus (MERS-CoV) pose a severe threat to humans because of high mortality. Despite the risk of coronavirus emerging in the human population there are no antiviral drugs or vaccines to combat coronavirus infection. The focus of my dissertation was to study the multifunctionality of papain-like proteases (PLPs) encoded within coronavirus genomes to facilitate the development of antiviral drugs and vaccines. The viral PLPs are critical for processing the amino-terminal end of the replicase during virus replication and are attractive targets for antiviral therapies. In my research, I analyzed the activities of PLPs from multiple coronaviruses to determine if those proteases can be targets for broad spectrum therapeutics.

I determined that SARS-CoV PLpro is an effective target of small molecule inhibitors by evaluating their efficacy and ability to inhibit SARS-CoV replication in cell culture. Further, I determined that the predicted PLpro domain from MERS-CoV is a multifunctional enzyme with protease, deubiquitinase, interferon antagonism and deISGylating activities. Despite low sequence identity, this multifunctionality of PLPs seems to be conserved among many coronaviruses since I demonstrated protease and
deubiquitinase activity of PLP2s from HCoV-OC43, HCoV-229E, HCoV-HKU1, and FIPV.

To evaluate the role of PLP during virus replication and pathogenesis I utilized a mouse model system mouse hepatitis virus (MHV). I determined that similar to other PLPs, MHV PLP2 is a multifunctional enzyme. Further, I used two approaches to investigate the role of the PLP in virus pathogenesis. First, I performed deletion analysis and mutagenesis of the ubiquitin-like (UBL) domain of the PLP2 and showed, for the first time, that the UBL domain is important for PLP2 stability and virus pathogenesis. The UBL mutant virus is attenuated in mice and protects the mice from the disease upon challenge with wild-type virus suggesting that this virus is a vaccine candidate. Secondly, based on the MHV PLP2 crystal structure and modeling of the PLP2 with an ubiquitin moiety, I identified residues on PLP2 that likely interact with ubiquitin. I tested multiple mutants of predicted PLP2-ubiquitin interaction sites and found several residues that are important for both protease and deubiquitinase function. Taken together my data suggest that PLP multifunctionality is conserved among coronaviruses and that it is a valuable target for vaccines and antiviral drugs development for existing and emerging coronaviruses.
CHAPTER I
INTRODUCTION

CORONAVIRUSES IN HUMAN DISEASE

Coronaviruses are positive sense RNA viruses that infect humans and other animals. Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) and Middle East Respiratory Syndrome coronavirus (MERS-CoV) pose a severe threat to humans because of high mortality (Peiris, Guan, & Yuen, 2004; Zaki, van Boheemen, Bestebroer, Osterhaus, & Fouchier, 2012). As of April 14, 2014, there have been 228 confirmed cases and 92 deaths of MERS (http://www.who.int/2014_03_20_mers/en/). MERS disease is characterized primarily by respiratory symptoms but several patients also developed renal failure (Drosten et al., 2013; Zaki et al., 2012). In most cases reported thus far, immunosuppression or other types of medical disorders have been associated with more severe disease (Assiri et al., 2013). The sequence of the RNA genome of MERS-CoV is most similar to bat coronaviruses HKU4 and HKU5 (van Boheemen et al., 2012); however, the origin of MERS-CoV is not known. A recent report showed that dromedary camels have high levels of neutralizing serum antibodies against MERS-CoV, suggesting a possible zoonotic source (Alagaili et al., 2014; Reusken et al., 2013). Moreover, MERS-CoV sequences with 99% nucleotide similarity to human MERS-CoV have been detected in dromedary camels (Chu et al., 2014). Further analysis of the
polymorphism signatures of virus sequences from infected camels suggested cross-species transmission of MERS-CoV (Memmish et al., 2014). In addition, analysis of fecal samples from bats identified the Egyptian tomb bat as a potential source of infection (Memish et al., 2013), but more work is needed to identify the animal reservoir(s) for MERS-CoV. Limited human-to-human transmission of MERS-CoV has been reported, which considering the high mortality, raises a concern that the virus has a potential to become a threat to public health similar to SARS-CoV (Assiri et al., 2013; Guery et al., 2013). The SARS-CoV pandemic from 2002-2003 with 10% mortality rate (Drosten, Preiser, Gunther, Schmitz, & Doerr, 2003; Drosten, Gunther, et al., 2003; Peiris et al., 2003), was controlled by public health measures of identification and isolation of infected, symptomatic individuals and their contacts which broke the chain of human-to-human transmission (Zhong, 2004). A SARS-CoV-like virus is endemic in Chinese horseshoe bats, but changes in the sequence of the spike glycoprotein are required for this virus to efficiently infect humans (Lau et al., 2005; Rockx et al., 2007). For MERS-CoV, it is unclear if the virus can jump directly from bats to humans, if there are any mutations in the viral genome that facilitate infection or disease in humans, and if there are both symptomatic and asymptomatic cases, which would make any potential epidemic more difficult to control by public health measures alone.

Additional endemic human coronaviruses that cause respiratory tract disease include: HCoV-229E and HCoV-OC43, which cause common colds; HCoV-NL63, which has been associated with croup in children; and HCoV-HKU1, associated with lower respiratory tract infection and pneumonia in the elderly. Coronaviruses are also
important pathogens of livestock and pets including transmissible gastroenteritis virus (TGEV) (pigs), porcine epidemic diarrhea virus (PEDV) (pigs); Feline Infectious Peritonitis Virus (FIPV) feline coronavirus (cats); bovine coronavirus (cows); infectious bronchitis virus (chickens); and canine coronavirus (dogs). Interestingly, we now recognize that bats harbor diverse strains of coronaviruses, and these bat viruses may be the ancestors of the “species-specific” viruses (Lau et al., 2013). The identification of a common therapeutic target in the genomes of coronaviruses may allow for the development of a broad spectrum antiviral therapy to combat existing and potentially emerging coronaviruses.

Despite the threat of coronaviruses emerging in the human population and the fact that coronavirus infections in domestic animals lead to significant economic losses, there are no FDA approved antiviral drugs or vaccines to combat coronavirus infection. Coronaviruses, similar to other viruses, have evolved multiple ways to delay and evade the induction of protective immune responses in the host upon infection. By understanding the mechanisms by which coronaviruses antagonize the innate immune response, we might be able to rationally design specific antiviral therapeutics.

CORONAVIRUSES STRUCTURE AND REPLICATION

Coronaviruses are enveloped, positive strand RNA viruses belonging to the order Nidovirales (De Groot et al., 2012). The viral particle is about 120-160 nm in diameter. The spike glycoproteins project out from the virus surface which looks like the crown
Figure 1. MHV life cycle. Upon entry virus RNA is translated into polyprotein. Viral replicase is processed and produced proteins assemble on double membrane vesicles which are required for the replication of viral RNA and production of the nested set of subgenomic RNA. Upon translation of structural genes the virions bud from the ER and through the Trans Golgi network translocate to the plasma membrane where the progeny virus is released.
surrounding the viral particle (*corona* is a Latin for crown). Coronaviruses are the biggest RNA viruses genome wise ranging in size from 28 to 32 kilobases. Coronaviruses enter the host cell via interaction of the spike glycoprotein and cell surface receptor. All coronaviruses replicate in the cytoplasm of infected cells through the action of the viral replicase complex. The coronavirus replicase is produced upon translation of the incoming RNA genome. The genomic RNA has a 5’-methyl cap and polyadenylated tail that resembles cellular mRNA and can be directly translated by cellular translation machinery in the cytoplasm. Two thirds of the genome encodes replicase and the rest of the genome encodes structural and accessory genes. The replicase gene contains two open reading frames, ORF1a and ORF1b, which are connected by a frame shift region allowing for translation of the ORF1ab polyprotein. The replicase polyproteins, designated pp1a and pp1ab, are processed into non-structural proteins (nsps) by two or three, depending on the coronavirus, viral proteases. The papain-like proteases (PLPs) are responsible for the cleavage of the amino-terminal portion of the polyprotein and 3C-like proteinase (3CLpro or Mpro) are required for polyprotein processing and virus replication. The detailed description of PLPs activity is presented in sections below. Proteolytic processing allows for the generation of 16 non-structural proteins that assemble on cellular membranes forming double membrane vesicles (DMVs) (Gosert, Kanjanahaluethai, Egger, Bienz, & Baker, 2002; Knoops et al., 2008). The DMVs are derived from cellular membranes and are the sites of viral RNA synthesis. Replication and “hiding” from cellular Pattern Recognition Receptors on DMVs is one of the hypotheses for the lack of induction of interferon response early during virus replication.
The replication cycle is completed upon translation of the structural genes when the viral particles assemble and bud from the endoplasmic reticulum. Following, viral particles traffic via Trans Golgi network to the plasma membrane, where the progeny virus is released (Figure 1) (Masters, 2006).

**MULTIFUNCTIONAL CORONAVIRUS PAPAIN-LIKE PROTEASES**

Packing a lot of information into a small space is a challenge for all microbes and analysis of viral genomes reveals interesting strategies of genetic economy. Positive strand RNA viruses employ genetic economy by encoding polyproteins that are processed by viral proteases during replication. This strategy minimizes genome size by allowing for the expression of multiple protein products from a single open reading frame. An additional twist to this genetic economy is that the viral protease itself may be multifunctional, i.e. the protease may act on both viral and host cell proteins. Hepatitis C virus encodes a polyprotein that is processed by the viral protease, NS3/4a, that also cleaves host cell mitochondrial associated viral sensor (MAVS), thus inactivating the innate immune response to viral infection (Li, Sun, Seth, Pineda, & Chen, 2005). Poliovirus 2A protease processes the viral polyprotein and host cell factor eIF4G, which shifts the ribosomes from cap-dependent to cap-independent translation, whereas 3C protease cleaves poly(A)-binding protein to facilitate the complete host translation shutoff (Gradi, Svitkin, Imataka, & Sonenberg, 1998; Kuyumcu-Martinez, Van Eden, Younan, & Lloyd, 2004). In the section below, I will discuss coronaviruses and arteriviruses, two families of positive strand RNA viruses in the order Nidovirales, and
review recent findings illuminating the multifunctionality of the papain-like proteases (PLPs) encoded in the replicase polyproteins. For these viruses, PLPs play a critical role in processing the amino-terminal portion of the replicase polyprotein and are attractive targets for antiviral drug development. In addition, structural studies have revealed a striking similarity of the viral PLPs to cellular deubiquitinating enzymes (DUBs), which are involved in the regulation of the innate immune response to viral infection. This raises the question of the multifunctional potential of Nidovirus PLPs and their role in antagonism of the innate immune response to viral replication (summarized in Table 1).

CORONAVIRUS PAPAIN-LIKE PROTEASES AND THEIR ROLE IN VIRUS REPLICATION

The PLPs are responsible for the cleavage of the amino-terminal portion of the polyprotein during coronavirus replication. Coronavirus PLP activity was identified by in vitro transcription/translation studies of genomic RNA and the recognition that the polyprotein product was processed by an encoded protease domain (Denison & Perlman, 1986). Site-directed mutagenesis and deletion studies revealed that, for the model coronavirus mouse hepatitis virus (MHV), there are two PLP domains with PLP1 processing the polyprotein between nsp1/nsp2 and nsp2/nsp3 (Baker et al., 1989, 1993; Denison et al., 1992; Teng, Piñón, & Weiss, 1999). Further expression studies of MHV ORF1a revealed that the PLP2 domain processes the polyprotein at the nsp3/nsp4 site using a recognition site of LXGG (Kanjanahaluethai & Baker, 2000). Similar studies of other coronaviruses revealed the existence of either one or two PLP domains that were
Table 1. Coronavirus and Arterivirus papain-like proteases characteristics. Table published in Mielech, Chen, Mesecar, & Baker, 2014.

<table>
<thead>
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<th>Virus</th>
<th>Pro tease characteristics</th>
<th>Reference</th>
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<td><strong>Coronaviridae</strong></td>
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<td>SARS-CoV PPLpro</td>
<td>Proteolytic activity</td>
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<td></td>
<td></td>
<td>Crystal structure</td>
<td>Ratia et al., PNAS 2006</td>
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<td></td>
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<td>DUB activity in cell culture</td>
<td>Frieman et al. J Virol 2009,</td>
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<td>Lindner et al., ABB, 2007</td>
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<td></td>
<td>MERS-CoV PPLpro</td>
<td>Proteolytic activity</td>
<td>Kilianski et al., J Virol., 2013</td>
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<td>DelSGylating activities in cell culture</td>
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required for processing the amino-terminal region of the replicase polyprotein (Figure 2). SARS-CoV encodes only one PLP domain termed PLpro within nsp3 which cleaves the replicase polyprotein at the junctions between nsp1/nsp2, nsp2/nsp3, and nsp3/nsp4 through recognition of a LXGG motif (Harcourt et al., 2004). For the recently emerged MERS-CoV the cleavage sites recognized by the PLpro are predicted and await experimental validation (van Boheemen et al., 2012). However, Kilianski and co-workers showed that MERS-CoV PLpro can recognize a LXGG motif similarly to SARS-CoV PLpro (Kilianski, Mielech, Deng, & Baker, 2013). It has been shown that when two PLP domains are encoded within the coronavirus replicase polyprotein, the PLP2 has similar characteristics to SARS-CoV PLpro and recognizes the LXGG motif (Figure 2). Coronavirus PLP2 domains are known to or predicted to cleave between nsp3 and nsp4, whereas the cleavage between nsp1/nsp2 and nsp2/nsp3 is usually mediated by the PLP1 domain. Interestingly, gamma- and deltacoronaviruses encode one PLpro domain within nsp2 that is predicted to cleave between nsp1/nsp2 and nsp2/nsp3.

FROM VIRAL PROTEASE TO VIRAL DEUBIQUITINASE

The multifunctionality of coronavirus PLPs was first proposed by Sulea and co-workers from their study of molecular modeling of the SARS-CoV PLpro domain (Sulea, Lindner, Purisima, & Menard, 2005). In their study, the authors predicted PLpro deubiquitinating (DUB) activity based on the observations that PLpro could be modeled onto the structure of the herpesvirus-associated ubiquitin-specific protease (HAUSP/USP18), which was a known cellular DUB.
Figure 2. Coronavirus and Arterivirus Papain-like Proteases. Schematic depiction of the N-terminal region of the replicase polyprotein of selected coronaviruses (A) and arteriviruses (B). The papain-like protease domains, termed PLpro, PLP1 or PLP2 for CoV, or Pa, Pβ or PLP2 for arteriviruses are depicted with correspondingly colored arrowheads indicating predicted or confirmed cleavage sites. Figure published in Mielech, Chen, Mesecar, & Baker, 2014.
DUBs are enzymes that can remove ubiquitin modifications from target proteins. Ubiquitination is a post translational modification of proteins that allows for the addition of ubiquitin molecules to a specific lysine residue within the target protein in an ATP-dependent reaction. This system is mediated by three enzymes: an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase. These enzymes might add one (monoubiquitination) or several (polyubiquitination) ubiquitin moieties to a target protein. The ubiquitin molecules in the polyubiquitin chain can be covalently linked via any of the seven lysine residues present within ubiquitin. Several types of polyubiquitination have been characterized [reviewed in (Komander & Rape, 2012)]. The form of ubiquitin linkage determines the fate of the modified protein and influences its function. Three types of polyubiquitination are known to be involved in the regulation of the innate immune signaling pathways: Lys48-linked Ub (K-48-Ub), Lys63-linked Ub (K-63-Ub), and linear polyubiquitination. K-63 linked ubiquitin and linear ubiquitin modifications are associated with activation of proteins in certain innate immune signaling cascades. In contrast, K-48 linked polyubiquitination is a signal that directs proteins for proteosomal degradation. Removal of ubiquitin from cellular targets is mediated by cellular enzymes called deubiquitinases (DUBs) (Komander, Clague, & Urbe, 2009). DUBs recognize the RLRGG motif that links ubiquitin chains and remove ubiquitin conjugates from cellular proteins.

The SARS-CoV PLpro DUB activity predicted by Sulea and co-workers was tested \textit{in vitro} and in transfected cells by several groups. Using purified enzyme, two groups showed SARS-CoV PLpro DUB activity \textit{in vitro} and that catalytic activity of the
protein is required for DUB activity. They showed that the mutation of the predicted catalytic cysteine or aspartic acid residue to alanine leads to a loss of DUB activity (Barretto et al., 2005; Lindner et al., 2005). Further studies revealed that PLpro is capable of processing both K-48-linked diubiquitin and heptaubiquitin, as well as K-63-linked heptaubiquitin chains in vitro (Lindner et al., 2007). These in vitro studies were the first to demonstrate the multifunctional nature of the coronavirus PLPs.

Structural studies have contributed to a detailed understanding of SARS-CoV PLpro (Ratia et al., 2006). The structure of SARS-CoV PLpro displays a thumb-palm-fingers architecture resembling cellular ubiquitin specific proteases (USPs) (Figure 3A) (Ratia et al., 2006). Structural superposition of SARS-CoV PLpro with USP14 resulted in a pairwise root-mean-square-deviation (RMSD) of 3.6 Å over 198 aligned backbone Cα, although the sequence identity between these two proteins is only around 10%. The active site of SARS-CoV PLpro consists of a canonical Cys-His-Asp catalytic triad, which is in a similar position to that of USP14 (Figure 3A). In contrast to some USPs, including USP14 and HAUSP, whose fingers domains consist of a Cys4-type-like zinc ribbon without zinc (Hu et al., 2005), the fingers domain of SARS-CoV PLpro contains a zinc finger with four cysteines tetrahedrally coordinating a zinc atom (Ratia et al., 2006). Moreover, the zinc-binding ability seems to be essential for SARS-CoV PLpro activity, as supported by mutagenesis studies (Barretto et al., 2005). Such dependence on zinc seems to be conserved among coronaviral PLPs as zinc binding has also been observed for other PLPs, including TGEV PLP1 and HCoV-229E PLP1 (Herold, Siddell, & Gorbalenya, 1999; Wojdyla et al., 2010).
Figure 3. Structures of SARS-CoV PLpro compared to USP14 and EAV PLP2 compared to yeast OTU1. (A) Structural superposition of SARS-CoV PLpro (magenta, PDB code 2FE8) to USP14 (cyan, PDB code 2AYO). The zinc atom from the zinc finger of SARS-CoV PLpro is shown as a gray sphere. (B) Close-up view of the active site in SARS-CoV PLpro with the catalytic triad residues shown as sticks. Numbering of the residues is based on (Ratia et al., 2006) (C) Structural overlay of EAV PLP2 (blue, PDB code 4IUM) with yeast OTU1 (yellow, PDB code 3BY4) bound to ubiquitin. The ubiquitin molecules have been omitted for clarity. The zinc atom from the zinc finger of EAV PLP2 is shown in gray sphere. (D) Close-up view of the active site in EAV PLP2. Numbering of the residues is based on (van Kasteren et al., 2013). Asn263 in EAV PLP2 catalytic triad has two alternative conformations. Images were generated using PyMOL. Figure published in Mielech, Chen, Mesecar, & Baker, 2014.
The structure of the PLP1 domain of TEGV is similar to SARS-CoV PLpro and several cellular USPs including USP 2, 7, 8, 14, and 21 (Wojdyla et al., 2010). Purified TGEV PLP1 has DUB activity in vitro. In addition, the authors showed that TEGV PLP1 has a slight preference for cleavage of K-48-linked over K-63-linked polyubiquitin chains. The role of DUB activity of TEGV PLP1 in cell culture or infected animals remains to be determined.

HCoV-NL63 PLP2 that has only 22% homology to SARS-CoV PLpro has also been shown to act as a DUB in vitro and in cell culture. Expression of PLP2, but not PLP2 catalytic mutant (C1678A) leads to a decrease in the level of ubiquitinated proteins in transfected cells. In addition, purified PLP2 can process both K-48-linked and K-63-linked hexaubiquitin chains (Chen et al., 2007; Clementz et al., 2010).

Similarly to HCoV-NL63, PEDV encodes two PLPs in the genome; however, only PLP2 has been shown to have the ability to deconjugate ubiquitin from cellular substrates (Xing et al., 2013). Moreover, the authors showed that alanine mutants of the active site residues (cysteine, histidine, or aspartic acid) led to the loss of DUB activity in PEDV PLP2-transfected cells. In addition, the authors showed that PEDV PLP2 can efficiently remove both K-48-linked and K-63-linked polyubiquitin conjugates from cellular proteins. Specifically, the authors showed that PLP2 can remove K-63-linked polyubiquitin chains from RIG-I and STING, two molecules important for induction of innate immunity. These results suggest a possible mechanism by which coronavirus PLP2 DUB activity can block induction of interferon β (IFNβ) (Xing et al., 2013).
DUB activity of MHV PLP2 has also been reported. Frieman and co-workers showed that MHV can deubiquitinate multiple cellular proteins conjugated with ubiquitin in infected cells (Frieman, Ratia, Johnston, Mesecar, & Baric, 2009). In addition, Zheng and co-workers showed that MHV PLP2 is capable of processing both K-48-linked and K-63-linked polyubiquitin chains from cellular substrates (Zheng, Chen, Guo, Cheng, & Tang, 2008). Further, Wang and co-workers determined that MHV PLP2 can deubiquitinate K-63-linked TBK1, a kinase required to activate transcription factor IRF3, which activates expression of proinflammatory cytokines such as IFNβ (Wang, Chen, Zheng, Cheng, & Tang, 2011). These results suggest that MHV PLP2 DUB activity may have a role in blocking the induction of the antiviral state.

Of note, the PLpro domain from the recently emerged, highly pathogenic MERS-CoV has been characterized as a protease, DUB and inhibitor of innate immune responses, including IFNβ (Kilianski, Mielech, Deng, & Baker, 2013; Mielech et al., 2014; Yang et al., 2013). Further work is required to determine if the DUB activity detected during the overexpression of the PLpro in transfected cells is also critical for viral replication or pathogenesis.

FROM VIRAL PROTEASE TO DEISGYLATING ENZYME

The recognition that coronavirus PLPs had the ability to recognize the LXGG motif and process both polyproteins and ubiquitin chains led to the hypothesis that PLP domains might also process ubiquitin-like molecules such as interferon stimulated gene 15 (ISG15). ISG15 is an interferon stimulated di-ubiquitin-like molecule that can be
linked to cellular targets via a mechanism termed ISGylation (Jeon, Yoo, & Chung, 2010). Similar to ubiquitination, ISGylation requires the activity of E1, E2, and E3 enzymes, and the process is reversible (deISGylation). Several screens have identified cellular targets of ISGylation including molecules important for the activation of an innate immune response, particularly RIG-I, JAK1, STAT1, PKR, and MxA [reviewed in (Lenschow, 2010)]. The exact mechanism of how modification with ISG15 influences these proteins’ activity is not well characterized; however, ISGylation is important for protection and clearance of viral infections. It has been shown that ISG15 knock-out mice are more susceptible to infection with influenza, herpes and Sindbis viruses, than are wild-type mice (Lenschow et al., 2005, 2007). Coronavirus encoded PLPs, in addition to DUB activity, have also been shown to be able to remove IGS15 conjugates from cellular targets, an activity which might also contribute to virus pathogenesis.

DeISGylating activity has been demonstrated for several coronavirus PLPs. DeISGylating activity of SARS-CoV PLpro and HCoV-NL63 PLP2 was demonstrated in vitro (Nicholson et al., 2008). In addition, Clementz and co-workers showed that HCoV-NL63 PLP2 has the ability to remove ISG15 conjugates from cellular proteins in a catalytically dependent manner (Clementz et al., 2010). Moreover, the PLpro domain from MERS-CoV has similar properties (Mielech et al., 2014; Yang et al., 2013) suggesting that deISGylating activity of PLPs might be conserved between PLPs from different coronavirus species.
CELLULAR INNATE IMMUNITY SIGNALING

In the human body, there is a constant battle between viruses that infect cells and the earliest immune response: the innate response. The innate immune response allows for the establishment of an antiviral state which prevents viruses from replicating. In addition, this early response signals through interferon to neighboring non-infected cells limiting spread of the virus. Coronaviruses are successful pathogens and, to become such, have had to evolve multiple mechanisms to inhibit innate immunity and replicate efficiently. PLPs are hypothesized to inhibit the induction of innate immune in infected cells.

In order to limit virus replication, cells evolved multiple mechanisms to activate their defense systems. The innate immune system is the first line of defense against pathogens. It plays very important roles in the recognition of viral infection and stimulation of the adaptive immune response which will activate T cells and induce production of antibodies (Le Bon & Tough, 2002; Theofilopoulos, Baccala, Beutler, & Kono, 2005). When the virus enters the cell its pathogen associated molecular patterns (PAMPs) can be recognized by pattern recognition receptors (PRRs) that will induce signaling cascades to stimulate the production of proinflammatory cytokines and chemokines, leading to establishment of an antiviral state in the cell. The schematic depiction of innate immune signaling critical for limiting RNA virus replication is presented in Figure 4.

For recognition of RNA viruses several PRRs are particularly important (Figure 4) (Le Bon & Tough, 2002). Toll-like receptors (TLRs) 3 and 7 are present in the
Figure 4. Innate immunity signaling. Diagram on the left shows the schematic depiction of recognition of RNA viruses and signaling cascades that lead to production of proinflammatory cytokines and chemokines, one of which is interferon β. On the right the diagram shows signaling by IFNβ binding to its receptor that leads to establishment of an antiviral state.
endosomes and can recognize RNA during virus entry (Alexopoulou, Holt, Medzhitov, & Flavell, 2001). Viral RNA can also be recognized by TLR-independent sensors like cellular helicases: retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (Mda5) (Kang et al., 2002; Yoneyama et al., 2004). Both RIG-I and Mda-5 contain caspase recruiting (CARD)-like domain which interacts with downstream mitochondria associated molecule MAVS (also known as IPS-1, Cardif, and VISA). This interaction triggers signaling cascades that eventually lead to activation of transcription factors IRF3, IRF7, and NF-κB (Kawai et al., 2005; Meylan et al., 2005; Seth, Sun, Ea, & Chen, 2005; Xu et al., 2005). Activation of IRF3 requires activation of TBK1 and IKKε kinases by MAVS. Kinases phosphorylate IRF3 and IRF7. Upon phosphorylation, IRF3 homodimerizes and translocates to the nucleus where it can act as a transcription factor. To activate the NF-κB pathway, MAVS signals to activate a complex of three kinases, IKKa/IKKβ/IKKγ, which phosphorylate IκBα. Upon phosphorylation, IκBα is poly-K-48 ubiquitinated which leads to its proteosomal degradation. This releases NF-κB which translocates to the nucleus and, along with the IRF3 dimer, binds to promoters and stimulate expression of proinflammatory cytokines [reviewed in (Yoneyama & Fujita, 2009)]. The major cytokines produced upon virus infection are type I interferons (IFN) alpha and beta. Secreted IFN stimulates neighboring cells by binding to IFN receptor (IFNAR) and activating the Jak/STAT pathway. Upon receptor binding Jak and Tyk2 kinases phosphorylate STAT1 and STAT2 respectively. This allows for the formation of the heterodimer and its translocation to the nucleus, where upon binding to multiple promoters, induce interferon inducible genes (ISGs) [reviewed in (Platanias, 2005)]. One
of the interferon-induced pathways is oligoadenylate synthetase (OAS) and RNaseL pathway. OAS is capable of catalyzing 2’.,5’- phosphodiester bonds allowing for the formation of ATP oligomers which bind to constitutively inactive monomer of RNaseL. This induces RNaseL homodimerization and activation. Upon activation RNaseL is capable of degradation of viral ssRNA (Sadler & Williams, 2008). Another important and well-studied interferon-induced molecule is ISG15 which is conjugated to multiple proteins which helps in the establishment of an antiviral state. The importance for ISG15 has been shown by the sensitivity of ISG15-/- mice to viruses such as Herpes virus or Influenza (Lenschow et al., 2007). OAS, ISG15, and other ISGs contribute the antiviral state which limits the virus replication and spread.

PAPAIN-LIKE PROTEASES AS ANTAGONISTS OF THE INNATE IMMUNE RESPONSE

DUB and deISGylating activities of the coronavirus PLPs have been proposed as a mechanism to block the induction of the cellular response to viral infection. DUB activity might be important for blocking induction of cellular antiviral response because ubiquitination controls innate immunity signaling (Figure 5). Devaraj and co-workers determined that SARS-CoV PLpro can inhibit polyI:C and Sendai virus-induced IFNβ, and that PLpro catalytic activity is important for antagonism. Co-expression of PLpro with stimulators of interferon activation such as RIG-I, MAVS, TRIF, TBK1 or IKKε reduced IFNβ induction. In contrast, PLpro was not able to inhibit IFNβ induced by expression of a dominant active form of IRF3. The authors showed that PLpro can down-
Figure 5. Ubiquitination of innate immunity signaling pathway. Schematic depiction of innate immunity signaling cascade and ubiquitintation involved in regulation of this process.
regulate phosphorylation of IRF3 and prevent IRF3 dimer formation. Furthermore, the authors were able to co-immunoprecipitate IRF3 with transiently expressed PLpro, and observed co-localization of IRF3 and nsp3/PLpro during SARS-CoV infection (Devaraj et al., 2007). Subsequently, Frieman and co-workers also showed that PLpro inhibits the IFN response. In addition, the authors showed that PLpro can inhibit NF-κB-luciferase activity by stabilizing IκBα, a molecule that has to be degraded in order to release the transcription factor NF-κB, which activates proinflammatory responses (Frieman et al., 2009). Later studies showed that PLpro can block TNFα-induced NF-κB activation, and further that this block can be removed when the cells are treated with a PLpro inhibitor, revealing a possible antiviral strategy (Clementz et al., 2010). Furthermore, a recent analysis showed that the expression of SARS-CoV or MERS-CoV PLpro decreases endogenous RNA levels of proinflammatory cytokines and chemokines in activated cells (Mielech et al., 2014).

MULTIFUNCTIONAL ARTERIVIRUS PAPAIN-LIKE PROTEASES

The Arterivirus family of positive strand RNA viruses contains genomes ranging from 10 to 14 kilobases. The Arterivirus family includes important pathogens such as Equine Arteritis virus (EAV) that causes disease in horses, and Porcine Reproductive and Respiratory Syndrome virus (PRRSV) that infects pigs and is lethal to piglets. These diseases can lead to significant economic loss and there are currently no effective vaccines or antiviral drugs available to treat infected animals.
Arteriviruses encode at least one PLP that is responsible for proteolytic processing of the virus polyprotein (Figure 2). The arterivirus genome has a similar organization to the coronavirus genome and is composed of 2 large ORFs that encode polyproteins pp1a and pp1ab, and 8-11 downstream ORFs that encode accessory and structural proteins. The amino-terminal region of the ORF1 is processed by two or three PLP domains. The PLP encoded within the amino-terminal region of nsp2, termed PLP2 or P2, has a similar function as SARS-CoV PLpro ([Snijder, Wassenaar, Spaan, & Gorbalenya, 1995] and reviewed in [Snijder & Kikkert, 2013]). The sequence recognized by this protease domain is only partially conserved across the Arterivirus species. For example, EAV PLP2 recognizes the sequence LIGG, whereas PRRSV P2 can recognize cleavage sites containing either TTGG or PSGG. The partial conservation of the site cleaved by the arterivirus PLP2s, and its similarity to motif recognized by cellular DUBs and deISGylating enzymes, led to the hypothesis that arterivirus P2s might be multifunctional enzymes.

Expression of the EAV PLP2 domain revealed that it indeed exhibits DUB activity in cell culture (Frias-Staheli et al., 2007). DUB activity of the amino-terminal region of nsp2 (Nsp2(N)) was confirmed in vitro. EAV Nsp2(N) can deubiquitinate K-48-linked and K-63-linked polyubiquitin chains in a catalytic dependent manner, as alanine mutants of the three predicted catalytic residues lost DUB activity (van Kasteren et al., 2012). To address the possible role for Nsp2(N) DUB activity in the inhibition of innate immunity, the authors determined the ubiquitination status of RIG-I which requires K-63-linked polyubiquitination for activation. Studies have shown that DUB activity of
Nsp2(N) from EAV can block RIG-I ubiquitination in a catalytic dependent manner (van Kasteren et al., 2012). This led to the hypothesis that EAV DUB activity might be important for blocking the innate immune response to viral infection. To test this hypothesis, it was critically important to obtain structural information on how the enzyme interacts with ubiquitin. With this information in hand, researchers can attempt to generate PLP2 mutants that retain protease activity but are impaired for interaction with ubiquitin.

The co-crystal structure of EAV PLP2 with ubiquitin was solved by van Kasteren and co-workers (van Kasteren et al., 2013). EAV PLP2 adopts a two-domain fold and shares a β-sheet core and two central helices with eukaryotic OTUs (Figure 3C). Structural overlay of EAV PLP2 with yeast OTU1 resulted in a RMSD of 3.3 Å over 81 aligned Cα with only around 9% sequence identity. The Cys-His-Asn catalytic triad of EAV PLP2 resides in a structurally conserved region of OTU DUBs (Figure 3D). What is unique about the structure of EAV PLP2 is the presence of a zinc finger as a part of the OTU domain. This zinc finger is critical for the structural integrity and catalytic activity of EAV PLP2. The presence of a zinc finger has not been observed in other known OTU-like proteases. Thus, it has been proposed that EAV PLP2 may represent the first member of a new class of zinc-dependent OTUs (van Kasteren et al., 2013).

The co-crystal structure of EAV PLP2 with ubiquitin revealed interaction sites between the enzyme and ubiquitin that are distal to the active site (van Kasteren et al., 2013). Using this structural information, the authors were able to successfully separate PLP2 protease and DUB activities. Mutation of the ubiquitin-interacting residues of EAV
PLP2 had no evident effect on protease activity but significantly reduced DUB activity in overexpression studies. To study the effect of the DUB mutant on virus replication and innate immunity, the authors used reverse genetics to recover two replication competent mutant viruses (a single mutant I353R and a triple mutant T312A/I313V/I353R in the EAV PLP2 domain) and showed that replication kinetics of these viruses were essentially identical to the wild-type virus. However, the viruses with the mutant PLP2 domain had lost the ability to deconjugate ubiquitin from cellular targets in virus-infected cells. In addition, cells infected with the EAV DUB mutant viruses generated an elevated innate immune response, particularly expression of IFNβ, IL8, and MX1, suggesting that DUB activity is important for inhibition of the innate immune response (van Kasteren et al., 2013). The EAV DUB mutant study was the first study to demonstrate that viral protease and deubiquitinase functions can be separated. This is an important contribution because it opens the door for the study of viral DUB activity in vaccines and as a target for antiviral therapeutics.

Interestingly, EAV is not the only member of the Arterivirus family for which multifunctional protease/DUB activity has been shown. PRRSV is a well-studied member of the family which causes disease of high economic importance in pigs. The proteolytic activity of PRRSV P2 has been well studied (Han, Rutherford, & Faaberg, 2010; Jun Han, Rutherford, & Faaberg, 2009) and P2 has the ability to cleave ubiquitin conjugates from cellular targets (Frias-Staheli et al., 2007). Sun and co-workers showed that P2 had deubiquitinating activity in vitro and in cell culture. The authors showed that PRRSV P2 can block Sendai virus-induced IFNβ and inhibit NF-κB by preventing IκBα degradation.
by its deubiquitination in cell culture. In addition, they generated several mutant versions of P2 that had reduced ability to inhibit NF-κB-activation. To test if P2 can block NF-κB activation in the context of the virus, the authors introduced these mutations into PRRSV. Although some of the mutations that altered P2 activity in vitro could not be recovered as viable virus, the authors did report two single amino acid P2 mutant viruses that showed decreased ability to inhibit NF-κB-reporter activity and a decrease in the level of IκBα in infected cells (Sun, Chen, Lawson, & Fang, 2010). However, the authors did not address whether protease or DUB activity was responsible for the effect they observed in infected cells. The authors noticed that the mutant viruses had severe replication defects suggesting that P2 protease activity, as well as DUB activity, may have been impaired (Sun et al., 2010).

Similar to studies on coronavirus PLP activities, groups characterizing arterivirus PLP domains investigated their deISGylating activity. Frias-Staheli and co-workers showed that EAV P2/PLP2 is capable of deconjugating IGS15 from cellular proteins in transfected cells (Frias-Staheli et al., 2007). Furthermore, a report by Sun and co-workers showed that PRRSV P2 decreases endogenous ISG15 protein levels upon Sendai virus stimulation, and that it has deISGylating activity in cell culture (Sun, Li, Ransburgh, Snijder, & Fang, 2012). Furthermore, the authors generated PRRSV P2 mutants that had reduced deISGylating activity. The first mutant had a 23 aa deletion in nsp2 (aa 402-424), the second mutant had a 19 aa deletion in nsp2 (aa 402-420), and the third mutant had the 19 aa deletion and additional point mutation (S462A). When cells were transfected with those mutants the endogenous levels of IGS15 was increased compared to cells
transfected with the wild-type version of P2. Interestingly, the processing of nsp2/nsp3 was not affected by the deletion or deletion/point mutations in the P2 domain of the virus. The authors were able to recover two viruses (19 aa deletion, and 19 aa deletion/S462A). The growth kinetics analysis showed that those two viruses do not replicate as efficiently as wild-type virus, which may be due to the inability to efficiently process the nsp2/nsp3 site in the replicase polyprotein (Sun et al., 2012). Further studies including solving the X-ray crystal structure of the PRRSV P2 domain and determining to role of P2 DUB/deISGylating mutants on viral pathogenesis in animal models are needed.

Viruses have evolved many different mechanisms to down-regulate innate immune response upon infection. The ability of a virus to block or delay the induction of the antiviral state in an infected cell is likely an important contributor to pathogenesis. Nidoviruses encode in their genomes several proteins that are capable of blocking or delaying innate immune signaling. One of them is the papain-like protease, the multifunctional enzyme that can act as a potent protease, DUB, deISGylating enzyme, and antagonist of the innate immune response. Separating protease and DUB activities is needed to fully understand the role of those proteins during virus replication. Thus far, separating protease activity from DUB and IFN antagonism activity has been shown only for the arterivirus EAV (van Kasteren et al., 2013). The specific mechanisms and the contributions of arteri- and coronavirus PLPs to interferon antagonism during virus infection and pathogenesis are an exciting new direction of research that may lead to the development of effective vaccines and novel antiviral therapeutics.
CORONAVIRUS INDUCTION OF INTERFERON AND INTERFERON ANTAGONISTS

As cellular innate immunity mechanisms evolved, viruses co-evolved and established multiple ways to inhibit the innate immune response. MHV and other coronaviruses encode, in addition to PLPs, multiple accessory and multifunctional proteins that can act as interferon antagonists. Below I will review the hypothesis and data regarding inhibition of innate immunity by model coronavirus MHV.

In contrast to many RNA viruses, which upon infection and recognition by PRRs induce robust type I interferon response, MHV-A59 and JHM strains have been shown not to induce interferon response in multiple fibroblast cell lines such as L2 fibroblasts and 17C11 cells (Roth-Cross, Martinez-Sobrido, Scott, Garcia-Sastre, & Weiss, 2007; Ye, Hauns, Langland, Jacobs, & Hogue, 2007; Zhou & Perlman, 2007). In addition, MHV is not capable of inducing IFNs in bone marrow derived dendritic cells which are known to produce high amounts of IFNα and IFNβ (Zhou & Perlman, 2006). Multiple groups observed no activation of IRF3 and NF-κB dependent promoters in MHV infected cells (Roth-Cross et al., 2007; Zhou & Perlman, 2007). Two main mechanisms have been proposed to explain the lack of interferon induction. The virus can either encode proteins that act as IFN antagonists and/or the virus “hides” from the recognition by PRRs. The proposed role of MHV PLP2 in inhibiting the induction of interferon is described above.

Several groups showed that MHV is resistant to IFN treatment in cell culture and proposed that the virus is capable of blocking signaling downstream of IFN receptors.
(Koetzner et al., 2010; Roth-Cross et al., 2007; Ye et al., 2007) (Figure 6). Zust and co-workers showed that expression of nsp1 can inhibit the induction of IFN genes. The virus lacking functional nsp1 was not attenuated in vitro. However, mice infected with nsp1 mutant virus fully recovered from infection. The attenuation in mice could be reversed in IFNAR-/- mice suggesting that nsp1 mediates IFN antagonism. The authors concluded that nsp1 might be blocking signaling downstream of the IFN receptor since IFNα pretreatment of macrophages led to dose-dependent inhibition of the mutant virus replication (Zust et al., 2007).

Two reports from the Weiss laboratory showed that MHV co-infection of L2 fibroblasts can inhibit IFNβ protein levels upon Sendai virus (SV) and New Castle’s Disease virus (NDV) infection. However, MHV can induce IFNβ mRNA levels late during infection. Furthermore, MHV infection cannot prevent Sendai Virus or Newcastle Disease Virus- induced IRF3 translocation to the nucleus (Rose, Elliott, Martinez-Sobrido, Garcia-Sastre, & Weiss, 2010; Roth-Cross et al., 2007). In the first report the authors showed that MHV cannot block IFN induction completely in vivo, and they detected significant amounts of IFNβ in the brains of infected mice. In addition, IFN pretreatment and subsequent infection with MHV and NDV resulted in infection with MHV only, suggesting that MHV does not block “general antiviral activities”. To investigate the possible mechanism the authors determined that interferon did not accumulate inside infected cells nor was degraded via proteosomal pathway. Thus, they suggested that the inhibition occurs between mRNA synthesis and protein translation but the exact mechanism remains to be determined (Roth-Cross et al., 2007). In a more recent
**Figure 6. Antagonists of innate immunity in MHV genome.** Schematic depiction of MHV genome, characterized IFN antagonists are depicted in green. Table below summarizes the mechanisms of inhibition.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Targeted innate immunity pathway/protein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nsp1</td>
<td>IFN activation</td>
<td>Züst et al., PLoS Pathogens, 2007</td>
</tr>
<tr>
<td>PLP2</td>
<td>TBK1 and IRF3</td>
<td>Zheng et al., Cell Res., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wang et al., PLoS ONE, 2011</td>
</tr>
<tr>
<td>ns2</td>
<td>2'-5' OAS/RNaseL</td>
<td>Roth-Cross et al., J. Virol. 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zhao et al., J. Virol., 2011</td>
</tr>
<tr>
<td>ORF5a</td>
<td>To be determined</td>
<td>Koetzner et al., J. Virol, 2010</td>
</tr>
<tr>
<td>Nucleocapsid (N)</td>
<td>2'-5' OAS/RNaseL</td>
<td>Ye et al., J. Virol, 2007</td>
</tr>
</tbody>
</table>
publication, the authors determined that MHV preinfection cannot only inhibit induction of interferon promoter but also delay expression interferon stimulated genes induced by Sendai virus infection. Interestingly, MHV preinfection did not affect STAT1 or STAT2 activation, phosphorylation or nuclear translocation (Rose et al., 2010).

One proposed mechanism which explains the lack of IFN induction upon infection is that MHV is not recognized by the typical PRRs known to recognize RNA viruses. Early reports showed that MHV preinfection cannot prevent polyI:C mediated activation of IFN, IRF3 and NF-kB dependent promoters. Moreover, the authors showed that the overexpression of RIG-I, Mda5 or TLR3 is sufficient for IFN promoter induction in the presence MHV infection suggesting that MHV is not recognized by PRRs and the mechanism may involve virus “hiding” on DMVs during replication, which makes RNA inaccessible for cytoplasmic sensors (Jackson et al., 2005; Schwartz, Chen, Lee, Janda, & Ahlquist, 2004; Sims, Ostermann, & Denison, 2000; Zhou & Perlman, 2007).

Ye et al., determined that upon MHV infection protein kinase R (PKR) is not phosphorylated and activated leading to a lack of phosphorylation of the transcription factor eIF2α which is required to inhibit protein translation, a known antiviral mechanism that limits virus replication. Further, the authors determined that 2′,5′-oligoadenylate synthetase (2′-5′ OAS), another dsRNA activated pathway is also not activated upon MHV infection. The authors concluded that the virus does not induce RNase L activity since no RNA degradation was observed in infected HeLa cells. These data support a model where MHV replication intermediates are not recognized by PKR and 2′-5′OAS sensors. Moreover, the authors showed that MHV A59 nucleocapsid N protein
contributes to virus IFN resistance and inhibits 2’-5’ OAS pathway activity (Ye et al., 2007). A report by Koetzner and co-workers proposed that the viral accessory protein ORF5a is an interferon antagonist. Koetzner et al., used the observation that MHV-A59 in contrast to MHV-S is IFN resistant in L2 cells and 929 cells. The authors determined that MHV-S, in contrast to all other strains of MHV, does not have ORF 5a in the genome. The authors generated a MHV-A59 virus that had the 5a gene knocked out and this virus was highly sensitive to IFN-α treatment. However; the chimeric virus was not as sensitive as MHV-S; even when the ORF5a knock out was combined with substitution of the nucleocapsid gene from MHV-S; suggesting that MHV A59 may encode additional IFN antagonists. The authors showed that chimeric virus does not inhibit activation of PKR and 2’-5’ OAS pathways and the exact mechanism by which ORF 5a mediates antagonism remains to be determined (Koetzner et al., 2010).

Roth-Cross and co-workers showed that MHV ns2 protein has 2’-5’ phosphodiesterase activity and that this activity is required for IFN antagonism. The authors generated a ns2 MHV mutant strain and determined that it can replicate in fibroblasts but is attenuated in vivo (Roth-Cross et al., 2009). Further studies revealed that the ns2 mutant is attenuated in macrophage cell lines (Roth-Cross et al., 2009). In a recent paper, the authors reported that cellular 2’-5’ oligoadenylate is a target for ns2 which prevents activation of the RNaseL pathway and inhibits innate immune response and the induction of an antiviral state in liver macrophages. This allows MHV to infect hepatocytes and induce hepatitis (Zhao et al., 2012).
In contrast to fibroblasts, MHV is capable of inducing significant interferon responses in plasmacytoid DCs, brain macrophages/microglia, and oligodendrocytes (Li, Liu, & Zhang, 2010; Roth-Cross, Bender, & Weiss, 2008; Zust et al., 2007). Li and co-workers showed that in oligodendrocytes, helicases RIG-I and Mda5 are important for virus detection. However; another report suggests that MHV is not recognized by RIG-I because RIG-I recognizes uncapped mRNA and MHV RNA is 5’ capped and polyadenylated. This report proposes that virus “hides” from the recognition by this PRR by mimicking cellular mRNA (Li et al., 2010). In agreement with this, is the observation that recognition of virus infection in microglia is mediated by Mda5 helicase (and not by RIG-I) which recognizes dsRNA, an intermediate present during virus replication.

MHV infection is organ restricted in mice. MHV A59 is hepatotropic and JHM is a neurotropic strain. However, IFNAR-/- mice are highly susceptible to MHV infection, and infection disseminates into multiple organs whereas upon infection of wild-type mice infection is organ limited (Cervantes-Barragan et al., 2007). All these data lead to the conclusion that IFN response limits virus replication in vivo. It is not clear yet if the virus encodes antagonists and/or is not recognized upon infection which prevents IFN induction in some cell types/organs.

ANTIVIRAL DRUGS TO CORONAVIRUS PAPAIN-LIKE PROTEASES

Despite the fact that SARS-CoV emerged over a decade ago and many efforts have been made to develop specific coronavirus antiviral drugs to this day there are no FDA approved anti-coronavirus therapeutics [reviewed in (Barnard & Kumaki, 2011)].
Many proteins known to facilitate coronavirus replication in infected cells have been shown to be valuable targets for antivirals [reviewed in Kilianski & Baker, 2014]).

Previous studies have shown that SARS-CoV papain-like protease (PLpro) which process viral polyprotein during virus replication is an effective antiviral target, because the protease activity is required for virus life cycle.

The first study reporting the usefulness of SARS-CoV PLpro as a drug target was done by Ratia and co-workers. The authors screened a library of 50,080 compounds in order to find drugs that inhibit SARS-CoV PLpro activity (Ratia et al., 2008). They performed *in vitro* screen using a fluorescent-based high-throughput screening system. The goal of this screen was to find non-covalent inhibitors that specifically target SARS-CoV PLpro, because previous studies on covalent inhibitors of other viral cysteine proteases have shown that covalent modification very often affects untargeted cellular cysteine residues thus leading to significant cytotoxicity of those compounds (Leung-Toung, Li, Tam, & Karimian, 2002). The screen performed by Ratia and co-workers identified a lead-compound with an IC₅₀=20.1µM which was used for further optimization. The generated optimized compound designated GRL0617 showed increased potency *in vitro* (IC₅₀=0.6µM) and was also effective in blocking SARS-CoV replication in cell culture (EC₅₀=14.5µM). Further, the authors showed that GRL0617 is SARS-CoV PLpro specific inhibitor that does not affect human cysteine proteases including cellular deubiquitinating enzymes (HAUSP, USP18, UCH-L1, UCH-L3). In addition, the drug did not block HCoV-NL63 PLP2 activity supporting its specificity towards SARS-CoV PLpro. Finally, the authors generated a co-crystal structure of
SARS-CoV PLpro with the inhibitor revealing the mechanism by which the drug inhibits protease activity. They found that the drug binds to the cleft that leads to the enzyme active site via two hydrogen bonds and several hydrophobic interactions with enzyme residues. The important finding came from the comparison of the structures of apo-enzyme and enzyme-inhibitor complex. Inhibitor binding leads to the changes in the flexible loop (Gly267-Gly272) of the enzyme. Binding of the drug closes the otherwise flexible loop, which leads to the clamping of the inhibitor to the body of the protein. This first study on SARS-CoV inhibitors was significant because it showed that PLpro is a valuable target for the design of specific SARS-CoV inhibitors, presented the efficacy of structure activity relationship in SARS-CoV PLpro drug design, and revealed drug candidates that could be further optimized to increase potency (Ratia et al., 2008).

Further structure-based optimization of the compound described above (GRL0617) led to two new SARS-CoV PLpro inhibitors of improved potency. Compound 2 had a significantly reduced IC$_{50}$ of 0.46µM, comparing to the lead-compound and also an improved ability to inhibit SARS-CoV replication (EC$_{50}$=6.0µM). The second compound generated had a slightly higher IC$_{50}$ of 1.3 but had significantly improved efficacy (EC$_{50}$=5.2µM). Based on the crystal structure described by Ratia and co-workers the model of compound 2 with SARS-CoV PLpro was generated facilitating optimization of the compounds in the future (Ghosh et al., 2009).

The same screen of 50,080 compounds which discovered GRL0617 lead-compound, identified the second best compound (compound 3) with an IC$_{50}$=59.2µM towards SARS-CoV PLpro (Ghosh et al., 2010). This compound was used in further
optimization and allowed for the synthesis of the compound termed 15g which is a very potent inhibitor of SARS-CoV PLpro. The IC$_{50}$ of this compound equals 0.32µM, and EC$_{50}$=9.1µM which is a significant improvement compared to the lead-compound 3. Furthermore, the authors generated a crystal structure of the compound 15g with SARS-CoV PLpro and found that even though the inhibitor binds to the same loop as the inhibitor described by Ratia and co-workers, the specific interactions between inhibitor and enzyme are significantly different, which gave new insights into drug-enzyme interactions and can be used in the future to design inhibitors that can prevent SARS-CoV replication more effectively (Ghosh et al., 2010).

Because none of the previously described studies reported inhibitors that had an EC$_{50}$ value that would suggest therapeutic application of the compound, further studies on SARS-CoV PLpro inhibitors were needed. The recent report by Baez-Santos and co-workers used the knowledge from above described study and co-crystal structure of SARS-CoV PLpro with inhibitor 15g to design new compounds that would block SARS-CoV replication. My work on this project regarding evaluating of the efficacy of the compounds to inhibit SARS-CoV replication is described in the results section. The new structure-guided design led to the evaluation of several new candidates and showed that compound 3k is the most effective in inhibiting the activity of purified enzyme (IC$_{50}$=0.2µM) and that it potently inhibits SARS-CoV replication. However, other drugs, particularly 3e and 5c, are more metabolically stable than 3k and are still very potent inhibitors of PLpro with EC$_{50}$ values of 8.3µM and 9.5µM respectively, which makes them more promising candidates for the future optimization. We also reported the crystal
structure of compound 3k in complex with SARS-CoV PLpro, which revealed that similar to compound 15g described by Ghosh and co-workers, 3k binds to a site adjacent to the active site of the enzyme. The flexible loop (Gly267-Gly272) of the enzyme changes its conformation upon binding of the compound which leads to the inhibition of the enzyme activity (Báez-Santos et al., 2014).

Overall, even though currently there are no compounds that target SARS-CoV PLpro that could be used in clinical trials due to relatively high EC\textsubscript{50} values, the studies that I described above gave a lot of information and directions for further optimization. Furthermore, they showed the potential and effectiveness of structure-based design of PLpro inhibitors and showed that non-covalent inhibitors of papain-like protease can be effective and less toxic than drugs that covalently bind to enzymes.

In addition to the screen described above, Frieman and co-workers reported on a new yeast-based screening technique that allowed them to identify inhibitors of SARS-CoV PLpro. The authors showed that one of the identified compounds had the ability to block specifically SARS-CoV replication (EC\textsubscript{50}<1µM), and was not effective against influenza virus (Frieman et al., 2011). In this report the authors screened a relatively small library of compounds (about 2,000) but showed the proof of principle that a yeast-based screen is a good assay to identify SARS-CoV PLpro inhibitors, and that it can be used in the future in combination with structure-based optimization to develop more potent drugs (Frieman et al., 2011).

Recently, Kilianski and co-workers tested compound 15g described by Ghosh and co-workers for its ability to inhibit the activity of MERS-CoV PLpro in transfected cells;
however, they found that this inhibitor is not active against MERS-CoV PLpro, which is probably due to the differences in the structures of the enzyme and particularly the flexible loop (Gly267-Gly272 in SARS-CoV structure) (Kilianski, Mielech, Deng, & Baker, 2013). Thus, further studies and structure-based optimization of compounds are needed to facilitate the development of inhibitors of MERS-CoV PLpro.

Taken together literature shows that there PLpro is a valuable target for anti-coronavirus drugs; however, all drugs designed so far need further optimization and validation before they can be used in the clinical settings. One of the challenges in the development of anti-coronavirus therapeutics is the lack of natural infection which makes it hard to evaluate the drugs’ efficacy. For this reason, and because a new coronavirus can emerge into the human population at any time, now the efforts are focused more on designing of the broad spectrum antivirals. The efficacy of this kind of drugs can be tested in the context of natural infection (against HCoV-NL63 which causes common colds). In addition, the availability of broad spectrum anti-coronavirus drugs and/or vaccines can be beneficial if a new coronavirus with pandemic potential emerges.
CHAPTER II
MATERIALS AND METHODS

CELLS AND TRANSFECTIONS

HEK293T and VeroE6 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum (FCS) and 2% glutamine. Transfections were performed with 70% confluent HEK293T cells in cell bind plates (Corning) using TransIT-LT1 Reagent (Mirus) according to manufacturer’s protocol. DBT cells were cultured in Minimal Essential Medium with 5% FCS, 2% L-glutamine, and 10% Tryptose Phosphate Broth (TPB). BHK-R cells were kindly provided by Dr. M. Denison (Vanderbilt University Medical Center) laboratory and maintained in DMEM media supplemented with 10% FCS, 2% L-glutamine, and 0.8mg/ml G418.

EXPRESSION PLP CONSTRUCTS AND MUTAGENESIS

The MERS-CoV PLpro (pcDNA-MERS-PLpro) expression plasmid and generation of catalytic mutant were described previously (Kilianski, Mielech, Deng, & Baker, 2013). Initial pcDNA-SARS-PLpro wild-type and catalytic mutant expression plasmids were described elsewhere (Clementz et al., 2010).

The constructs expressing HCoV-HKU1 (amino acids), HCoV-OC34, HCoV-229E, SARS-CoV PLpro and FIPV PLP2s with in frame V5 epitope tag in pCDNA-
V5/His-B were codon optimized and synthetized (Genscript). To generate catalytic mutants Gibson Assembly (New England Biolabs) technique according to manufacturer’s protocol was used with primers indicated in Table 2. The sequences of all constructs are shown in Appendix 1.

**MHV PLP2 EXPRESSION CONSTRUCTS AND MUTAGENESIS**

To generate WT PLP2 expression constructs we obtained synthetic codon-optimized MHV PLP2 sequences (amino acids 1611-1970 PLP2-A, and amino acids 1525-1911 PLP2-B of MHV-A59) (GeneScript). The PLP2-A sequence was amplified with primers which introduced restriction sites facilitating cloning into pCAGGS vector as well as in frame V5 tag. Two step PCR was used to generate pCAGGS-PLP2-A construct. First, reaction was performed with forward primer F1 and reverse primer R1. Reverse primer R2 was used in secondary reaction (Table 3). The product was generated using Pfu polymerase. Upon verification on 1% agarose gel, the PCR products were purified using Wizard SV Gel and PCR Clean Up System (Promega). The products were digested with SacI, and XmaI (Fermentas) and introduced into pCAGGS vector. To generate deletion mutants two additional restriction sites (KpnI and MluI) were introduced using PCR that facilitated generating multiple truncations. Mutagenic primers used are shown in Table 3. Codon-optimized synthetic PLP2-B sequence was cloned into pCAGGS vector bearing KpnI and MluI sites generated in steps described above. For mutagenesis of PLP2-B construct the overlapping PCR strategy was used for each mutant. The primary reactions were performed with combination of forward mutagenic
Table 2. Primers used to generate catalytic mutants of PLP2 constructs

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Forward Mutagenic Primer</th>
<th>Reverse Mutagenic Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCoV-HKU1 C113A</td>
<td>5’ CAG AGT CAC AAC AAT GCC TAC GTC AAC GTG</td>
<td>5’ CAC GTT GAC GTA GGC ATT GTT GTG ACT CTG</td>
</tr>
<tr>
<td>HCoV-229E C106A</td>
<td>5’ CA AGC GAT AAC AAT GCC TGG GTG AAT GCT GTC</td>
<td>5’ GAC AGC ATT CAC CCA GGC ATT GTT ATC GCT TG</td>
</tr>
<tr>
<td>HCoV-OC43 C114A</td>
<td>5’ G GCC AAC AAT AAC GCC TTC GTC AAC GTG TCC TGT</td>
<td>5’ ACA GGA CAC GTT GAC GAA GGC GTT ATT GTT GCC C</td>
</tr>
<tr>
<td>FIPV C113A</td>
<td>5’ CAG ACA GAC AAC AAT GCC TGG GTC AAT GCC</td>
<td>5’ GGC ATT GAC CCA GGC ATT GTT GTC TGT CTG</td>
</tr>
<tr>
<td>SARS-CoV C112A</td>
<td>5’ TGG GCA GAT AAC AAT GCC TAT CTG AGC TCC GTG</td>
<td>5’CAC GGA GCT CAG ATA GGC ATT GTT ATC TGC CCA</td>
</tr>
</tbody>
</table>

For. out primer: 5’ CAC GTT GAC GTA GGC ATT GTT GTG ACT CTG
Rev. out primer: 5’ CT AGT CCA GTG TGG TGG AAT TCA CCATG
Table 3. Primers used to generate MHV PLP2-A mutants

<table>
<thead>
<tr>
<th>Name</th>
<th>Genome aa</th>
<th>Mutation</th>
<th>Forward Mutagenic Primer</th>
<th>Reverse Mutagenic Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP2-A</td>
<td>1611-1970</td>
<td>WT</td>
<td>5’ CAGAGCAACATACCGCTGAAATGCA 3’</td>
<td>5’ GCCACGTTATGATACGGGTAATCTG 3’</td>
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<tr>
<td>PLP2-A1</td>
<td>1611-1970</td>
<td>C1716A</td>
<td>5’ CCGGTACCAGCTGTTGG 3’</td>
<td>5’ GCCATGGTATGATACGGGTAATCTG 3’</td>
</tr>
<tr>
<td>PLP2-A2</td>
<td>1630-1970</td>
<td>Δ1</td>
<td>5’ CCGGTACCAGCTGTTGG 3’</td>
<td>5’ GCCATGGTATGATACGGGTAATCTG 3’</td>
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<tr>
<td>PLP2-A3</td>
<td>1651-1970</td>
<td>Δ2</td>
<td>5’ CCGGTACCAGCTGTTGG 3’</td>
<td>5’ GCCATGGTATGATACGGGTAATCTG 3’</td>
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<tr>
<td>PLP2-A4</td>
<td>1671-1970</td>
<td>Δ3</td>
<td>5’ CCGGTACCAGCTGTTGG 3’</td>
<td>5’ GCCATGGTATGATACGGGTAATCTG 3’</td>
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<tr>
<td>PLP2-A5</td>
<td>1691-1970</td>
<td>Δ4</td>
<td>5’ CCGGTACCAGCTGTTGG 3’</td>
<td>5’ GCCATGGTATGATACGGGTAATCTG 3’</td>
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<tr>
<td>PLP2-A6</td>
<td>1711-1970</td>
<td>Δ5</td>
<td>5’ CCGGTACCAGCTGTTGG 3’</td>
<td>5’ GCCATGGTATGATACGGGTAATCTG 3’</td>
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<tr>
<td>PLP2-A7</td>
<td>1611-1991</td>
<td>Δ6</td>
<td>5’ CCGGTACCAGCTGTTGG 3’</td>
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<tr>
<td>PLP2-A8</td>
<td>1611-1911</td>
<td>Δ7</td>
<td>5’ CCGGTACCAGCTGTTGG 3’</td>
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<tr>
<td>PLP2-A9</td>
<td>1611-1931</td>
<td>Δ8</td>
<td>5’ CCGGTACCAGCTGTTGG 3’</td>
<td>5’ GCCATGGTATGATACGGGTAATCTG 3’</td>
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<td>PLP2-A10</td>
<td>1611-1951</td>
<td>Δ9</td>
<td>5’ CCGGTACCAGCTGTTGG 3’</td>
<td>5’ GCCATGGTATGATACGGGTAATCTG 3’</td>
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<tr>
<td>PLP2-A11</td>
<td>1615-1970</td>
<td>Δ1-A</td>
<td>5’ CCGGTACCAGCTGTTGG 3’</td>
<td>5’ GCCATGGTATGATACGGGTAATCTG 3’</td>
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<tr>
<td>PLP2-A12</td>
<td>1615-1970</td>
<td>Δ1-B</td>
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<td>Δ1-C</td>
<td>5’ CCGGTACCAGCTGTTGG 3’</td>
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<td>PLP2-A14</td>
<td>1629-1970</td>
<td>Δ1-D</td>
<td>5’ CCGGTACCAGCTGTTGG 3’</td>
<td>5’ GCCATGGTATGATACGGGTAATCTG 3’</td>
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<td>PLP2-A15</td>
<td>1611-1970</td>
<td>Δ1-D</td>
<td>5’ CCGGTACCAGCTGTTGG 3’</td>
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<tr>
<td>PLP2-A17</td>
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<td>Δ1-D</td>
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<tr>
<td>PLP2-A18</td>
<td>1611-1970</td>
<td>Δ1-D</td>
<td>5’ CCGGTACCAGCTGTTGG 3’</td>
<td>5’ GCCATGGTATGATACGGGTAATCTG 3’</td>
</tr>
</tbody>
</table>

Forward outside primer for A15-A18: 5’ GAGGTCACGGTGCGCGCTGATCGTCGCTG 3’
Reverse outside primer for A15-A19: 5’ GCCATGGTATGATACGGGTAATCTG 3’

F1: 5’ GCCGTACCAGCTGTTGG 3’
R1: 5’ GCCGTACCAGCTGTTGG 3’
R2: 5’ GCCGTACCAGCTGTTGG 3’
primer and reverse outside primer, and reverse mutagenic primer and forward outside primer (Table 4). Upon verification on 1% agarose gel, purified PCR products were used in the secondary reaction with forward and reverse outside primers. The products were verified and purified as described above, and digested with restriction enzymes KpnI and MluI (Fermentas). Following purification PCR products were ligated with digested and purified pCAGGS vector using T4 ligase (New England Biolabs) for 2 hours at RT. Then the ligation mix was transformed into One Shot TOP 10 Competent Cells (Life Technologies). To generate the deletion mutants single PCR reaction was performed using primers described in Table 4. Gibson Assembly technique according to manufacturer’s protocol was used to generate PLP2-ubiquitin interaction mutants. The introduced mutations were verified by sequencing. Sequences of all used constructs are gathered in Appendix 1. Primers used to generate DUB mutants are shown in Table 5.

**EXPRESSION PLASMIDS**

For the luciferase assay experiments we used IFNβ-Luc provided by John Hiscott (Jewish General Hospital, Montreal, Canada), Renilla-luciferase expression plasmids pRL-TK (Promega), NF-κB reporter expression plasmid pGL4 32 [luc2 NF-κB-RE Hyrgo] (Promega), N-RIG-I expression plasmid was provided by Ralph Baric (University of North Carolina). The pEF-BOS MDA5 (Addgene #27225) and pEF-BOS MAVS (Addgene #27224) expression plasmids were gifts of Kate Fitzgerald (University of Massachusetts Medical School). pcDNA3.1-Flag-Ub was kindly provided by Dr. Adriano Marchese (Loyola University Medical Center), pcDNA3-myc6-mISG15 was a
### Table 4. Primers used to generate MHV PLP2-B mutants

<table>
<thead>
<tr>
<th>Name</th>
<th>Genome aa</th>
<th>Mutation</th>
<th>Forward Mutagenic Primer</th>
<th>Reverse Mutagenic Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP2-B</td>
<td>1525-1911</td>
<td>WT</td>
<td>Codon optimized synthetic sequence</td>
<td></td>
</tr>
<tr>
<td>PLP2-B1</td>
<td>1525-1911</td>
<td>C1716A</td>
<td>5’ G TCA AAC AAG AAC GCC TCA TCT GAC GAA GAC G</td>
<td>5’ CCG TGA TGT AGG CGT TTG TGT TTG ACG</td>
</tr>
<tr>
<td>PLP2-B2</td>
<td>1525-1911</td>
<td>Δ1611-1614</td>
<td>5’ GCT GGC CAA TAA GTG CAC CTT CTA CTA CCA GGA GGT</td>
<td>5’ ACC CCG TCG AGG GTG CAC CTA TTA TTG GGC AGC</td>
</tr>
<tr>
<td>PLP2-B3</td>
<td>1525-1911</td>
<td>VDVVLSSS (1611-1614)</td>
<td>5’ GCC CAA TAA GAG GAG TAG TAG TAG TG CAC CTT CCA GGA CGG</td>
<td>5’ CCG TCG ACG GTG CAA CTA CTA CTA CTT TTA TTG GGC</td>
</tr>
<tr>
<td>PLP2-B4</td>
<td>1525-1911</td>
<td>VDVSS (1611-1612)</td>
<td>5’ GCC CAA TAA G AGT GAT GTG CTA CAC CTT CCA GGA CGG</td>
<td>5’ CCG TAA CAG TGC ACA GCA CAC TAG TAC TAT TTG CTG CC</td>
</tr>
<tr>
<td>PLP2-B5</td>
<td>1525-1911</td>
<td>DVSSS (1612-1613)</td>
<td>5’ GCC CAA TAA G GTG AGT TCG CTG TG CAC CTT CCA GGA CGG</td>
<td>5’ CCG TCA CAG TGC ACA GCA CAC TAG TAC TAT TTG CTG CC</td>
</tr>
<tr>
<td>PLP2-B6</td>
<td>1525-1911</td>
<td>VVSSS (1613-1614)</td>
<td>5’ GCC CAA TAA G ACG GAT TCA TGA CAC CTT CCA GGA CGG</td>
<td>5’ CCG TCA CAG TGC ACA GCA CAC TAG TAC TAT TTG CTG CC</td>
</tr>
<tr>
<td>PLP2-B7</td>
<td>1525-1911</td>
<td>VDVVLSS (1611-1614)</td>
<td>5’ GCC CAA TAA G ACG GAT GAT GTG CTA CAC CTT CCA GGA CGG</td>
<td>5’ CCG TCA CAG TGC ACA GCA CAC TAG TAC TAT TTG CTG CC</td>
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<tr>
<td>PLP2-B8</td>
<td>1525-1911</td>
<td>V1611S</td>
<td>5’ GCC CAA TAA G ACG GAT GTG CTA CAC CTT CCA GGA CGG</td>
<td>5’ CCG TCA CAG TGC ACA GCA CAC TAG TAC TAT TTG CTG CC</td>
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<tr>
<td>PLP2-B9</td>
<td>1525-1911</td>
<td>V1613S</td>
<td>5’ GCC CAA TAA G GTG AGT TCG CTG TG CAC CTT CCA GGA CGG</td>
<td>5’ CCG TCA CAG TGC ACA GCA CAC TAG TAC TAT TTG CTG CC</td>
</tr>
<tr>
<td>PLP2-B10</td>
<td>1525-1911</td>
<td>Δ1612K</td>
<td>5’ GCC CAA TAA G GGA GTG CTG TG CAC CTT CCA GGA CGG</td>
<td>5’ CCG TCA CAG TGC ACA GCA CAC TAG TAC TAT TTG CTG CC</td>
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<tr>
<td>PLP2-B11</td>
<td>1525-1911</td>
<td>Δ1612L</td>
<td>5’ GCC CAA TAA G GGA GTG CTG TG CAC CTT CCA GGA CGG</td>
<td>5’ CCG TCA CAG TGC ACA GCA CAC TAG TAC TAT TTG CTG CC</td>
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Forward outside primer: 5’ CCGTACCCAGTGGCTGGCTGGG
Reverse outside primer: 5’ CGG TAC CGT CCT CAG GTT TTT
Table 5. Primers used to generate MHV PLP2-B mutants

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Forward Mutagenic Primer</th>
<th>Reverse Mutagenic Primer</th>
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</thead>
<tbody>
<tr>
<td>R253A</td>
<td>5' GC ATT GAC TTC ATG GGG GTG CTG AGA GAA C</td>
<td>5' GCT CTC TCA GGA CGG GCA TGA AGT CAA TGC</td>
</tr>
<tr>
<td>R257A</td>
<td>5' G AGG GTG GTG CTG GCA GAA GCT GAT CTG TC</td>
<td>5' GAC AGA TCA CTA GCT TCT GCC AGC AGC ACC CTC</td>
</tr>
<tr>
<td>E279A</td>
<td>5' GTGGCGTGGAGCAG GCA CAG CCG AAA GGA GTC</td>
<td>5' GAC TCC TTT CCG CTG TGC CTT CAC GCC AC</td>
</tr>
<tr>
<td>F290A</td>
<td>5' GAC GCA GTG ATG CAC GCT GCC ACC CTG GAT AAG</td>
<td>5' CTT ATC CAG GCT GCC AGC GTG CAT CAC TGC GTC</td>
</tr>
<tr>
<td>Y302A</td>
<td>5' CTG GTG CGC GGG GCC ACC ATC GCC TGC A</td>
<td>5' TGC AGG CGA TGT TGG CCC CCG GCA GCA GAC</td>
</tr>
<tr>
<td>R253/257A</td>
<td>5' CGG TCG TGC TGG CAG AAG CTG ATC TGT C</td>
<td>5' GAC AGA TCA GCT TCT GCC AGC ACC G</td>
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<tr>
<td>V313A</td>
<td>5' GT GGA TCT AAG CTG GCT CAT TGC ACC CAG TTC</td>
<td>5' GAA CTG GGT GCA ATG AGC CAG CTT AGA TCC AC</td>
</tr>
<tr>
<td>Y302F</td>
<td>5' CTC GTG CGC GGG TCC AAC ATC GCC TG</td>
<td>5' CAG GCG ATG TGT AAC CCG CGC ACC AG</td>
</tr>
<tr>
<td>I249R</td>
<td>5' A GCC CTCA GAT AGC CTT GAC TTC ATG AG</td>
<td>5' CTC ATG AAG TCA CGG CTG TCT GAG GCC T</td>
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<tr>
<td>I304A</td>
<td>5' TG CCG GGT TAC AAC GCA GCC TGC ACT TG</td>
<td>5' CAA GTG CAG GCT GCC TGT TAC CCG CG</td>
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<tr>
<td>F270A</td>
<td>5' TGC AAT GTG GAG GTG GCT TGC AAG TGT G</td>
<td>5' CAG ATT TGC AGA CAG CCT CCA GAT TGC A</td>
</tr>
<tr>
<td>R281E</td>
<td>5' G CAG GAA CAG GAG AAA GGA GTC GAC G</td>
<td>5' CGT CCA CTC CCT TCT CCT GCT TGT GC</td>
</tr>
<tr>
<td>F290W</td>
<td>5' CAG GTG ATG CAC TGG GCC ACC CTG GAT AAG</td>
<td>5' CTT ATC CAG GGT GCC CCA GTG CAT CAC TG</td>
</tr>
<tr>
<td>I244V</td>
<td>5' A GCC CTCA GAT AGC TGC GAC TTC ATG AG</td>
<td>5' CTC ATG AAG TCG AGC CTA TCT GAG GCC T</td>
</tr>
<tr>
<td>T328A</td>
<td>5' TC TGT AGT AAC GCA CCC GAG GGG AGA AAG C</td>
<td>5' GCT TCC CCT CGG GTG GTC TAC TAC AGA</td>
</tr>
<tr>
<td>Forward outside primer</td>
<td>5' GAA TTC GAG CTC ACC ATG GCC GG T ACC GAG</td>
<td></td>
</tr>
<tr>
<td>Reverse outside primer</td>
<td>5' GGA GAG GTG TAG GGA TAG GCT TAC CAC GCG</td>
<td></td>
</tr>
</tbody>
</table>
gift of Dr. Min-Jung Kim (Pohang University of Science and Technology, Pohang, Republic of Korea). pcDNA3-Ube1L, pcDNA3-UbcH8, and pcDNA-Herc5 were provided by Dr. Robert M. Krug (University of Texas).

**DEISGYLATING ACTIVITY ASSAY**

HEK293T cells in 12-well plates were transfected with 10, 25, 50, 100 ng of indicated PLP expression construct wild-type or catalytic mutant, and 250 ng pISG15-myc, 125 ng pUbcH8, 125 ng pUbe1L, and 125 ng pHerc5. At 20 hours post-transfection, cells were lysed with lysis buffer (20mM Tris (pH 7.5), 150mM NaCl, 1mM EGTA, 1mM EDTA, 1% Triton X-100, 2.5mM Na pyrophosphate, 1mM beta-glycerophosphate, 1mM Na ortho-vanadate, 1µg/ml leupeptin). Proteins were separated by SDS-PAGE, and transferred to PVDF membrane using a semi-dry transfer apparatus (BioRad). Following transfer, the membrane was blocked using 5% dried skim milk in TBST buffer (0.9%NaCl, 10mM Tris-HCl, pH=7.5, 0.1% Tween 20) over night at 4°C. The membrane was incubated with mouse anti-myc antibody (MBL) at the dilution of 1:2500. The membrane was washed 3 times for 15 minutes in TBST buffer. Following the membrane was incubated with secondary goat-anti-mouse-HRP antibody at the dilution 1:5000 (Amersham). Then the membrane was washed 3 times for 15 minutes in TBST buffer. The detection was performed using Western Lighting Chemiluminescence Reagent Plus (PerkinElmer) and visualized using FluoroChemE Imager (Protein Simple). To verify expression of the PLpro the membrane was probed with mouse anti-V5
antibody (Invitrogen) at the dilution 1:5000. Mouse anti-calnexin antibody (Cell Signal) at the dilution 1:2000 was used to determine loading standard.

DEUBIQUITINATING (DUB) ACTIVITY ASSAY

To assess DUB activity, HEK293T cells in 12-well plates were transfected with 400 ng pcDNA3.1-Flag-Ub and 0.25, 0.5, or 1µg of indicated PLP expression construct wild-type or catalytic mutant. At 18 hours post-transfection, cells were lysed with 100µl of lysis buffer, SDS-PAGE and transferred to PVDF membrane as described above. Membrane probing was performed using mouse anti-Flag M2 antibody (Sigma) at the dilution of 1:2000.

LUCIFERASE REPORTER ASSAY

HEK293T cells in 24-well plates were transfected using with 50ng Renilla-luciferase, 100ng IFN-β-luc or 100ng ISRE-luc, and indicated PLP expression plasmids. As a stimulation 150ng pEF-BOS MDA5, or 50ng pEF-BOS MAVS, or 50ng N-RIG-I per well was transfected. Empty pCDNA3.1-V5/His-B vector plasmid was used to standardize the total amount of DNA used for transfection. At 16 hours-post transfection cells were lysed using 1X Passive Lysis buffer (Promega). Alternatively, the cells were transfected with 50ng pGL4 32 [luc2 NF-κB-RE Hyrgo], 100ng IFN-β-luc and wild-type or catalytic mutant PLP expression plasmids for 12 hours and then treated with 10ng/µl TNFα (Roche) for 4 hours, and lysed. For all experiments Firefly and Renilla luciferase were measured using Dual Luciferase Reporter Assay System (Promega) and
luminometer (Veritas). Results were normalized to Renilla luciferase expression control. Experiments were performed in triplicate. Remaining lysates were incubated with Lysis buffer A and analyzed by SDS-PAGE as described above. The presence of PLPs was detected using mouse anti-V5 antibody (Invitrogen) at the dilution 1:5000.

**pGlo ENDPOINT ASSAY**

HEK293T in cells in 24-well CellBind plates (Corning) were transfected with 150ng pGlo (Promega) pGlo-RLKK construct, 25ng pRL-TK (Promega), and increasing amounts of protease expression plasmid. After 20 hours, cells were lysed with 1X passive lysis buffer (Promega) and 25ul of lysate was taken and assayed for luciferase activity using dual luciferase activating reagents (Promega).

**pGlo LIVE-CELL ASSAY**

HEK293T were transfected with 37.5ng pGlo (Promega) pGlo-RLKGG construct and 50ng of PLP expression plasmids. 18 hours post-transfection GloSensor (Promega) reagent diluted 1:50 in DMEM+10% FCS was added. Plates were imaged using a luminometer (Veritas) every hour for 5 hours.

**qRT-PCR ANALYSIS**

HEK 293T cells in 12-well plates were transfected with 300ng pEF-BOS MDA5 or 100ng N-RIG-I and 200ng of plasmids expressing wild-type, catalytic mutant or indicated PLP mutants. Empty vector plasmid pCDNA3.1-V5/His-B vector was used to
standardize the total amount of DNA in each sample. The cells were lysed 18 hours-post transfection with Buffer RLT (Qiagen) and RNA was extracted using RNeasy Mini (Qiagen). Reverse transcription was performed using 1 μg of total RNA and the RT2 First Strand Kit (Qiagen) according to manufacturer’s protocol. 1 μl of cDNA was used to set up qRT-PCR reaction according to the manufacturer’s protocol using Single Primer Assay for IFNβ, CXCL10, and CCL5 (SABiosciences) or Human Innate and Adaptive immune response gene array (SABiosciences). CT values were normalized to housekeeping gene (RPL13).

**GENERATING THE NSP3 MUTANT VIRUS**

To generate a mutant MHV-A59 a previously described method was used (Yount, Denison, Weiss, & Baric, 2002). Briefly B plasmid was mutagenized using primers described Table 6. All plasmids encoding complete virus genome were digested with restriction enzymes (New England Biolabs) and gel purified. Further, the fragments were ligated using T4 ligase at 4°C overnight. The ligation reaction was isopropanol precipitated and *in vitro* RNA transcription was performed using mMESSAGE mMASCHINE Kit (Applied Biosystems) according to the following protocol: 40.5°C for 25 min, 37.5°C for 50 min, 40.5°C for 25 min. RNA was electroporated into BHK-R cells, and seeded into DBT cells. The detailed protocol is described in Appendix 2.
Table 6. Primers used to generate mutant viruses

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Forward Mutagenic Primer</th>
<th>Reverse Mutagenic Primer</th>
</tr>
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<tbody>
<tr>
<td>AM1</td>
<td>5' CTC GCT AAT AAG AGT TCG TCC TCG TGT ACT GTT GAT GAG GAA CTC TTA TTA GCG AG</td>
<td></td>
</tr>
<tr>
<td>AM2</td>
<td>5' CTC GCT AAT AAG AGT TCG TCC TCG TGT ACT GTT GAT GAG GAA CTC TTA TTA GCG AG</td>
<td></td>
</tr>
<tr>
<td>F290A</td>
<td>5' GAC GCT GTT ATG CAT GCT GGT AGT GAT AAA GG</td>
<td>5' CCT TTA TCC AAG GTA CCA GCA TGC ATA ACA GGG GC</td>
</tr>
<tr>
<td>Forward outside primer for AM1 and AM2</td>
<td>5' GAA TGC CCG GGC GGG ATT TTT GTA TCC</td>
<td></td>
</tr>
<tr>
<td>Reverse outside primer for AM1 and AM2</td>
<td>5' CCA CAA GAT CTG CCT CGG ACA AAT C</td>
<td></td>
</tr>
<tr>
<td>Forward outside primer for F290A</td>
<td>5' CTT TCA GTA CAG TGA TTT GTC CGA GGC AG</td>
<td></td>
</tr>
<tr>
<td>Reverse outside primer for F290A</td>
<td>5' GCT TCC TCA TGG CCA AGC CAG ACA ACC GG</td>
<td></td>
</tr>
</tbody>
</table>
TEMPERATURE SHIFT EXPERIMENT

DBT cells in 6-well plates were infected with 0.1 MOI of icMHV-A59 or AM2. The supernatant was collected every 2 hours and the virus titer was determined using plaque assay. Plaque assay was performed as follows: DBT cells seeded in 6 well-plates were infected with 300 µl of serial dilution of virus stock. The plates were incubated for 1 hour at 37°C, with rocking every 15 minutes. Upon infection the inoculum was removed and 2ml of media/agar (1:1 of 2xMEM 2%FCS and 0.8% Noble agar) layer was applied. The plates were incubated at 37°C for 48 hours and fixed with 10% formaldehyde for at least 1 hour. Upon that time the cells were stained with crystal violet.

EXPRESSION AND PURIFICATION OF V1613S MUTANT PROTEIN

Sequence encoding MHV PLP2 domain (residues 1525-1910 of polyprotein 1ab from MHV strain A59) was cloned into LIC vector pEV-L8, which is a modified pET-30. The expression of wild type PLP2 and V1613S mutant was performed with Escherichia coli strain BL21 (DE3) cells. Cultures were grown in LB medium at 37°C until the optical density at 600nm (A600) reached 0.6, and then induced with 0.1mM isopropyl-β-D-thiogalactopyranoside (IPTG). WT PLP2 was induced at 25°C for 6h, while V1613S mutant was induced at 18°C overnight. Cells were harvested by centrifugation at 5,000 rpm for 20min (4°C) and frozen at -80°C. The cell pellet from 1L culture was then resuspended in 40ml buffer A (25mM Tris pH 7.0, 500mM NaCl, 20mM imidazole, 5mM βME) supplemented with flakes of lysozyme and DNase, lysed through sonication and centrifuged at 13,000 rpm for 30min (4°C). The supernatant was filtered through a
0.45μm membrane (Millipore) and loaded onto a 5ml Ni HisTrapHP column (GE healthcare) pre-equilibrated with buffer A. Then column was washed with buffer A supplemented with 5% buffer B (25mM Tris pH 7.0, 500mM NaCl, 500mM imidazole, 5mM βME) until the UV was back to the baseline. The protein was eluted through a gradient of 5%-100% buffer B in 30 column volumes (CV). Fractions collected were pooled upon activity and purity assessment, followed by TEV protease (His-tagged) cleavage overnight at 4°C along with dialysis into buffer C (25mM Tris pH 7.0, 100mM NaCl, 10mM βME). Then His-tag cleaved PLP2 was separated from uncleaved PLP2 and TEV through another step of Ni HisTrap column. The flowthrough collected was concentrated using Millipore Micron concentrators to a volume of less than 2ml, and loaded onto a Superdex-75 Hiload 26/60 column (GE Healthcare) pre-equilibrated with buffer D (50mM HEPES pH 7.0, 100mM NaCl, 10mM DTT), and eluted at a flow rate of 2ml/min. Fractions were pooled upon activity and purity assessment, and flash-frozen in 2% glycerol and stored at -80°C. This experiment was performed by Y. Chen, Purdue University.

**ENZYMATIC ACTIVITY OF V1613S PURIFIED PROTEIN**

Wild-type PLP2 and the V1613S mutant were incubated at 25°C for different time periods (5-50min). At each time point, the activity of both enzymes was measured at 25°C with 50μM RLRGG-AMC as the substrate and 5μM enzyme. The experiments were performed in triplicate. Similar experiments were carried out when the enzymes were incubated at 30°C. To analyze the data, the ratio of the reaction rate at time t to the
rate at time 0 was plotted in logarithmic scale against incubation time. Kinetic data of the V1613S PLP2 incubated at 30°C are fitted to a first-order exponential decay model (\(\frac{\text{Rate}}{\text{Rate}_0} = e^{-kt}\)), from which the inactivation rate constant \(k_{\text{inact}}\) and half-life \(t_{1/2}\) can be determined. Other data are fitted to a line since no significant temperature inactivation is observed. This experiment was performed by Y. Chen, Purdue University.

**THERMAL MELT ANALYSIS USING CIRCULAR DICHROISM (CD)**

Thermal melting analysis of wild-type PLP2 and V1613S mutant was carried out with a Chirascan circular dichroism (CD) spectrometer (Applied Photophysics) equipped with a temperature control system (Quantum Northwest Inc.) by monitoring CD at 220nm while increasing the temperature at step interval 0.4°C and rate 0.5°C/min. Protein sample was hold in a 10mm cell (Starna Cells) with magnetic stirring. Thermal scan was performed with wild-type PLP2 or V1613S in three independent experiments. The melting temperature (Tm) was calculated as the first derivative peak determined using SigmaPlot. This experiment was performed by Y. Chen, Purdue University.

**MOUSE STUDIES**

C57BL/6 mice were purchased from The Jackson Laboratory. The mice were maintained and performed at Loyola University Chicago in accordance with all federal and university guidelines. 4-week old C57BL/6 male mice were anesthetized with ketamine/xylazine prior to intracranial injection with 600 pfu of icMHV-A59 or AM2. The body weight loss was monitored daily over time. The mice were humanely sacrificed.
when they have lost 25% of the initial body weight. The mice infected with AM2, and age-matched controls were challenged with 6,000 pfu of icMHV-A59 9 weeks post primary infection. The body weight loss was monitored daily over time. The mice were humanely sacrificed when they have lost 25% of the initial body weight.

SARS-CoV ANTIVIRAL ACTIVITY ASSAY

VeroE6 cells in 96 well plates were infected with SARS-CoV Urbani strain (provided by the Centers for Disease Control and Prevention) MOI of 10 or mock infected with serum-free media. The plates were incubated for 1 hour at 37°C, 5% CO2. After that time virus inoculum was removed and replaced with DMEM 2% FCS and serial dilutions of antiviral compounds (from 40µM to 1.8µM). The cell viability was determined after 48hours using The CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to manufacturer’s protocol. The experiments were performed in triplicates. Work was performed in BSL-3 facility according to the BSL-3 protocol and all liquid infectious waste was treated with bleach. All solid waste was autoclaved.
CHAPTER III
RESULTS

INVESTIGATING THE ABILITY OF SMALL MOLECULE PAPAIN-LIKE PROTEASE SELECTIVE INHIBITORS TO BLOCK SARS-CoV REPLICATION IN INFECTED CELLS

SARS-CoV is a highly pathogenic virus with 10% mortality. Thus far there are no FDA approved antiviral drugs against SARS-CoV. Previous studies showed that the papain-like protease (PLpro) from SARS-CoV is required for virus replication because it cleaves viral polyprotein to generate replication complexes. Moreover, SARS-CoV PLpro has a possible role in virus pathogenesis because of its reported deubiquitinating, deISGylating and interferon antagonism activities. All these properties make SARS-CoV PLpro a good target for small molecule inhibitors. Previous high-throughput screens identified several compounds that had the ability to inhibit SARS-CoV replication with micromolar EC\textsubscript{50} values (Ghosh et al., 2010; Ratia et al., 2008). However, the potencies of these PLpro inhibitors were not low enough to have a therapeutic potential. Thus further optimization and structure-activity relationship (SAR) analysis based on crystal structure, was performed to generate a new series of potent SARS-CoV PLpro inhibitors.
My goal was to evaluate the newly designed compounds’ ability to inhibit SARS-CoV replication in cell culture and determine their EC₅₀ values. Out of over 30 new small molecule inhibitors that were tested in vitro, I evaluated 10 drugs that had the lowest IC₅₀ values ranging from 0.2 to 12.7µM against purified enzyme. All experiments with SARS-CoV were performed in the Biosafety Level 3 (BSL-3) laboratory according to approved protocols. To determine the EC₅₀ of tested compounds, I infected VeroE6 cells in 96 well plates with SARS-CoV for one hour. After that time the media was replaced with media containing serial dilutions of compounds. The plates were incubated for 48 hours and the cells were lysed. The cells viability was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). The data are summarized in the Table 7 and representative cell viability graphs for two compounds are shown in Figure 7. I found that several compounds with the highest IC₅₀ values (>1.6 µM) did not show inhibitory activity towards SARS-CoV replication in infected cells. In contrast, the compounds that had IC₅₀ lower that 1.0 µM were able to inhibit SARS-CoV replication. The determined EC₅₀ values for those compounds ranged from 14.45 µM for the least active inhibitor to 5.5 µM for the most active compound CCG 203 888. My results indicate that the SAR analysis was very effective and that new compounds have improved potency. Moreover, I found that IC₅₀ value is a good indicator of the probable efficacy of the drug in infected cells. Finally, I determined that compound CCG 203 888 is a very good candidate for further optimization. Additionally, our collaborators found that this drug has relatively low cytotoxicity suggesting that this small molecule inhibitor is worth further evaluation and optimization (Báez-Santos et al., 2014).
Figure 7. SARS-CoV PLpro inhibitor CCG 203 888 inhibits the replication of SARS-CoV. VeroE6 cells were infected with SARS-CoV. After 1 hour incubation, inoculum was replaced with media containing compound CCG203888 (A) or CCG203494 (B) in serial dilution concentration. Cell viability was measured 48 hours post infection using CellTiter-Glo Luminescent Cell Viability Assay (Promega). The error bars represent SD of triplicates within a single experiment. The figure shows representative data from at least two independent experiments.
Table 7. Potency of selected SARS-CoV inhibitors. The table shows IC<sub>50</sub> values for selected compounds. VeroE6 cells were infected with SARS-CoV for 1 hour and then incubated in the presence of the serial dilutions of the drug. Cell viability was measured 48 hours post infection using CellTiter-Glo Luminescent Cell Viability Assay (Promega). EC<sub>50</sub> values were determined in triplicates in at least two independent experiments using GraphPad Prism software. These data are published in (Báez-Santos et al., 2014).

<table>
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<tr>
<th>Drug</th>
<th>Corresponding Name Baez-Santos et al., 2014</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; [µM]</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; [µM]</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; [µM]</th>
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<tbody>
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<td>CCG 206 492</td>
<td>3h</td>
<td>12.7 ± 0.3</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>CCG 206 554</td>
<td>4a</td>
<td>7.0 ± 0.7</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>CCG 206 494</td>
<td>4c</td>
<td>6.8 ± 0.3</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>CCG 202 841</td>
<td>2a</td>
<td>2.2 ± 0.1</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>CCG 203 887</td>
<td>2e</td>
<td>1.9 ± 0.1</td>
<td>9.91 ± 0.54</td>
<td>13.49 ± 0.41</td>
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<tr>
<td>CCG 203 843</td>
<td>15g</td>
<td>0.7 ± 0.03</td>
<td>14.45 ± 0.71</td>
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<tr>
<td>CCG 203 885</td>
<td>3j</td>
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<td>13.06 ± 0.91</td>
<td>10.07 ± 0.36</td>
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<tr>
<td>CCG 206 553</td>
<td>5c</td>
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<td>9.91 ±0.54</td>
<td>9.07 ± 2.69</td>
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<tr>
<td>CCG 206 552</td>
<td>3e</td>
<td>0.4 ± 0.01</td>
<td>7.77 ± 0.14</td>
<td>8.78 ± 0.70</td>
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<tr>
<td>CCG 203 888</td>
<td>3k</td>
<td>0.2 ± 0.01</td>
<td>5.48 ± 0.26</td>
<td>5.27 ± 0.35</td>
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INVESTIGATING MULTIFUNCTIONALITY OF PAPAIN-LIKE PROTEASE (PLpro) FROM A NOVEL CORONAVIRUS MIDDLE EAST RESPIRATORY SYNDROME CORONAVIRUS (MERS-CoV)

MERS-CoV PLpro has deISGylating and deubiquitinating activities

MERS-CoV is a recently emerged coronavirus with about 45% mortality in human population. Unfortunately there are no FDA approved antiviral drugs or vaccines against this coronavirus. In order to facilitate the development of antiviral strategies I evaluated whether a predicted papain-like protease (PLpro) domain from MERS-CoV is a valuable target for protease inhibitors and vaccines.

MERS-CoV PLpro is encoded within nonstructural protein 3 (nsp3) of the replicase polyprotein. To gain insight into the potential activity of MERS-CoV PLpro, our collaborators at Purdue University used the high-resolution X-ray structure of SARS-CoV PLpro in apo-enzyme form (PDB: 2Fe8, chain C) to generate a homology model of MERS-CoV PLpro. The homology model displays several conserved structural features between MERS-CoV and SARS-CoV PLpro; including the ubiquitin-like domain (UBL), a catalytic triad consisting of C1594–H1761–D1776, and the ubiquitin-binding domain at the zinc finger. To model ubiquitin (Ub) into the zinc finger of MERS-CoV PLpro, the electron density of SARS-CoV PLpro in complex with Ub aldehyde (Ubal) for refinement and energy minimization of the model in complex with Ub was used. The model displays a nearly perfect fit of the Ub moiety in the zinc finger with orienting Ub C-terminal extension towards the enzymes subsites and catalytic triad (Figure 8).
Figure 8. Modeling MERS-CoV PLpro onto the SARS-CoV PLpro-ubiquitin-aldehyde structure. Homology model of MERS-CoV PLpro (blue cartoon and gray surface) aligns with the overall structural architecture found in SARS-CoV PLpro-Ubiquitin-aldehyde complex PDB:4MM3 (beige cartoon), including the ubiquitin binding domain at the zinc finger and the extended Ub-like (Ubl) domain. Ubiquitin (red) modeled into the zinc finger domain of MERS-CoV PLpro, with its C-terminus reaching the active site. An enlargement of predicted MERS-CoV PLpro active site superimposed onto the SARS-CoV PLpro active site suggests that the MERS-CoV PLpro catalytic triad is composed of C1594–H1761–D1776 and the putative oxyanion hole residue is L1590 (Mielech et al., 2014).
From this model, I hypothesized that the PLpro domain from MERS-CoV, like SARS-CoV is a multifunctional enzyme with protease, deubiquitinating and deISGylating activity.

To determine if PLpro from recently emerged MERS-CoV is a multifunctional enzyme I designed a codon-optimized synthetic construct to express wild-type PLpro. The synthetic MERS-PLpro extends from amino acids 1485 to 1802 of ORF1a, with the addition of 2 amino acids at the N-terminus to allow efficient translation (methionine and alanine) and a V5 epitope tag on the C-terminus (Figure 9A). Using Gibson Assembly technique (New England Biolabs) I generated a catalytic mutant where the predicted catalytic cysteine C1594 (PLproCA) residue is mutated to alanine. Cysteine 1594 is predicted to be the active site cysteine nucleophile that attacks the substrate peptide bond and mutation to alanine should significantly reduce or abolish enzymatic activity (Figure 8). First, I tested the protease activity of MERS-CoV PLpro using transcleavage assay. HEK293T cells were transfected with plasmids expressing increasing doses of MERS-CoV wild-type PLpro, or PLproCA, along with a plasmid DNA expressing the SARS-CoV nsp2-3-GFP substrate. The nsp 2-3-GFP substrate is commonly used to assess the cleavage ability of transiently expressed CoV PLPs. I detected evidence of processing of the nsp2-3-GFP substrate in the presence of the catalytically active form of MERS-PLpro, but not in the presence of the catalytic mutant MERS-PLpro (Figure 9B). These data confirm that the putative PLP domain located within nsp3 of the MERS-CoV genome indeed functions as a PLP capable of cleaving LXGG-containing polyprotein substrates. These data have been described in (Kilianski, Mielech, Deng, & Baker, 2013).
Figure 9. Proteolytic activity of MERS-CoV PLpro. A) Schematic diagram of MERS-CoV ORFs and the papain-like protease (PLpro) domain within nonstructural protein 3 (nsp3). Expression plasmid pcDNA-MERS-PLpro (amino acids 1485-1802) and the predicted catalytic cysteine residue 1594 are indicated. B) To determine protease activity HE293T cells were transfected with nsp2/nsp3-GFP plasmid and plasmids expressing wild-type (WT) or catalytic mutant (CA) MERS-CoV PLpro. Cell lysates were analyzed by western blot 24 hours post-transfection. Figure shows representative data from at least two independent experiments (Kilianski, Mielech, Deng, & Baker, 2013; Mielech et al., 2014).
To assess the DUB activity of MERS-CoV PLpro, I transfected HEK293T cells with plasmid expressing Flag-Ub and increasing amounts of wild-type PLpro or PLproCA. I harvested cell lysates at 18 hours post-transfection to evaluate the presence of ubiquitinated proteins using western blot. I determined that wild-type PLpro can deubiquitinate multiple cellular substrates, and that PLpro catalytic activity is required for DUB activity (Figure 10B). This DUB activity is also observed with expression of SARS-CoV PLpro, consistent with previous reports (Barretto et al., 2005; Frieman et al., 2009; Lindner et al., 2005, 2007; Ratia et al., 2006). In these experiments, I noticed the difference in the expression levels of SARS-CoV and MERS-CoV PLpros in transfected cells, which may be due to differences in codon optimization in the MERS-CoV PLpro construct. Further in vitro studies using purified enzymes are needed to determine the relative kinetics of SARS-CoV and MERS-CoV PLpro DUB and deISGylating activities.

To determine the deISGylating activity of MERS-CoV PLpro, HEK293T cells were transfected with c-myc-ISG15 plasmid, ISG15 conjugation machinery, and increasing amounts of plasmids expressing MERS-CoV PLpro wild-type and catalytic mutant C1594A (PLproCA). In addition, the cells were transfected with plasmids expressing SARS-CoV PLpro wild-type or catalytic mutant (C1651A). 20 hours post-transfection cell lysates were evaluated for the presence of ISGylated proteins. Both MERS-CoV and SARS-CoV PLpro can deconjugate ISG15 from multiple cellular substrates in a dose-dependent manner. In contrast, PLpro catalytic mutants did not deconjugate ISG15, indicating that catalytic activity of PLpro is required for its
Figure 10. Enzymatic activities of SARS-CoV PLpro and MERS-CoV PLpro. A) DeISGylating activity of SARS-CoV PLpro and MERS-CoV PLpro. HEK293T cells were transfected with myc-ISG15, E1, E2, E3 ISGylating machinery plasmids, and wild-type (WT) or catalytic mutant (CA) PLpro expression plasmids. At 18 hours post transfection, cells were lysed and analyzed by Western blotting. B) Deubiquitinating activity of SARS-CoV PLpro and MERS-CoV PLpro. HEK293T cells were transfected with Flag-Ub expression plasmid, and wild type (WT) or catalytic mutant (CA) PLpro. Cells were lysed 18 hours post transfection and analyzed by Western blotting. Figure shows representative data from at least two independent experiments (Mielech et al., 2014).
deISGylating activity (Figure 10A). Thus, MERS-CoV PLpro like SARS-CoV PLpro (Lindner et al., 2007) has deISGylating activity.

Taken together, my data indicate that MERS-CoV PLpro is a potent deISGylating enzyme that also exhibits DUB activity and that both activities require cysteine 1594 for catalysis, likely in the context of the predicted catalytic triad (Figure 8).

**MERS-CoV PLpro is an interferon antagonist**

Coronavirus PLPs have been shown to block interferon β (IFNβ) induction in transfected cells (Clementz et al., 2010; Devaraj et al., 2007; Frieman et al., 2009). In addition, the deubiquitinase function of an arterivirus PLP has been shown to have a role in interferon antagonism during virus infection (van Kasteren et al., 2013). Therefore, I assessed the ability of MERS-CoV PLpro to antagonize interferon production. First, I addressed if MERS-CoV PLpro can inhibit MDA5 induced IFNβ reporter, since MDA5 has been implicated in recognition of coronaviruses during virus infection (Zust et al., 2011). I transfected HEK293T cells with plasmids expressing IFN-β-luciferase, Renilla luciferase, pEF-BOS-MDA5 (Rothenfusser et al., 2005) and increasing amounts of wild-type PLpro or PLproCA. At 16 hours post-transfection I assessed luciferase reporter activity. I determined that MERS-CoV PLpro can potently inhibit MDA5 mediated induction of IFNβ in a dose-dependent manner and that catalytic activity of MERS-CoV PLpro is required for IFNβ antagonism (Figure 11A). Using overexpression of an active form of RIG-I, I determined that MERS-CoV PLpro can also inhibit N-RIG-I induced IFNβ reporter. Similarly to the experiment with MDA5 stimulation, the catalytic activity of MERS-CoV PLpro is necessary for IFNβ antagonism upon N-RIG-I stimulation.
Figure 11. Interferon antagonism activity of MERS-CoV PLpro. HEK293T cells were transfected with plasmids expressing wild-type (WT) or catalytic mutant PLpro (CA), plasmids expressing IFNβ-luc (A, B, and C), or NF-κB-luc (D), Renilla-luc, and the stimulator indicated at the top of the figure. For A-C, at 16 hours post-transfection, cells were lysed and luciferase activity was measured. For D, at 10 hours post-transfection cells were treated with TNFα for 4 hours, lysed and luciferase activity was measured. Experiments were performed in triplicate. Error bars represent standard deviation of the mean (Mielech et al., 2014).
Upon recognition of viral RNA by pattern recognition receptors (PRRs) such as MDA5 or RIG-I, the signal is transmitted downstream via mitochondrial antiviral signaling protein (MAVS). Thus, I tested if PLpro is able to inhibit MAVS-induced IFNβ reporter. To stimulate the IFNβ reporter, I overexpressed pEF-BOS-MAVS in HEK293T cells, co-expressed reporters, and either the wild-type PLpro or PLproCA. I found that PLpro, but not PLproCA inhibits MAVS-induced IFNβ reporter (Figure 11C).

In addition, experiment performed by Andy Kilianski, tested the ability of MERS-CoV PLpro to inhibit NF-κB reporter activity as observed with SARS-CoV PLpro. HEK293T cells were transfected with plasmids expressing NF-κB luciferase, Renilla luciferase, and MERS-CoV wild-type PLpro or PLproCA, treated cells with TNFα to activate the NF-κB pathway, and harvested cell lysates at 4 hours post-treatment to assess luciferase activity. Wild-type PLpro reduced induction of NF-κB reporter in a dose-dependent manner and the catalytic cysteine residue is required for this activity (Figure 11D). Taken together these results indicate that MERS-CoV PLpro is an interferon antagonist and that catalytic activity is required for the antagonism. In addition, PLpro can reduce TNFα-mediated induction of NF-κB reporter activity and catalytic activity is also required.

MERS-CoV PLpro and SARS-CoV PLpro inhibit expression of proinflammatory cytokines.

To further investigate the role of coronavirus PLpros in inhibiting innate immune responses I tested the effect of MERS-CoV PLpro on the expression of endogenous
cytokines. First, using the Human Innate & Adaptive Immune Responses PCR Array (SABiosciences) I determined that in HEK293T cells CCL5 (RANTES), IFNβ, and CXCL10 (IP-10) mRNA levels are upregulated more than 20-fold upon MDA5 stimulation and therefore selected these genes for further analysis. To determine the effect MERS-CoV PLpro and SARS-CoV PLpro on cytokine expression, I performed qRT-PCR to measure mRNA levels encoding CCL5, IFNβ, and CXCL10 levels in the presence of CoV PLpros. HEK293T cells were transfected with pEF-BOS-MDA5, and wild-type or catalytic mutants of MERS-CoV or SARS-CoV PLpros. At 18 hours post-transfection the total RNA was extracted and qRT-PCR was performed. I found that both MERS-CoV and SARS-CoV PLpro can potently inhibit (over 3-fold reduction) expression of CCL5 upon MDA5 stimulation and that catalytic activity is required for this inhibition (Figure 12A). In agreement with the results from luciferase reporter assays, I observed that expression of IFNβ in MDA5 stimulated cells is inhibited in the presence of wild-type MERS-CoV PLpro and SARS-CoV PLpro (Figure 12B). CXCL10 mRNA levels were also significantly reduced (p<0.0005) when wild-type, but not catalytic mutant versions of MERS-CoV PLpro and SARS-CoV PLpro were expressed (Figure 12C). These findings show for the first time that both MERS-CoV PLpro and SARS-CoV PLpro can reduce induction of endogenous proinflammatory cytokines in cells, and that the mechanism requires catalytic activity.
Figure 12. Proinflammatory cytokine expression in the presence of SARS-CoV PLpro or MERS-CoV PLpro. HEK293T cells were transfected with plasmids expressing MDA5 and wild-type (WT) or catalytic mutants (CA) of MERS-CoV PLpro or SARS-CoV PLpro. At 18 hours post-transfection, cells were lysed and mRNA levels of CCL5, IFNβ and CXCL10 were determined using qRT-PCR. Data represents fold increase of mRNA levels compared to unstimulated cells ($2^{-\Delta\Delta C_{t}}$). The figure shows the results from representative experiments performed in triplicates and are shown as means, error bars represent SEM. Experiments were performed in duplicate. * p<0.0005, **p<0.001, ***p<0.01 (Mielech et al., 2014).
INVESTIGATING MULTIFUNCTIONALITY OF PREDICTED PAPAIN-LIKE PROTEASE (PLP) DOMAINS FROM SELECTED ALPHA AND BETACORONAVIRUSES

Generating expression constructs of HCoV-HKU1, HCoV-OC43, HCoV-229E, and FIPV PLPs

To investigate whether PLP multifunctionality is a conserved feature among coronaviruses I wanted to test the activities of PLPs from selected alpha and betacoronaviruses. Particularly, I tested PLPs from HCoV-HKU1, HCoV-OC43, HCoV-229E, and feline coronavirus Feline Infectious Peritonitis Virus (FIPV). All mentioned coronaviruses contain two predicted PLP domains within their genome. PLP2 domains were used to design expression constructs because PLP2, not PLP1, domains from HCoV-NL63 and MHV, as well as SARS-CoV and MERS-CoV PLpros, are multifunctional enzymes. In addition, PLP2 domains have higher similarity to PLpros than PLP1 domains. The PLPs sequences were predicted based on sequences of SARS-CoV and MERS-CoV PLpros. The goal was to keep the predicted catalytic residues and extend the constructs so they include possible N-terminal ubiquitin-like (UBL) domains. The full lengths of the constructs were determined by counting the amino acids following predicted catalytic aspartic acid residue at the C-terminal end the constructs. Next, the synthetic expression constructs of putative PLPs with in frame V5 epitope tags were synthesized by Genscript. Further, I used site-directed mutagenesis and Gibson Assembly
Technique to generate catalytic mutants of predicted catalytic cysteine residues of all constructs. The successful mutagenesis was confirmed by sequencing.

**HCoV-HKU1, HCoV-OC43, HCoV-229E, and FIPV PLPs are multifunctional enzymes**

To determine the catalytic activity of HCoV-HKU1, HCoV-OC43, HCoV-229E, and FIPV PLPs Andy Kilianski from our laboratory performed transcleavage assay and found that all PLP2s can cleave SARS-CoV PLpro substrate nsp2/nsp3-GFP in transfected cells in catalytic dependent manner presumably by the recognition of conserved LXGG motif (Kilianski, dissertation). In addition, to test the catalytic activity of these PLP2s I used end-point pGLO assay described by our group previously. HEK293T cells were transfected with plasmid expressing pGLO substrate (pGLO-RLKGG) recognized by the PLP2, Renilla-luciferase, wild-type PLP2s, PLP2s catalytic mutants, and SARS-CoV PLpro or SARS-CoV PLpro catalytic mutant as a control. 18 hours post-transfection cells were lysed and luciferase activity was measured. I determined that all PLPs are capable of cleaving of the pGLO substrate with LKGG motif (Figure 13). Interestingly, none of the PLP2s was as efficient at the cleavage as SARS-CoV PLpro. In addition, expression of HCoV-229E PLP2 and HCoV-OC43 PLP2 led to only small but statistically significant increase in luciferase activity over mock. These data suggest that HCoV-HKU1, HCoV-OC43, HCoV-229E, and FIPV PLPs cleave LXGG motif. Further studies with species specific-substrates are needed to evaluate if there are in fact quantitative differences in catalytic activities of those PLP2s.
Figure 13. Proteolytic activity of HCoV-HKU1, HCoV-OC43, HCoV-229E, and FIPV PLPs. HEK293T cells were transfected with pGLO-RLKGG plasmid and either wild-type (WT) or catalytic mutant (CA) of indicated PLPs. At 18 hours post-transfection cells were lysed and luciferase activity was determined using Dual Luciferase Assay. Experiments were performed in triplicate. Error bars represent standard deviation. The figure shows representative data from two independent experiments.
Next, I determined if HCoV-HKU1, HCoV-OC43, HCoV-229E, and FIPV PLPs have DUB activity by transfecting HEK293T cells with plasmids expressing PLP2s, their corresponding catalytic mutants and Flag-Ub. As a control for DUB assay, I used SARS-CoV PLpro and HCoV-NL62 PLP2 which are well characterized DUBs. Upon western blot analysis I found that, even though there is less than 10% identity between tested PLPs, the DUB activity is a conserved PLpro and PLP2 function (Figure 14). All tested PLP2s showed DUB activity in a dose dependent manner and catalytic activity is required for their DUB activity. Overall my results suggest that PLPs’ multifunctionality is conserved among alpha and betacoronaviruses.

EVALUATING THE ROLE OF PAPAIN-LIKE PROTEASE IN PATHOGENESIS

PART I: MHV-A59 NSP3 UBIQUITIN-LIKE DOMAIN IS REQUIRED FOR PROTEIN STABILITY AND VIRUS PATHOGENESIS

Generating expression construct of MHV PLP2

SARS-CoV and MERS-CoV are human respiratory pathogens that require BSL-3 conditions for research. Other human coronavirus such as HCoV-NL63, HCoV-HKU1, HCoV-OC43 and HCoV-229E replicate poorly in cell culture. To facilitate my research, I used murine model coronavirus, mouse hepatitis virus (MHV) in order to evaluate the
Figure 14. Deubiquitinating activity of HCoV-HKU1, HCoV-OC43, HCoV-229E, and FIPV PLPs. HEK293T cells were transfected with Flag-Ub expression plasmid, and wild-type (WT) or catalytic mutant (CA) PLP2 or PLpro. Cells were lysed 18 hours post-transfection and analyzed by western blotting. (A) HCoV-OC43 PLP2, (B) HCoV-229E PLP2, (C) HCoV-HKU1 PLP2, (D) FIPV PLP2, (E) SARS-CoV PLpro, (F) HCoV-NL63 PLP2. Figure shows representative data from two independent experiments.
pathogenesis of the mutants affecting papain-like protease. First, I had to determine if similar to other coronaviruses, MHV papain-like protease (PLP) is a multifunctional enzyme.

MHV encodes two PLP domains in its genome. PLP1 is required for the cleavage of the polyprotein between nsp1/nsp2 and nsp2/nsp3. PLP2 has more sequence similarity to SARS-CoV and MERS-CoV PLpro as it cleaves polyprotein at the nsp3/nsp4 junction by the recognition of the LXGG motif (Kanjanahaluethai & Baker, 2000). For those reasons I focused my research on PLP2 domain. I designed a PLP2 construct with in frame V5 epitope tag extending from amino acids 1611 to 1970 of the MHV-A59 genome. The construct was codon optimized; splice sites were removed, and synthesized by Genscript. I cloned the construct into a pCAGGS-MCS vector to facilitate the expression of protein in mammalian cells (PLP2-A). Next, I used site-directed mutagenesis to mutate predicted catalytic cysteine residue (C1716) to alanine to generate the catalytic mutant (PLP2-A CA).

**MHV PLP2 is a protease and deubiquitinase**

To determine if MHV PLP2 is a multifunctional enzyme I tested its protease, deubiquitinase and interferon antagonism activities. To determine proteolytic activity of MHV PLP2, I transfected HEK293T cells with PLP2-A and PLP2-A CA in the presence plasmid expressing SARS-CoV nsp2/nsp2-GFP substrate which contains the LXGG sequence recognized by the protease during virus infection. The GFP tag allows for the detection of the substrate and the cleavage product using western blot. I determined that wild-type PLP2 has the ability to cleave the nsp2/nsp3 substrate (Figure 15A). In
Figure 15. Enzymatic activities of MHV PLP2. (A) To determine protease activity HE293T cells were transfected with nsp2/nsp3-GFP plasmid and plasmids expressing wild-type (WT PLP2-A) or catalytic mutant (C1716A) PLP2. Cell lysates were analyzed by western blot 24 hours post-transfection. (B) HEK293T cells were transfected with Flag-Ub expression plasmid, and wild-type (WT PLP2-A) or catalytic mutant (C1716A) PLP2 Cells were lysed 18 hours post-transfection and analyzed by western blot. Figure shows representative data from at least two independent experiments.
addition, catalytic activity of this protein is required for the cleavage as the catalytic mutant failed to cleave to substrate.

Since MHV PLP2 recognizes LXGG, next I tested if it can remove ubiquitin molecules in transfected cells similar to cellular DUBs that recognize the RLRGG motif. I transfected HEK293T cells with plasmids expressing PLP2-A or PLP2-A CA, and the plasmid expressing Flag-Ub. 20 hours post-infection I lysed the cells and determined the levels of ubiquitinated proteins by western blot. I found that MHV PLP2 is a potent deubiquitinase since the levels of ubiquitinated proteins were significantly reduced upon expression of wild-type PLP2-A (Figure 15B). In contrast, the expression of catalytic mutant resulted in no change in ubiquitinated proteins compared to cells transfected with the plasmid expressing Flag-Ub alone, supporting the multifunctionality of MHV PLP2.

MHV PLP2 is an interferon antagonist

To further evaluate MHV PLP2 multifunctionality, I determined if MHV PLP2 can act as an interferon antagonist using luciferase assay. First, I tested if MHV PLP2 can inhibit a broad IFN induction signal generated by infecting the cells with a known IFN inducer Sendai virus (SV). HEK293T cells were transfected with increasing doses of PLP2-A or PLP2-A CA, plasmids expressing IFN-β-luciferase and Renilla luciferase. At 4 hours post-transfection cells were infected with SV, and 6 hours post-infection the cells were lysed and luciferase activity was determined. As predicted, infection with SV led to the induction of IFN-β promoter. The MHV PLP2 was able to inhibit this induction in dose dependent manner and its catalytic activity is required for IFNβ antagonism (Figure
Next, I determined if MHV PLP2 can block IFN induction upon stimulation by overexpression of specific pattern recognition receptors (PRR) such as active form of RIG-I (N-RIG-I) and MDA5. I found that MHV PLP2 can inhibit IFN induction in both cases. MHV PLP2 inhibits RIG-I induced IFN in a dose dependent manner and catalytic activity is required MHV PLP2 IFN antagonism (Figure 16B). PLP2 can also potently inhibit MDA5 induced IFN; however, in this case the catalytic activity of the protein is not required (Figure 16C). Expression of the PLP2-A CA reduced induction of IFN upon MDA5 stimulation but not to the same level as wild-type protein which suggests the existence of catalytic independent mechanism of inhibition of IFN activation upon MDA5 stimulation.

To evaluate at which step in innate immune signaling cascade PLP2 might work as antagonist, I performed luciferase assay using MAVS overexpression as a stimulator. MAVS is an adaptor protein that functions downstream of RIG-I and MDA5, and transmits the signal to activate expression of proinflammatory cytokines and chemokines. I found that MHV PLP2 inhibits IFN induction upon MAVS stimulation in a dose dependent manner and that catalytic activity is not required for antagonism (Figure 16D). These data suggest that MHV PLP2 inhibits IFN activation downstream of MAVS in the signaling cascade.

In addition, to evaluate the IFNβ promoter activation, I tested if MHV PLP2 can inhibit interferon stimulated response element (ISRE) and specific NF-κB activation. To determine if MHV PLP2 blocks ISRE activation, I transfected HEK293T cells with plasmids expressing ISRE luciferase, Renilla luciferase, N-RIG-I, and PLP2-A
Figure 16. Interferon antagonism activity of MHV PLP2. HEK293T cells were transfected with plasmids expressing wild-type (WT) or catalytic mutant PLP2 (CA), plasmids expressing IFNβ-luc, Renilla-luc, and the stimulator is indicated at the top of the figure. At 16 hours post-transfection, cells were lysed and luciferase activity was measured. Experiments were performed in triplicate. Error bars represent standard deviation. The figure shows data from at least two independent experiments.
Figure 17. Interferon antagonism activity of MHV PLP2. HEK293T cells were transfected with plasmids expressing wild-type (WT) or catalytic mutant PLP2 (CA), plasmids expressing ISRE-luc (A), NF-κB-luc (B), and Renilla-luc. (A) Cells were stimulated by overexpression of N-RIG-I. At 16 hours post-transfection, cells were lysed and luciferase activity was measured. (B) 10 hours post-transfection cells were treated with TNFα for 4 hours, lysed and luciferase activity was measured. Experiments were performed in triplicate. Error bars represent standard deviation. The figure shows representative data from at least two independent experiments.
wild-type or CA. When luciferase activity was assessed 16 hours post-transfection, I found that MHV PLP2 can inhibit induction of ISRE, and that catalytic activity of MHV PLP2 is required for this inhibition (Figure 17A). To test if MHV PLP2 can inhibit NF-κB activation, HEK293T cells were transfected with plasmids expressing NF-κB luciferase, Renilla luciferase, and PLP2-A wild-type or CA, treated cells with TNFα to activate the NF-κB pathway, and at 4 hours post-treatment cell lysates were harvested to assess luciferase activity. Wild-type PLP2 reduced induction of NF-κB reporter in a dose-dependent manner and catalytic cysteine residue is required for this activity (Figure 17B).

To determine if MHV PLP2 can inhibit induction of endogenous proinflammatory cytokines and chemokines I used qRT-PCR. First, I investigated the effect of MHV PLP2 on the expression of IFNβ. I transfected HEK293T cells with PLP2-A or PLP2-A CA and N-RIG-I expression plasmid. Using qRT-PCR I determined the levels of IFNβ mRNA in transfected cells. I found that MHV PLP2 can reduce endogenous IFNβ levels in catalytic dependent manner (Figure 18A). Further, I used Human Innate & Adaptive Immune Responses PCR Array (SABiosciences) to determine which, other than IFNβ, genes expression is inhibited in the presence of MHV PLP2 upon RIG-I and MDA5 overexpression. I found that upon N-RIG-I stimulation 9 genes were upregulated over 4 fold, compared to mock transfected cells, including: CCL5, CXCL10, DDX58, IFNβ, IL8, IRF7, MX1, STAT1, and TLR3 (Figure 18B). MHV PLP2 was able to reduce the levels of expression of all those genes. In contrast, overexpression of MDA5 led to upregulation of only 4 genes in array: CCL5, CXCL10, DDX58, and IFNβ (Figure 18C).
Figure 18. Interferon antagonism activity of MHV PLP2. HEK293T cells were transfected with plasmids expressing wild-type PLP2 (WT) and N-RIG-I, or Mda5 as stimulator. At 16 hours post-transfection cells were lysed and total RNA was isolated. (A) qRT-PCR for IFNβ was performed. Data are normalized to RPL13A expression levels. Experiments were performed in triplicate. Error bars represent standard deviation. (B and C) qRT-PCR for human Innate and Adaptive Immune response (SABiosciences) was performed. Data are presented as fold change over non-stimulated cells. The figure shows data from two independent experiments.
Similar to the results with N-RIG-I overexpression, PLP2 expression reduced mRNA levels of all those cytokines and chemokines (Figures 18B and 18C). Taken together my data suggest that MHV PLP2 is an interferon antagonist. Those results and the observation that MHV PLP2 is a multifunctional enzyme with protease and deubiquitinase activities allowed me to use MHV PLP2 as a model for other coronavirus PLPs in further studies.

**PLP2 deletion analysis and mutagenesis**

To determine the minimal domain of MHV PLP2 required for multifunctionality of the enzyme I performed deletion analysis of MHV PLP2-A construct (Figure 19). Using overlapping PCR and subsequent cloning I generated N-terminal and C-terminal 19 amino acid truncations of the PLP2 (termed Δ1-Δ9) keeping catalytic residues intact. All introduced mutations were confirmed by sequencing. The mutants were tested for their ability to cleave a polyprotein substrate using the transcleavage assay, and for their ability to antagonize innate immune response using the luciferase assay.

To test catalytic activity of the mutants I transfected HEK293T cells with plasmids expressing wild-type PLP2 (PLP2-A), PLP2-A CA, or various PLP2 mutants along with a plasmid expressing the SARS-CoV nsp2-3-GFP substrate. The presence of the cleavage product was not detected for the mutants Δ4, Δ5, and Δ6, suggesting that those residues are absolutely required for PLP2 protease activity. Mutants Δ1-Δ3 had reduced protease activity compared to wild-type PLP2. In contrast mutants Δ7-Δ9 showed similar catalytic activity to wild-type proteins suggesting that those residues are
Figure 19. Schematic diagram of MHV PLP2. Diagram of MHV ORFs and the papain-like protease (PLP2) domain within nonstructural protein 3 (nsp3). Expression plasmid pCAGGS-MHV PLP2-A (amino acids 1611-1970) and PLP2-B (amino acids 1525-1911), and the predicted catalytic cysteine residue 1716 are indicated.
dispensable for PLP2 protease activity (Figure 20A). Following, I determined the ability of deletion mutants to antagonize IFNβ induction. I transfected HEK293T cells with increasing doses of PLP2-A or PLP2-A CA, or various PLP2 deletion mutants, and plasmids expressing IFNβ-luciferase, Renilla luciferase, and N-RIG-I as a stimulator. I determined luciferase activity 16 hours post-transfection. Since mutants 47-Δ6 did not show proteolytic activity I did not test those mutants in the luciferase assay. I found that the ability of deletion mutants to antagonize interferon induction correlated well with their proteolytic activity. The Δ7-Δ9 mutants which had intact catalytic activity efficiently inhibited IFN induction upon N-RIG-I stimulation, whereas Δ1-Δ3 mutants have lost the ability to antagonize induction of IFN (Figure 20B).

Because mutant Δ1 have reduced protease activity I wanted to evaluate whether all amino acids from this region were required for the protease activity. To do that I generated N-terminal 4 amino acid truncations of the PLP2-A construct (termed Δ1-A – Δ1-D). I also mutated all charged residues within Δ1 region to alanine to determine if those residues are required for PLP2 activities (D1612A, D1618A, R1623A, and E1629A). To evaluate these deletion mutants I performed the transcleavage assay and luciferase assay as described above. I found that all deletion mutants Δ1-A to Δ1-D had decreased proteolytic activity and lost the ability to block induction of interferon suggesting that 4 amino acids at the N-terminal of PLP2 construct are required for both efficient processing of the substrate as well as PLP2’s ability to act as an IFN antagonist (Figures 21A-C). Interestingly, charged residues within 20 N-terminal amino acids are also not required for PLP2 catalytic activity and IFN antagonism. The alanine mutants
Figure 20. Activities of MHV PLP2 deletion mutants. (A) To determine protease activity HE293T cells were transfected with nsp2/nsp3-GFP plasmid and plasmids expressing wild-type (WT PLP2-A) or catalytic mutant (C1716A), or indicated PLP2 mutants. Cell lysates were analyzed by western blot 24 hours post-transfection (B) HEK293T cells were transfected with plasmids expressing wild-type (WT) or catalytic mutant PLP2 (CA), or indicated PLP2 mutants, plasmids expressing IFNβ-luc, Renilla-luc, and N-RIG-I. At 16 hours post-transfection, cells were lysed and luciferase activity was measured. Experiments were performed in triplicate. Error bars represent standard deviation. The figure shows data from two independent experiments.
Figure 21. Activities of MHV PLP2 mutants. (A) To determine protease activity HE293T cells were transfected with nsp2/nsp3-GFP plasmid and plasmids expressing wild-type (WT PLP2-A) or catalytic mutant (C1716A), or indicated PLP2 mutants. Cell lysates were analyzed by western blot 24 hours post-transfection (B) HEK293T cells were transfected with plasmids expressing wild-type (WT) or catalytic mutant PLP2 (CA), or indicated PLP2 mutants, plasmids expressing IFNβ-luc, Renilla-luc, and N-RIG-I. At 16 hours post-transfection, cells were lysed and luciferase activity was measured. Experiments were performed in triplicate. Error bars represent standard deviation. The figure shows data from two independent experiments.
D1612A, D1618A, R1623A, and E1629A had catalytic activity and were able to block induction of IFN to the same levels as wild-type PLP2. Taken together these data suggest that 4 N-terminal amino acids of the PLP2 are critical for PLP2 proteolytic activity and IFN antagonism (Figures 21A-C).

MHV PLP2 crystal structure revealed that 60 N-terminal amino acids make ubiquitin-like domain (UBL). This fold is conserved between SARS-CoV PLpro and MHV PLP2 (Figure 22). To evaluate if the N-terminal region of PLP2 UBL is truly required for the PLP2 activities, and to determine if the effects that I observe are not due to overall misfolding, since those are 4 N-terminal residues of the protein, I generated a new PLP2 construct termed PLP2-B (Figure 19). I decided to extend the construct at the N-terminus by including residues from MHV-A59 genome, and also to shorten the C-terminus since I saw no change in PLP2 activities for Δ7 construct. The new construct extended from amino acid 1525 to 1911 of MHV-A59 genome with in frame V5 epitope tag, and it was codon optimized, splice sites removed, and synthesized by Genscript. I cloned the ORF into the mammalian expression plasmid pCAGGS-MCS. Next, I generated the catalytic mutant in which the catalytic cysteine C1716 was mutated to alanine (PLP2-B CA).

Next, to determine protease activity of the new PLP2 construct I transfected HEK293T cells with plasmid expressing nsp2/nsp3-GFP, and plasmids expressing either wild-type PLP2-B, catalytic mutant PLP2-B CA (C1716A), or PLP2-A. 24 hours post-transfection cells were lysed and analyzed by western blot. I found that wild-type PLP2-B is efficiently cleaving the substrate with comparable activity to PLP2-A, whereas the
Figure 22. Alignment of SARS-CoV PLpro and MHV PLP2 UBL domains. A) Linear alignment of SARS-CoV, MERS-CoV, and MHV PLP2 UBL domains. V1613 is indicated by arrow B) Alignment of crystal structures of SARS-CoV PLpro UBL (beige) and MHV PLP2 UBL (purple). The alignments were generated by Y. Chen, Purdue University.
catalytic mutant PLP2-B CA lost the ability to cleave nsp2/nsp3-GPF substrate (Figure 23A). The new construct upon expression showed ability to antagonize IFN induction to the same extent as PLP2-A (Figures 23 C-D). Further, I tested the ability if new PLP2-B construct had DUB activity. To validate that I transfected HEK293T cells with plasmids expressing Flag-Ub, and wild-type PLP2 (PLP2-B), PLP2-B CA. Using western blot analysis I determine the levels of ubiquitinated proteins in transfected cells. I found that wild-type PLP2-B is efficient DUB and that catalytic activity of the protein is required for DUB activity (Figure 23B).

With the knowledge that the new PLP2-B construct expresses multifunctional PLP2 I performed deletion analysis and mutagenesis of 4 amino acid (1611-1614, VDVL) span within PLP2 UBL domain. To do that I generated 4 amino acid deletion (VDVL 1611-1614, and termed the construct PLP2-B2), quadruple mutant of VDVL region to SSSS (PLP-B3), and a set of double mutants where 2 amino acids of VDVL region were mutated to serine (termed PLP2-B4 to PLP2-B7) (see Table 8 for the reference). I also made single mutants: V1611S, D1612K, and V1613S. All mutations were confirmed by sequencing.

To determine the effect of the mutations on the PLP2 multifunctionality, I tested the mutants using transcleavge assay to determine proteolytic activity, luciferase assay to determine the mutants ability to act as IFN antagonists, and DUB assay to determine their DUB activity. The experiments were performed as described above. I found that all mutants had reduced but detectable proteolytic activity compared to wild-type PLP2 (Figures 24A-B). In addition, all of the mutants had decreased DUB activity and lost the
Figure 23. Activities of MHV PLP2-B construct. (A and B) To determine protease and DUB activity HEK293T cells were transfected with nsp2/nsp3-GFP plasmid (A) or Flag-Ub expression plasmid (B) and plasmids expressing wild-type (WT PLP2-A or PLP2-B), or catalytic mutant (CA), or indicated PLP2-B mutants. Cell lysates were analyzed by western blot. (C, D) HEK293T cells were transfected with plasmids expressing wild-type (WT) or catalytic mutant PLpro (CA), or PLP2-B mutants, plasmids expressing IFNβ-luc, Renilla-luc, and N-RIG-I (C), or MDA5 (D). At 16 hours post-transfection, cells were lysed and luciferase activity was measured. Experiments were performed in triplicate. Error bars represent standard deviation. The figure shows data from two independent experiments.
Figure 24. Activities of MHV PLP2-B UBL mutants. (A) To determine protease activity HE293T cells were transfected with nsp2/nsp3-GFP plasmid and plasmids expressing wild-type (WT PLP2-A or PLP2-B), or catalytic mutant (CA), or indicated PLP2-B mutants. Cell lysates were analyzed by western blot 24 hours post-transfection. (B) HEK293T cells were transfected with Flag-Ub expression plasmid, and wild-type (WT PLP2-B) or catalytic mutant (C1716A) PLP2, or indicated PLP2-B mutants. Cells were lysed 18 hours post-transfection and analyzed by western blot. The figure shows representative data from at least two independent experiments.
Figure 25. Interferon antagonism activity of MHV PLP2-B UBL mutants. HEK293T cells were transfected with plasmids expressing wild type (WT) or catalytic mutant PLP2 (CA), or indicated PLP2-B mutants, plasmids expressing IFNβ-luc, Renilla-luc, and N-RIG-I. At 16 hours post-transfection, cells were lysed and luciferase activity was measured. Experiments were performed in triplicate. Error bars represent standard deviation. The figure shows representative data from at least two independent experiments.
Table 8. Enzymatic activities of MHV PLP2 mutants.

<table>
<thead>
<tr>
<th>Name</th>
<th>Genome aa</th>
<th>Mutation</th>
<th>Protease Activity</th>
<th>IFN antagonism (LUC Assay)</th>
<th>DUB Activity</th>
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</tr>
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ability to inhibit induction of IFN (Figures 24C-D, and 25A-B). Overall, these results suggest that VDVL region of PLP2 UBL domain is required for PLP2 activity. Interestingly, this region is homologous among coronaviruses including SARS-CoV, and specifically V1613 residue is highly conserved (Figure 22).

**V1613S mutant virus is temperature sensitive**

To determine the role of PLP2 UBL domain for virus replication and virus pathogenesis I introduced 1611-1614 deletion or V1613S mutation into the infectious clone MHV-A59 (icMHV-A59) using reverse genetics described by Yount and co-workers (Yount et al., 2002). First, using site-directed mutagenesis I engineered deletion or point mutation within B fragment of icMHV-A59 genome. I sequenced the whole B plasmid to make sure the construct contained only the desired deletion or point mutation. Next, I digested plasmids that correspond to whole MHV-A59 genome with restriction enzymes, and ligated to generate icMHV-A59 mutant genomes. Then, I performed *in vitro* RNA transcription, electroporated RNA into BHK-R cells and applied the on the monolayer of DBT cells. I monitored the cells for syncytia formation. I was able to recover both viruses; however, syncytia in V1613S mutant (AM2) appeared within 24 hours post-electroporation (Figure 27A), whereas, for the 1611-1614 deletion mutant (AM1), the syncytia appeared upon 5 days post-electroporation (Figure 26A). In addition, AM1 virus generated very small plaques, it grew only to the titer of 1X10^3 (upon several passages), and replicated poorly at 37°C (Figure 26B-C). Thus, in my further studies I focused on V1613S mutant virus. I observed that when wild-type or AM2 virus is used in
Figure 26. Generation of AM1 (VDVL/SSSS) virus. A) Syncytia present on BKH-R and DBT cells 5 days upon RNA electroporation. B) Plaques assay results. DBT cells were infected with AM1 or icMHV-A59. Cells were fixed and stained 48 hours post-infection. C) Growth kinetics of AM1 in comparison with wild-type virus. DBT cells were infected with MOI 0.1 and virus titer was determined at the indicated time points by plaque assay. The figure shows representative data from at least two independent experiments.
Figure 27. Plaque Assay of DBT cells infected with icMHV-A59 or AM2 virus. A) Syncytia present on BKH-R and DBT cells 5 days upon RNA electroporation. (B-D) DBT cells were fixed and stained 48 hours post-infection. Figure shows plaque assay of the supernatant performed at 37°C (B), 39.5°C (C), and 40.5°C (D). *p<0.05, **p<0.001. Figure shows representative data from at least two independent experiments.
plaque assay at 37°C, AM2 generates slightly smaller plaques that icMHV-A59 (Figure 27B). In contrast, when the plaques assay was performed at 39.5°C, AM2 virus generated much smaller plaques (Figure 27C). Interestingly, when the plaque assay was performed at 40.5°C the AM2 virus was not able to produce any plaques suggesting the replication defect at higher temperature (Figure 27D).

To determine the replication kinetics of the AM2, I infected DBT cells with wild-type isogenic icMHV-A59 or AM2 virus with the MOI 0.1. The cells were incubated at 37°C and the supernatant was harvested at the indicated time points. The virus titer in the supernatant was determined using plaque assay on DBT cells. I determined that AM2 replicated with similar kinetics, and to similar titers as icMHV-A59 (Figure 28A). Further, I determined if AM2 is impaired when grew at higher temperature. To do this, I infected DBT cells with MOI 0.1 with icMHV-A59 or AM2 at 37°C. At 6 hours post-infection, cells were moved to 39.5°C. The virus titer was determined at indicated time points by plaque assay on DBT cells at 37°C. I found that replication kinetics is delayed for AM2 as compared to icMHV-A59 (Figure 28B). At 14 hours post infection AM2 has a significantly lower titer (about half a log) than wild-type virus, suggesting that AM2 has a replication defect at higher temperature.

Next, I evaluated if incubation time at 37°C was important for the ability of the virus to replicate at higher temperature. To do that, I performed a temperature shift experiment by infecting DBT cells with AM2 or icMHV-A59 and incubating the cells at 37°C for 2, 4, or 6 hours. After the indicated time the cells were moved to 40.5°C. The virus titer was determined using plaque assay at 14 hours post-infection. I found that the
Figure 28. Replication kinetics of AM2 virus. DBT cells were infected with AM2 mutant or icMHV-A59 at the MOI 0.1. At indicated time points virus titers were determined by plaque assay. B) DBT cells were infected at the MOI 0.1 at 37°C with AM2 or icMHV-A59. 6 hours post-infection cells were moved to 39.5°C Virus titer was measured at the indicated time points. C) DBT cells were infected at the MOI 0.1 at 37°C with AM2 or icMHV-A59. At the indicated time points cells were moved to 40.5°C Virus titer was measured at 14 hours post-infection. The error bars represent standard deviation of the mean of two independent plaque assays. *p<0.05.
shorter incubation time at 37°C, the bigger the difference between the titer of AM2 compared to icMHV-A59. When the cells were incubated at 37°C for 6 hours the difference between the titers was about half a log, whereas when the cells were incubated at 37°C for only two hours the AM2 has a two log difference in final titer compared to icMHV-A59 (Figure 28C). Taken together these data suggest that AM2 virus encoding V1613S mutation has a temperature sensitive phenotype.

**V1613S mutation impairs protein activity and stability**

The observation that the time of incubation at 37°C had a dramatic impact on the replication of the virus and in order to understand the temperature sensitive phenotype of the AM2 mutant, our collaborators at Purdue University purified wild-type and V1613S mutant PLP2 (Figure 29A). Next, they incubated purified proteins at 25°C or 30°C. Each enzyme activity was determined at 25°C at indicated time points using RLRGG-AMC as a substrate. When both wild-type and V1613S mutant PLP2s were incubated at 25°C both enzymes had similar activity over the time course at the level of 100% (Figure 28B). In contrast, when both proteins were incubated at higher temperature (30°C) the activity of V1613S mutant protein decrease over time whereas wild-type protein maintained 100% activity over time (Figure 29B).

To determine stability of the mutant protein our collaborators performed circular dichroism (CD) analysis of wild-type and V1613S mutant proteins. They monitored CD at 220nm while increasing the temperature at step intervals. The determined melting temperature was 6.8°C lower for the mutant protein than for the wild-type protein (Figure
Figure 29. Stability of V1613S purified protein. A) Expression and purification of V1613S protein separated by SDS-PAGE. B) Temperature-dependent inactivation of the V1613S mutant. The activity of WT PLP2 and the V1613S were measured after incubation at 25°C and 30°C for different time periods, and then normalized to the activity at 0 min (Rate_t/Rate_0: rate at time t over initial rate). C) Thermal stability of WT and V1613S mutant PLP2. CD at 220nm was monitored over a range of temperature. Three independent experiments were performed for both WT PLP2 (grey) and V1613S mutant (black). Data generated by Y. Chen, Purdue University.
29C), suggesting that the mutant protein is less stable and unfolds at the lower temperature than the wild-type PLP2. Taken together these data suggest that V1613S mutation lowers the overall protein stability which leads to a decrease enzymatic activity at higher temperature (30°C).

**AM2 has reduced pathogenesis in infected mice**

To determine the effect of the UBL mutation on virus pathogenesis, I infected 4 week old male C57B/L6 mice with 600 pfu of wild-type and AM2 virus by intracranial injections. I monitored weight loss and the mice were humanly sacrificed when they lost 25% of initial body weight. I found that mice infected with wild-type virus succumb to infection by day 7. In contrast, the mice infected with AM2 survived the infection (Figure 30A). These data suggest that AM2 has reduced pathogenesis compared to wild-type virus.

**AM2 immunized mice are protected from challenge with wild-type virus**

To determine if primary infection with AM2 virus can protect mice from challenge with wild-type virus I infected AM2 and age-matched naïve control C57B/L6 mice with 6000 PFU of icMHV-A59 9 weeks post-primary infection. I monitored body weight lost over the course of infection and I found that naïve mice lost a significant percent of initial body weight starting at day 3 post-infection (Figure 30B). In contrast, AM2 immunized mice did not lose any weight and did not have any symptoms of disease upon challenge (Figure 30B). These data indicate that immunization with AM2 mutant
Figure 30. AM2 is attenuated in mice and generates protective immune response. A) C57BL/6 mice were infected with 600 pfu icMHV-A59 or AM2 intracranially and monitored for survival (N=7 for each group). Survival was monitored over the time. B) C57BL/6 mice immunized with AM2 mutant and naïve age-matched controls were challenged with 6000 pfu icMHV intracranially 9 weeks post-primary infection. The mice were monitored for body weight loss. Error bars represent SEM.
can protect the mice from wild-type infection suggesting that AM2 is a possible vaccine candidate.

PART II: SEPARATING MHV PAPAIN-LIKE PROTEASE PROTEASE AND DEUBIQUITINASE ACTIVITIES

MHV PLP2 crystal structure reveals ubiquitin-specific protein fold of the enzyme

To understand the role of PLP2 DUB activity in virus pathogenesis and to facilitate the development of the vaccines toward coronaviruses it is necessary to separate protease and DUB activity of PLP. This information is a key to generate DUB deficient viruses that are still able to replicate and process viral polyprotein during virus replication. To enable this process a crystal structure of the enzyme is required to visualize interaction sites between the enzyme and a ubiquitin or polyubiquitin chain. Our collaborators at Purdue University tried to crystalize MHV PLP2 using the PLP2-A protein. It was not until I generated and tested the PLP2-B construct that they were able to obtain crystals of MHV PLP2 which allowed for the generation of the crystal structure of the enzyme. The crystal structure revealed that similar to SARS-CoV PLpro, and despite the low amino acid sequence identity (28%), MHV PLP2 belongs to the ubiquitin specific protein (USP) family of deubiquitinating enzymes. The predicted catalytic triad consisting of C1716, H1873, and D1887 was confirmed. Additionally, the crystal structure showed that upstream of PLP2 there are the ubiquitin-like domain (UBL), and
Figure 31. X-ray Crystal structure of MHV PLP2. A ribbon representation of the overall structure of MHV PLP2. The catalytic triad and VDVL residues in the UBL domain are represented in balls. UBL - ubiquitin-like domain, SUD-C-like – SARS unique domain C. Structure was generated by Y. Chen, Purdue University (Chen et al., in preparation).
SARS unique domain (SUD). The presence of SUD in PLP2-B construct was likely the key factor that enabled protein crystallization (Figure 31) (Chen et al., in preparation).

**Separating MHV PLP2 protease and deubiquitinase (DUB) activities**

Obtaining the crystal structure of MHV PLP2 was an important step in order to separate protease and DUB activities of MHV PLP2. To facilitate the mutagenesis of MHV PLP2 to separate protease and DUB activity the model of MHV PLP2 with ubiquitin was generated (Figure 32). The model was prepared using a known co-crystal structure of SARS-CoV PLpro with ubiquitin aldehyde (Ratia et al., in press, Chou et al., 2014). The model of MHV PLP2 and ubiquitin identified residues on PLP2 that likely interact with ubiquitin. Using site-directed mutagenesis and the Gibson Assembly technique I generated 5 alanine mutants of PLP2: R253, R257, E279A, F290, and Y302. All mutations were confirmed by sequencing. First, I tested if the generated mutants had protease activity. To do this I transfected HEK293T cells with plasmids expressing wild-type PLP2 (PLP2-B), PLP2-B CA, or various PLP2 mutants along with a plasmid DNA expressing the SARS-CoV nsp2-3-GFP substrate as described earlier. I detected evidence of cleavage of the nsp2-3-GFP substrate in the presence of the catalytically active form of MHV PLP2, but not in the presence of the catalytic mutant. The expression of PLP2 mutants led to cleavage of the substrate at various degrees. R257A mutant cleaves the substrate to the similar levels as wild-type PLP2; R253A, E279A, and F290A mutants have reduced protease activity; whereas Y302A mutant is not able to cleave the substrate similar to catalytic mutant of the PLP2 (Figure 33A). All mutant proteins, except R257A,
Figure 32. Model of ubiquitin-PLP2 interaction. Modeled MHV-PLP2 (beige) interaction with ubiquitin (green), residues of PLP2 that interact with ubiquitin are shown in sticks. Model generated by Y. Chen, Purdue University.
Figure 33. Activities of MHV PLP2 mutants. (A) To determine protease activity HE293T cells were transfected with nsp2/nsp3-GFP plasmid and plasmids expressing wild-type (WT PLP2-B), catalytic mutant (CA), or indicated PLP2-B mutants. Cell lysates were analyzed by western blot 24 hours post-transfection. (B) HEK293T cells were transfected with Flag-Ub expression plasmid, and wild-type (WT PLP2-B) or catalytic mutant (CA), or indicated PLP2-B mutants. Cells were lysed 18 hours post-transfection and analyzed by western blot. (C) HEK293T cells were transfected with plasmids expressing wild-type (WT) or catalytic mutant (CA), or indicated PLP2-B mutants, plasmids expressing IFNβ-luc, Renilla-luc, and N-RIG-I. At 16 hours post-transfection, cells were lysed and luciferase activity was measured. Experiments were performed in triplicate. Error bars represent standard deviation. The figure shows representative data from at least two independent experiments.
were expressed to the same levels as wild-type PLP2 as seen on the western blot. Even though I was not able to detect the expression of R257A on a western blot I think the protein was expressed because I could observe its catalytic activity. Detailed sequencing analysis revealed a point mutation in the sequence of V5 epitope tag which explains the inability to detect this protein on a western blot using mouse anti V5 antibody. Importantly, the sequence of the PLP2 ORF was intact and included the desired R257A mutation.

To further characterize the mutants I determined their DUB activity by transfecting HEK293T cells with plasmid expressing Flag-Ub, PLP2-B, PLP2-B CA, or various PLP2 mutants. I detected potent DUB activity of wild-type PLP2 but not catalytic mutant (as described in the previous sections). The E279A mutant had similar DUB activity to wild-type PLP2. R253A, R257A, and Y302A mutants had intermediate phenotype between DUB activity of wild-type PLP2 and complete lack of DUB activity of catalytic mutant. Interestingly, the F290A mutant lost the ability to deubiquitinate cellular proteins to the level of catalytic mutant (Figure 33B). To test if DUB assay data correlate with the ability of mutant PLP2s to work as interferon antagonists, I performed luciferase assay. I transfected HEK293T cells with increasing doses of PLP2-B or PLP2-B CA, plasmids expressing IFN-β-luciferase, Renilla luciferase, and N-RIG-I as a stimulator. I found that the R253A and R257A mutants can potently inhibit induction of interferon. The E279A mutant slightly reduced interferon induction upon N-RIG-I stimulation. Interestingly, the Y302A and F290A mutants lost the ability to inhibit induction of interferon (Figure 33C). The Y302A did not exhibit protease activity and for
this reason was not a good candidate for introduction of this mutation into the virus. However, the F290A mutant has protease activity (although slightly reduced compared to wild-type PLP2), lacks DUB activity and interferon antagonism activity making it a good candidate to test the role of DUB activity on virus pathogenesis.

To evaluate the effect of the F290A mutation in the context of the virus I used reverse genetics technique to generate mutant virus. I introduced the F290A mutation using site-directed mutagenesis and Gibson Assembly assay into B fragment of icMHV-A59 genome. Upon restriction digest of all fragments I ligated them to generate a whole genome. This ligation product was purified and used for an \textit{in vitro} RNA transcription reaction. Next, I electroporated RNA into BHK-R cells and applied them on a monolayer of DBT cells and incubated the cells at 37\textdegree C. Unfortunately, I was not able to recover the mutant virus. To make sure there were no mistakes in the procedure I generated wild-type icMHV-A59 using the same fragments (except that I used wild-type B fragment) and procedure. The protocol was effective and I could recover the wild-type virus. To test if the reason why I did not generate the F290A mutant was the fact that the virus is temperature sensitive, I repeated the procedure but this time upon electroporation I incubated the cells at 30\textdegree C. Unfortunately, this time I did not also recover the virus. These data suggest that the F290A mutation prevents virus replication. This might be due to the defect in proteolytic activity of the F290A that I observed in transfected cells. In addition, our collaborators at Purdue University purified the F290A protein and found that DUB activity of the F290A mutant with ubiquitin as substrate is much lower than for wild-type protein, and the F290A mutant does not have activity with peptide substrate
suggesting that the F290A mutant virus might not be able to process the polyprotein thus explaining why I could not recover this mutant virus.

The results that I gathered facilitated refinement of the MHV PLP2 - ubiquitin model and allowed me to predict new sites of hydrophobic interaction between MHV PLP2 and ubiquitin (Figure 34). Using site-directed mutagenesis and the Gibson Assembly technique I generated the following mutants: I249R, F270A, R281E, Y302F, I304A, and V313A. In addition, based on previous analysis I generated R253A/R257A double mutant, because catalytic activity of single mutants was comparable to wild-type PLP2, and their DUB activity was slightly reduced. I expected that by combining the single mutants I would generate a DUB deficient mutant that maintains good protease activity. The presence of all mutations was confirmed by sequencing.

To test catalytic activity of the newly generated mutants HEK293T cells were transfected with plasmids expressing wild-type PLP2 (PLP2-B), PLP2-B CA, or various PLP2 mutants along with a plasmid DNA expressing the SARS-CoV nsp2-3-GFP substrate. The presence of the cleavage product was detected by western blot. The I249R cleaved the substrate more efficiently that wild-type PLP2. F270A, I304A, and the R253A/R257A double mutant had catalytic activity similar to wild-type PLP2. Two mutants V313A and Y302F had reduced catalytic activity. Finally, in the presence of R281E mutant the band of the cleavage product was almost non-detectable (Figure 35A).

Although described above transcleavage assay in transfected cells gave me qualitative understanding of PLP2 mutants’ catalytic ability I wanted to obtain quantitative results, especially because previously found F290A mutant had catalytic
Figure 34. Model of ubiquitin-PLP2 interaction. Top modeled MHV-PLP2 interaction with ubiquitin (surface representation, and residues of PLP2 that interact with ubiquitin are shown in sticks). Bottom representation of SARS-CoV PLpro with ubiquitin (surface representation, and residues of PLpro that interact with ubiquitin are shown in sticks). The model was generated by Dr. S. Savinov, Purdue University.
Figure 35. Proteolytic activity of MHV PLP2 mutants. (A) HE293T cells were transfected with nsp2/nsp3-GFP plasmid and plasmids expressing wild-type (WT PLP2-B), or catalytic mutant (CA), or indicated PLP2 mutants. Cell lysates were analyzed by western blot 24 hours post-transfection. (B) HEK293T cells were transfected with pGLO-RLKGG plasmid and either wild-type (WT) or catalytic mutant (CA) of indicated PLP2 mutants. At 18 hours post-transfection media was change and cell permeable substrate was added. Luciferase activity was measured at indicated time points. Experiments were performed in triplicate. The figure shows representative data from two independent experiments.
activity in transcleavage assay but failed to generate mutant virus. For this reason I decided to test the mutants using recently described by our laboratory live-cell pGLO assay (Kilianski, Mielech, Deng, & Baker, 2013). HEK293T cells were transfected with plasmid expressing substrate (pGLO-RLKGG) recognized by the PLP2, wild-type PLP2 (PLP2-B), PLP2-B CA, or various PLP2 mutants. 20 hours post-transfection cell permeable luciferase substrate was added to the cells, and luciferase activity was measured every hour for 5 hours. I found that the kinetics of substrate cleaved by wild-type PLP2 was similar to the cleavage kinetics of F270A and R253A/R257A double mutants. The R281E mutant, even though I observed slight catalytic activity using transceleavge assay, did not cleave pGLO substrate, similar to catalytic mutant PLP2. Y302F, I304A, and V313A mutants had intermediate ability to cleave the substrate (Figure 35B). Interestingly, F290A mutant which was used here as a control, did not cleave pGLO substrate supporting previous data and explaining the inability to recover the mutant virus with this mutation.

To evaluate DUB activity of the new mutants I transfected HEK293T cells with plasmids expressing Flag-Ub, and wild-type PLP2 (PLP2-B), PLP2-B CA, or various PLP2 mutants. Using western blot analysis I determined the levels of ubiquitinated proteins in transfected cells. I found that the I249R mutant, which had increased protease activity, had also increased DUB activity. In contrast, the R281E mutant that did not have catalytic ability did not also have DUB activity and its phenotype resembled catalytic mutant of PLP2. Additionally, the F290A, Y302F, I304A, V313A, and R253A/R275A
double mutant had slightly reduced DUB activity compared to wild-type PLP2 (Figure 36A).

To determine the effect of the PLP2 mutations on PLP2 deISGylating activity, HEK293T cells were transfected with c-myc-ISG15 plasmid, ISG15 conjugation machinery, and plasmids expressing PLP2-B, PLP2-B CA, and various PLP2 mutants. 20 hours post-transfection cell lysates were evaluated for the presence of ISGylated proteins. Wild-type PLP2 can deconjugate ISG15 from multiple cellular substrates. In contrast, PLP2 catalytic mutant did not deconjugate ISG15. Interestingly the PLP2 mutations seemed to have a stronger effect on PLP2 deISGylating than DUB activity. The I249R mutant had more robust deISGylating activity than wild-type PLP2. In contrast, all other mutants tested (F270A, R281E, Y302F, I304A, V313A, and R253A/R257A) showed decreased deISGylating activity comparable to PLP2 catalytic mutant (Figure 36B).

Finally, I tested the interferon antagonism activity of the PLP2 mutants. I transfected HEK293T cells with increasing doses of PLP2-B or PLP2-B CA, or, PLP2 mutants, plasmids expressing IFNβ-luciferase, Renilla luciferase, and N-RIG-I as a stimulator. 16 hours post-transfection the cells were lysed and luciferase activity was measured. I determined that three mutants had reduced ability to antagonize induction of IFN upon RIG-I stimulation: I249R, F270A, Y302F. In addition, the remaining mutants: R253A/R257A, R281E, I304A, and V313A have lost the ability to antagonize IFN induction (Figure 37).
Figure 36. Activities of MHV PLP2 mutants. HEK293T cells were transfected with Flag-Ub expression plasmid (A) or Myc-ISG15 and ISGylating machinery plasmids (B), and wild-type (WT PLP2-B) or catalytic mutant (C1716A) PLP2, or indicated PLP2 mutants. Cells were lysed 18 hours post-transfection and analyzed by western blot. The figure shows representative data from at least two independent experiments.
Figure 37. Interferon antagonism activity of MHV PLP2 mutants. HEK293T cells were transfected with plasmids expressing wild-type (WT) or catalytic mutant (CA), or indicated PLP2 mutants, plasmids expressing IFNβ-luc, Renilla-luc, and N-RIG-I. At 16 hours post-transfection, cells were lysed and luciferase activity was measured. Experiments were performed in triplicate. Error bars represent standard deviation. The figure shows representative data from at least two independent experiments.
Taken together these data suggest that thus far no mutant has allowed me to separate the protease and DUB activity of MHV PLP2 completely. Three mutants: F270A, I304A, and R253A/R257A have reduced DUB activity and maintain protease activity suggesting that those might have stronger effect on DUB activity when combined together in triple or quadruple mutants.

In addition to testing mutants that potentially disrupted the hydrophobic interaction between ubiquitin and PLP2, I generated a PLP2 mutant that targeted the predicted interaction of PLP2 with isoleucine (I44) patch on the ubiquitin molecule. The I44 patch is known to be one of the main interaction sites between ubiquitin and its interaction partners. In addition, van Kastern and co-workers showed that interaction with this path is critical for the interaction between ubiquitin and PLP2 from Equine Arteritis Virus (EAV). The overall fold of EAV PLP2 is much different (EAV PLP2 belongs to ovarian tumor domain (OTU) family of deubiquitinating enzymes) from MHV PLP2 thus these data obtained from EAV studies cannot be extrapolated to coronavirus PLP2.

However, I used the knowledge that the I44 patch is important for interactions with ubiquitin partners and model of MHV PLP2 to predict the residues that likely interact with the I44 patch residues: I44, V70, and L8. I predicted that I44 interacts with F290, V70 interacts with I249, and L8 interacts with T328 (Figure 38).

To test this hypothesis I generated MHV PLP2 triple mutant: I249V/F290W/T328A (termed IFT) using site-directed mutagenesis and Gibson Assembly technique. The triple mutation was confirmed by sequencing. Using the transcelavage assay I determined that I249V/F290W/T328A have protease activity
Figure 38. Predicted interaction between MHV PLP2 and ubiquitin Ile44 patch. The modeling of interaction between Ile 44 patch on ubiquitin (in green) with MHV PLP2 (in beige). Model was generated by Y. Chen, Purdue University.
Figure 39. Proteolytic activity of MHV PLP2 triple mutant (I249V/F270W/T328A).

(A) To determine protease activity HE293T cells were transfected with nsp2/nsp3-GFP plasmid and plasmids expressing wild-type (WT), or catalytic mutant (CA), or PLP2 triple mutant (IFT). Cell lysates were analyzed by western blot 24 hours post-transfection. (B) HEK293T cells were transfected with pGLO-RLKGG plasmid and either wild-type (WT) or catalytic mutant (CA) or PLP2 triple mutant (IFT). At 18 hours post-transfection media was change and cell permeable substrate was added. Luciferase activity was measured at indicated time Experiments were performed in triplicate.
comparable to wild-type PLP2 (Figure 39A). To determine how efficient is the I249V/F290W/T328A mutant in the cleavage of the pGLO-RLKGG substrate I transfected HEK293T cells with plasmid expressing the substrate, PLP2-B, PLP2-B CA, and I249V/F290W/T328A mutant. As additional controls, I transfected the cells with F290A and V1613S mutant expression plasmids. I found that the I249V/F290W/T328A mutant is as efficient at cleaving the substrate as wild-type PLP2. In contrast, F290A and PLP2 catalytic mutant were not able to cleave the substrate (Figure 39B). These data suggest that the I249V/F290W/T328A mutant is an efficient protease. To validate if this mutant has a deficiency in DUB activity, I transfected HEK293T cells with plasmids expressing Flag-Ub, and wild-type PLP2 (PLP2-B), PLP2-B CA, or various PLP2 mutants. Using western blot analysis I determined the levels of ubiquitinated proteins in transfected cells. I found that I249V/F290W/T328A mutant was as efficient DUB as wild-type PLP2 (Figure 40). These data suggest that this triple combination of mutations is not sufficient to separate PLP2 protease and DUB activities. However, further testing of various mutants that likely disrupt interaction between PLP2 and the I44 patch on ubiquitin is necessary to determine if the I44 patch is a site required for PLP2 – ubiquitin interaction. Table 9 summarizes data generated for all the mutants.
Figure 40. Deubiquitinating activity of MHV PLP2 triple mutant. HEK293T cells were transfected with Flag-Ub expression plasmid and wild-type (WT) or catalytic mutant (CA), or indicated PLP2 mutants. Cells were lysed 18 hours post-transfection and analyzed by western blot.
Table 9. Enzymatic activity profile of MHV PLP2 mutants. The table summarizes the results obtained for generated mutants.

<table>
<thead>
<tr>
<th>Protein</th>
<th>SARS-PLpro corresponding site</th>
<th>Protease Activity</th>
<th>DUB Activity</th>
<th>IFN Antagonism</th>
<th>DeSGylating Activity</th>
<th>Reverse Genetics Virus</th>
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<tr>
<td>WT</td>
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<td>+</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>Lethal</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>Viable</td>
</tr>
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<td>+/-</td>
<td>+</td>
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<td>N/A</td>
</tr>
<tr>
<td>R257A</td>
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<td>F290A</td>
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</tr>
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<tr>
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<td>-</td>
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<tr>
<td>I249V/F290W/T328A</td>
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VALIDATING SMALL MOLECULE INHIBITORS OF SARS-CoV REPLICATION

The recent emergence of MERS-CoV highlighted the importance of the development of antiviral drugs that can block coronavirus replication. Thus far, many efforts have been made to generate inhibitors of SARS-CoV replication. Papain-like proteases are attractive targets for the design of anti-coronavirus drugs because PLPs activity is required for virus replication. Previously described high-throughput screens identified candidate compounds that block SARS-CoV replication (Ghosh et al., 2009, 2010; Ratia et al., 2008). However, because of quite high EC_{50} values for those drugs, further optimization of those compounds was needed to facilitate the introduction of the drugs into clinical settings. During my research I evaluated the ability of second generation compounds to inhibit SARS-CoV replication. My results indicate that optimized compounds have lower EC_{50} values than previously tested SARS-CoV PLpro inhibitors (Table 7). The best compound in terms of ability to inhibit SARS-CoV replication is compound CCG 203888 with EC_{50} of 5.27µM. This compound, as determined by Y. Baez-Santos (Purdue University) is the most effective in inhibiting the activity of purified enzyme (IC_{50}=0.2µM). However, the analysis of stability of this drug
during metabolism showed that CCG 203888 stability is reduced compared to other compounds such as CCG 206552 and CCG 206553 (Báez-Santos et al., 2014). Interestingly, those two compounds also have a potent ability to inhibit SARS-CoV replication with EC$_{50}$ values of 8.78µM and 9.07µM respectively. Thus, CCG 206552 and CCG 206553 are now the most promising candidates and should be further optimized. In addition, co-crystal structures of SARS-CoV PLpro with compounds CCG 203888 and CCG 203885 have been resolved which allows for the further optimization of the inhibitors to design even more potent drugs (Báez-Santos et al., 2014).

Since MERS-CoV emerged into the human population many previously described compounds that have the ability to inhibit SARS-CoV PLpro, have been tested for their ability to inhibit MERS-CoV PLpro. Unfortunately, they were not effective against MERS-CoV PLpro. Kilianski and co-workers showed that potent SARS-CoV PLpro inhibitor, benzodioxolane derivative (compound 15g described in Gosh et al., 2010) is not capable of inhibiting MERS-PLpro activity in transfected cells. The authors hypothesized that the lack of inhibition was due to differences in structure of MERS-CoV and SARS-CoV PLpros at the drug binding site (Kilianski, Mielech, Deng, & Baker, 2013). This hypothesis needs to be further evaluated when the crystal structure of MERS-CoV PLpro is available. In addition, the ability of other SARS-CoV PLpro inhibitors to block MERS-CoV activity remains to be determined. Thus far, no broad-spectrum inhibitor for coronavirus PLPs has been described. However, it is critical to work and optimize not only specific SARS-CoV PLpro and MERS-PLpro inhibitors but
also broad-spectrum inhibitors that could be available in case of the emergence of a new coronavirus with pandemic potential.

**INVESTIGATING MULTIFUNCTIONAL VIRAL PROTEASES**

Viruses must “do more with less” because of the compact nature of their genomes. One example of this is the multifunctional papain-like protease (PLP) domain encoded in all members of the order *Nidovirales*. *Nidoviruses*, including those in the coronavirus and arterivirus families, encode one or more PLP domain. These PLPs are critical for proteolytic processing of the viral replicase polyprotein. In addition to protease activity, many of these PLPs have also been shown to act as viral DUBs, able to deconjugate ubiquitin and ISG15 from cellular substrates. Coronavirus DUB activity was first proposed by molecular modeling of the SARS-CoV PLpro domain which predicted that the protease would be multifunctional (Sulea et al., 2005). Indeed, analysis of the DUB activity of purified coronavirus PLPs and the X-ray crystal structure of SARS-CoV PLpro fully support the initial prediction of viral DUB activity (Barretto et al., 2005; Chen et al., 2007; Lindner et al., 2005; Ratia et al., 2006, 2008). Analysis of PLPs from coronaviruses and arteriviruses have revealed conserved DUB activity; although the enzymes in the coronavirus family fall into the Ubiquitin Specific Protease (USP) family whereas the arterivirus PLPs are in the Ovarian Tumor (OTU) domain family of enzymes (Figure 3). The identification of a newly emerged coronavirus, Middle East Respiratory Syndrome CoV (MERS-CoV), provided an opportunity to evaluate PLpro enzymatic
activity and develop new hypotheses about how this protease/DUB may contribute to viral pathogenesis.

SARS-CoV PLpro is a deISGylating and deubiquitinating enzyme (Lindner et al., 2005, 2007; Ratia et al., 2006). HCoV-NL63 encodes two papain-like proteases PLP1 and PLP2 in the genome. Only PLP2, which has 22% homology to SARS-CoV PLpro, is a multifunctional enzyme with deISGylating and DUB activities (Clementz et al., 2010). The modeling of the MERS-CoV PLpro domain onto the structure of SARS-CoV led to the prediction of viral DUB/deISGylating activity (Figure 8). Although the enzyme is only ~30% identical to SARS-CoV PLpro at the amino acid level my data suggest that multifunctionality of MERS-CoV PLpro and other coronavirus PLPs is conserved. First, I showed that predicted PLP domains from various alpha and betacoronaviruses can be expressed in mammalian cells and that they are catalytically active. I showed that not only MERS-CoV PLpro, but also HCoV-229E, HCoV-OC43, HCoV-HKU1, MHV and FIPV can recognize LXGG motif and cleave a polyprotein substrate (Figures 9A, 13, and 15A). The differences in the efficiency of substrate cleavage between those PLPs are noticeable. However, the substrate that I used in the assays is a native substrate recognized by SARS-CoV during virus replication. For many PLPs the cleavage sites have not been determined previously, only predicted. It needs to be further verified if there are true differences in the cleavage efficiency and whatever they contribute to the pathogenesis associated with a particular virus.

Further studies showed that DUB activity of PLPs is also a conserved feature. All tested alpha and betacoronavirus PLPs showed an ability to cleave polyubiquitin
chains in transfected cells in a catalytic dependent manner (Figures 10B, 14, and 15B). The degree of PLPs’ DUB activity varies; however, because western blotting is not a quantitative assay I cannot directly compare PLPs’ DUB activities with each other. Further in vitro studies with purified enzymes are needed to quantitatively compare PLPs DUB activities. Nevertheless, all tested PLPs including MERS-CoV, HCoV-229E, HCoV-OC43, HCoV-HUK1, MHV and FIPV showed efficient DUB activity. DUB activity of MERS-CoV PLpro and MHV PLP2 has been also shown by other groups supporting my conclusions (Wang, Chen, Zheng, Cheng, & Tang, 2011; Yang et al., 2013; Zheng et al., 2008). DeISGylating activity of MERS-CoV PLpro and MHV PLP2 is also conserved (Figures 10A and 35B). The deISGylating activity of HCoV-229E, HCoV-OC43, HCoV-HUK1, and FIPV remains to be determined.

It is interesting that the multifunctionality of coronavirus PLPs is conserved while there is less than 10% identity between their sequences. However, the overall polarity of the PLP domain is conserved suggesting that the structure might facilitate protein functions (Figure 41). Moreover, the variability of the PLP domain sequences suggests high flexibility of the structure that maintains its functions. The crystal structures of HCoV-229E, HCoV-OC43, HCoV-HUK1, and FIPV PLPs remain to be solved. However, the recently obtained by our collaborators crystal structure of MHV PLP2 revealed structural similarity between SARS-CoV PLpro and MHV PLP2 (Chen et al., in preparation). Both structures have similar ubiquitin specific protein (USP) fold, even though the sequence identity between these two proteases is about 28%, supporting the idea that the overall structure facilitates enzyme multifunctionality (Figure 31).
Figure 41. Alignment of coronavirus papain-like proteases and hydrophobicity characteristics of amino acid side chains. According to the hydrophobicity table of Kyte & Doolittle, 1982. The most hydrophobic residues according to this table are colored red and the most hydrophilic ones are colored blue. Figure generated by A. Kilianski, Loyola University Chicago.
INVESTIGATING PAPAIN-LIKE PROTEASE ABILITY TO INHIBIT INNATE IMMUNE RESPONSE AND THE ROLE OF DUB/DEISGYLATING ACTIVITY IN INNATE IMMUNE EVASION

Because ubiquitination controls innate immunity signaling and that ISGylation is important for antiviral responses upon infection, the DUB activity and deISGylating activity of PLPs have been implicated to have a role in antagonism of the innate immune response. As discussed above, the deISGylating and DUB activities of PLPs are conserved. In addition, SARS-CoV PLpro, HCoV-NL63 and porcine epidemic diarrhea virus PLP2s have been shown to act as IFN antagonists (Clementz et al., 2010; Devaraj et al., 2007; Frieman et al., 2009; Xing et al., 2013).

Coronaviruses have been shown to modulate immune responses upon infection, however, the mechanisms involved in the regulation are not yet clear (Totura & Baric, 2012). Cytokine and chemokine responses to SARS-CoV in non-lymphatic cells and infected patients results in low levels of several cytokines, including CCL5 and IFNβ (Spiegel & Weber, 2006; Wong et al., 2004). In addition, CXCL10, CCL5, and IFNβ among others, are not induced in cloned bronchial epithelial cell line and human alveolar type II cells infected with SARS-CoV early post infection (Qian et al., 2013; Yoshikawa et al., 2010). The innate immune response to MERS-CoV is also intriguing. Microarray analysis of MERS-CoV infection of Calu-3 cells results in a distinct immune response compared to SARS-CoV infection. Expression of multiple genes involved in activation of adaptive immune responses, such as MHC class I and II, are downregulated in MERS-CoV infected cells (Josset et al., 2013). The ability of SARS-CoV and MERS-CoV to
modulate early immune responses is likely due to multiple proteins encoded within virus genomes that may act as interferon antagonists.

Previous reports showed that several coronavirus proteins can block the activation of innate immune responses, particularly the interferon β (IFNβ) response (Totura & Baric, 2012). Structural proteins, such as SARS-CoV nucleocapsid (N) and membrane protein (M), in addition to being critical elements of the viral particles, have been shown to block the IFN response. Several accessory proteins (SARS-CoV ORF3b, ORF6, and Mouse Hepatitis Virus ns2) are known to act as antagonists of innate immunity (Kopecky-Bromberg, Martínez-Sobrido, Frieman, Baric, & Palese, 2007; Zhao et al., 2012). Moreover, MERS-CoV accessory protein 4 has been reported to block IFN induction (Niemeyer et al., 2013). In addition, nonstructural proteins including nsp1, nsp7, nsp15 have been implicated as IFN antagonists (Frieman et al., 2009; Kamitani, Huang, Narayanan, Lokugamage, & Makino, 2009). As described above PLPs encoded within nsp3 have been shown to block IFN induction as well.

In my dissertation I determined that MERS-CoV PLpro and MHV PLP2 are interferon antagonists and that they block induction of interferon elicited by various stimuli including RIG-I, MDA5, MAVS, and Sendai virus suggesting that the protease activity is important for inhibition of innate immunity downstream of the pattern recognition receptors (Figures 11 and 16). Further, I found that both MERS-CoV and SARS-CoV PLpros can modulate exogenous levels of proinflammatory cytokines and chemokines (Figure 12) (Mielech et al., 2014). Moreover, reports from other groups
support these findings (Yang et al., 2013; Zheng et al., 2008). It remains to be determined at exactly which step during activation of innate immune response PLPs function.

PLPs from arteriviruses are also known to block IFN responses. The N terminal region of nsp2 encodes the PLP in porcine reproductive and respiratory syndrome virus (PRRSV). This PLP has been characterized as an ovarian tumor domain (OTU) with deubiquitinating and deISGylating ability (Frias-Staheli et al., 2007). In addition, Sun et al., showed that PRRSV PLP domain can block Sendai virus induced IFNβ, and can also inhibit NF-κB by preventing IκBα degradation by its deubiquitination (Sun et al., 2010). A more recent report showed that PLP also has deISGylating activity which suggests multiple roles of PRRSV PLP in antagonism of innate immunity (Sun et al., 2012). The nsp2 of another member of the *Arteriviridae*, equine arteritis virus (EAV), has deubiquitinating and deISGylating activities as well (Frias-Staheli et al., 2007). The deubiquitinating ability of EAV PLP can block RIG-I induced IFN by inhibiting ubiquitination of RIG-I which is required for its activation (van Kasteren et al., 2012). Co-crystal structure of EAV PLP with ubiquitin revealed potential interaction sites between those molecules, and mutagenesis studies showed that PLP DUB activity is required for inhibition of innate immunity in infected cells (van Kasteren et al., 2013). Specific deubiquitinating and deISGylating activities have been shown for Crimean–Congo hemorrhagic fever virus (CCHFV) which is a highly pathogenic negative strand virus belonging to the family *Bunyaviridae*. The L protease of CCHFV contains an OTU domain with the ability to cleave ISG15 modification. L protease can remove ISG15-
mediated immune protection of IFNAR-/- mice and make them highly susceptible to Sindbis virus infection (Frias-Staheli et al., 2007).

Overall, my results and reports from multiple laboratories studying a variety of coronavirus, arterivirus and bunyavirus proteases indicate that the deubiquitinating and deISGylating activity of viral proteases play an important role in inhibition of innate immune responses and possibly virus pathogenesis. In my dissertation, I characterized the PLPs from MERS-CoV and MHV revealing the deISGylating and deubiquitinating activities, and that they can act as interferon antagonists. Moreover, I determined that protease multifunctionality is a conserved feature of PLPs from various alpha and betacoronaviruses including HCoV-OC43, HCoV-229E, HCoV-HKU1, and FIPV. Further, I determined for the first time that SARS-CoV PLpro and MERS-CoV PLpro can block induction of several endogenous proinflammatory cytokines, which are critical for establishing an antiviral state in infected cells. My data show that the antagonism of innate immune responses mediated by MERS-CoV and SARS-CoV PLpros is not limited to IFNβ, but may affect the expression of many cellular cytokines. Further, my results suggest that PLpro might contribute to the modulation of innate immune responses upon SARS-CoV and MERS-CoV infection, however, the exact mechanism and the role of coronavirus PLpro DUB and deISGylating activity in this process remains to be determined. My efforts towards understanding the role of PLP2 DUB activity in the inhibition of innate signaling are presented in the following sections.
INVESTIGATING THE ROLE OF UBIQUITIN-LIKE DOMAIN IN VIRUS PATHOGENESIS

Viruses evolved multiple strategies to facilitate productive infection. Highly pathogenic viruses such as coronaviruses, encode multiple proteins in their genomes that contribute to their pathogenesis. In my dissertation I investigated the role of the ubiquitin like (UBL) domain upstream of PLP2 from MHV in virus pathogenesis. Bioinformatic analysis predicts the presence of a UBL domain upstream of the PLP in all sequenced coronaviruses suggesting that it might play an important role in virus life cycle and making it a valuable target for antiviral drugs and vaccines development.

As my results and others show, PLPs are multifunctional enzymes and their protease, deubiquitinase, deISGylating and interferon antagonism activities have been reported. However, the role of the UBL domain located upstream of PLPs is poorly understood. Only the role of the UBL domain upstream of SARS-CoV PLpro has been investigated. It has been shown that the UBL domain is required for PLpro ability to inhibit the induction of interferon β but not for PLpro protease and DUB activity (Frieman et al., 2009). However, another group showed contradicting result that SARS-CoV PLpro domain is dispensable for IFN antagonism (Clementz et al., 2010). My data reveal a physiological role for the UBL domain in the virus life cycle and indicate that the UBL of MHV PLP2 is important for efficient protease activity and virus pathogenesis.

To determine the role of the UBL domain I performed deletion analysis and site-directed mutagenesis of the UBL domain in the context of two PLP2 expression
constructs. I performed site-directed mutagenesis of the N-terminal residues of UBL domain (amino acids 1611 to 1614 of MHV-A59 genome) and I found that the mutation of those residues to serine residues led to a decrease in protease activity in transfected cells (Figures 20A, 21A, and 24A). Even a single amino acid substitution in this region is able to decrease proteolytic activity of the PLP2 (Figure 24B). To determine the role of the UBL domain in virus pathogenesis I generated two mutant viruses using reverse genetics. The first mutant had four amino acids mutated to serine (1611-1614), termed AM1. Another virus had a single amino acid substitution (V1613S), termed AM2. I decided to generate this single amino acid mutant because V1613 is a conserved residue between UBL domains of MHV and highly pathogenic human coronaviruses such as SARS-CoV and MERS-CoV (Figure 22A).

I found that AM1 produced pinpoint plaques on DBT cells. Further, I found that AM1 replicated with slower kinetics than wild-type virus and never reached high titer that is classical for wild-type virus. AM1 replicated only to low titer of 1x10^3 pfu/ml. In contrast, AM2 replicated with efficiency similar to wild-type virus. For this reason and because V1613 residue is conserved among coronaviruses I decided to focus the remaining work on this mutant. The plaque assays performed for this virus showed that it forms slightly smaller plaques at 37°C. Further analysis showed that AM2 is a temperature sensitive virus and produces significantly smaller plaques at 39.5°C, and no plaques at 40.5°C (Figure 27B). AM2 growth kinetics was slightly, but significantly impaired at 39.5°C. In addition, temperature shift experiments showed that AM2 titers are significantly reduced upon temperature shift, supporting the temperature sensitive
Interestingly, the virus growth ability was dependent upon the time infected cells were incubated at 37°C before they were moved to 40.5°C, i.e., the longer the cells were incubated at 37°C, the lower the difference between AM2 and wild-type virus final titers. These observations could suggest that the incubation at 37°C allows for the accumulation of functional PLP2 in infected cells that could perform the function \textit{in trans} to cleave the polyprotein when the cells were moved to 40.5°C, which could mask the true defect associated with reduced PLP2 activity.

To address the mechanism of AM2 temperature sensitivity, biochemical analysis of purified enzyme containing V1613S mutation was performed at Purdue University. It was determined that compared to wild-type counterpart, V1613S mutant protein had reduced protease activity upon incubation at 30°C, but not at 25°C, supporting temperature sensitivity of the mutant virus (Figure 29B). Of note, the enzyme was unstable at 37°C, excluding the ability to determine enzyme activity at this temperature (Y. Chen, unpublished observation). To gain insights into the underlying cause of reduced activity of the enzyme at higher temperature, the circular dichroism analysis of the purified enzyme was performed. The analysis showed that the mutant has a significantly lower than the wild-type protein melting temperature indicating stability defect associated with the V1613S mutation (Figure 29C). These results can explain the effect that I saw in the temperature shift experiments. The incubation at 37°C might allow for the generation of pool of stable PLP2 that can act \textit{in trans} upon temperature shift. The longer the time at 37°C, the more of properly folded enzyme is available later
leading to smaller defect in virus growth. In contrast, when the infection occurs at 37°C for a shorter time the pool of properly folded and active PLP2 is smaller and cannot facilitate robust virus replication. The activity of PLP2 and processing of the polyprotein in the context of virus replication remains to be determined.

The availability of MHV PLP2 crystal structure determined at Purdue University facilitates the discussion regarding the role of V1613 and UBL domain in the PLP2 stability (Figure 31) (Chen et al., in preparation). The UBL domain is located upstream of the PLP2 domain, similar to the UBL domain of SARS-CoV (Ratia et al., 2006). Residues V1611 to L1614 are building a β-sheet within the MHV PLP2 UBL domain. Interestingly, the residues V1611 and V1613 are buried inside the UBL domain structure (Figure 22B). This could explain why AM1 is severely impaired and why AM2 is temperature sensitive. The quadruple mutant (AM1) disrupted the entire β-sheet structure and overall structure of the UBL domain. Further, a single amino acid mutation V1613S was sufficient to have a negative impact on UBL domain structure that resulted in an overall stability defect of the entire enzyme. It remains to be determined if the stability of UBL domain of other coronaviruses, including SARS-CoV and MERS-CoV where the valine residue is conserved, is required for PLpro overall stability.

My results show for the first time that PLP2 stability is important for MHV pathogenesis and show the physiologic role of the UBL domain in virus virulence. I determined that in contrast to infection with wild-type virus, AM2 is attenuated in a lethal model of intracranial inoculation in mice (Figure 30A). My data also suggest that infection with AM2 induces a protective immune response in infected mice. Mice
infected with AM2 are not susceptible to the disease upon challenge with wild-type virus (Figure 30B).

Overall my results highlight the importance of the UBL domain in maintaining PLP2 stability and virus pathogenesis. Thus far, literature has not described the role of the UBL domain in MHV or any other coronavirus infection. What is more, there has been only one report indicating the role of only one other nsp3 encoded protein in viral pathogenesis. An ADP-ribose-1’’-phosphatase (ADRP) domain was shown to be important for MHV virulence (Eriksson, Cervantes-Barragán, Ludewig, & Thiel, 2008). An ADRP domain inactive mutant virus does not cause acute hepatitis but replicates efficiently in infected animals. Infection with the ADRP mutant virus leads to reduced serum ALT levels compared to infection with wild-type virus. The proposed mechanism involves induction of proinflammatory cytokines such as IL-6 and TNFα. It remains to be determined if infection with AM2 leads to changes in inflammatory responses in infected mice.

The ability of AM2 to efficiently replicate in infected mice and its attenuated phenotype make this virus a potential vaccine candidate. Thus far, there are no FDA approved vaccines against any coronavirus. The ADRP mutant virus mentioned above has not been tested for its protective ability. Several live-attenuated virus vaccine candidates for SARS-CoV have been reported but their efficacy remains to be determined. SARS-CoV lacking exonuclease (ExoN) activity is an attenuated vaccine candidate. ExoN activity is important for proofreading of the RNA-dependent RNA polymerase and virus that lacks this activity acquires mutations during passages (Graham
et al., 2012). Another example of a live-attenuated vaccine candidate is a recently described mutant lacking 2’-O-methyltransferase activity that has been shown to have an attenuated phenotype in infected mice. Infection with this mutant virus generated a protective immune response in immunized animals (Menachery et al., 2014). The resistance to reversion to the virulent phenotype of those vaccine candidates remains to be determined and is a key factor in evaluating live-attenuated vaccines.

The knowledge gained from my results can be extrapolated to other coronaviruses, especially human pathogens such as SARS-CoV and MERS-CoV. The valine residue that is mutated in an attenuated AM2 virus is conserved among the UBL domains of SARS-CoV and MERS-CoV PLpro making it a possible target for future vaccine development. It is likely that a single amino acid mutation will not be sufficient to maintain attenuated phenotype due to the high mutation rate in RNA viruses. Long-term studies are needed to determine the likelihood of acquiring mutations that will result in reversion to the virulent phenotype. In the case of AM2, two nucleotides were changed when the V1613S mutation was introduced into AM2 (GTC codon was changed into TCC), which changed the polarity of the residue. Two nucleotide changes make it more difficult for the virus to revert; however, only one nucleotide would have to be changed in order to change serine to phenylalanine. Phenylalanine, similar to valine, is a non-polar amino acid thus it is possible that this substitution could facilitate correct conformation of the UBL domain, and reversion to the virulent phenotype. On the other hand, introduction of phenylalanine with significantly larger side chain compared to valine could also disrupt the overall UBL domain folding, thus this hypothesis remains to be tested.
Overall, it might be necessary to combine the AM2 mutant with other mutations, for example, ExoN mutant, to generate a fully efficacious and resistant to reversion vaccine candidate.

Overall, my results for the first time show the biological importance of the coronavirus UBL domain. I showed that the UBL domain confers PLP2 stability and is important for virus pathogenesis in vivo and that targeting UBL domain is a new potential way to generate attenuated viruses and vaccine candidates.

**SEPARATING MHV PAPAIN-LIKE PROTEASE PROTEASE AND DEUBIQUITINASE ACTIVITIES**

The multifunctionality of PLPs has been described in the literature and the previous sections of the discussion. The intriguing question is why are PLPs multifunctional? What is the role of DUB and/or deISGylating activities in virus replication and pathogenesis? Several efforts have been made to answer those questions by studies designed to separate the protease and DUB activities of PLPs. Studies on arterivirus PLP2 showed that it was possible to generate a mutant virus that was able to replicate, but did not have DUB activity (van Kasteren et al., 2013). This virus is able to induce proinflammatory response in infected cells; however, the role of DUB activity in virus pathogenesis has not been determined. Furthermore, the knowledge from this study cannot be extrapolated to coronaviruses because of the significant differences between structures of arterivirus PLPs which belong to OTU family of DUBs, and coronavirus PLPs which belong to USP family of DUBs (Mielech, Chen, Mesecar, & Baker, 2014).
In order to separate the protease and DUB activities of coronavirus PLPs co-crystal structures of SARS-CoV PLpro with a ubiquitin moiety have been obtained by two groups (Chou et al., 2014; Ratia et al., 2014). Both groups made efforts to separate the protease and DUB functions of PLpro using the information obtained from co-crystal structures and mutagenesis. However, neither one tested the role of DUB activity in virus pathogenesis. That is because it is very hard to study mutants of SARS-CoV because it is a BSL-3 pathogen. For this reason it is beneficial to work towards separating protease and DUB activities of a model coronavirus MHV, which has a convenient BSL-2 reverse genetics system and also allows for studying the effect of the mutagenesis on virus pathogenesis in the context of infection in the natural host.

For a long time, it was almost impossible to try to separate MHV PLP2 DUB and protease activities because the crystal structure of the enzyme was not available, and with only 28% identity between SARS-CoV PLpro and MHV PLP2 the alignments failed to generate an accurate model of MHV PLP2. Importantly, the crystal structure of MHV PLP2 was generated giving the hope of separating PLP2 protease and DUB activities (Figure 31) (Chen et al., in preparation). The obtained crystal structure facilitated the modeling of MHV PLP2 with SARS-CoV PLpro-ubiquitin co-crystal structure and allowed for predicting the residues on MHV PLP2 that likely interact with ubiquitin that are distal to the catalytic triad. If those interaction sites were mutated the interaction between PLP2 and ubiquitin would be disrupted and I could separate protease and DUB activity of PLP2.
The goal was to generate a mutant that maintains protease activity to facilitate virus replication but has decreased DUB activity to test the role of DUB activity in virus pathogenesis. First modeling revealed five residues that could be important for the interaction between MHV PLP2 and ubiquitin namely R253, R257, E279, F290, and Y302. I generated mutants of all of those residues to alanine with the goal of disrupting the interaction mediated by either charge of the amino acid (arginine or glutamic acid), polarity (tyrosine), or large side chain (phenylalanine). I found that the R253A, R257A E279A, and Y302A mutations only moderately disrupted PLP2 DUB activity. The reduction with DUB activity correlated with a reduction in interferon antagonism (Figure 33). Further, all mutants except R257A had decreased protease activity, or no protease activity as in the case of Y302A. Interestingly, F290A had significantly reduced DUB activity comparable to catalytic mutant of the protein and maintained protease activity to some extent. Those observations suggested that the F290A might be a valuable candidate to be tested in the context of the virus. Thus, I tried to generate the mutant virus using reverse genetics; however, I was not able to recover viable virus at 37°C or, predicting possible temperature sensitive phenotype, at 30°C. This suggested that F290 is required for virus replication and presumably proteolytic processing of the polyprotein, or PLP2 structure. Further in vitro analysis using purified protein performed at Purdue University supported this conclusion because F290A mutant does not exhibit proteolytic activity (Y. Chen, unpublished observation). These data suggest that mutating the F290 residue to alanine resulted in too dramatic change for the virus; however, it is possible that mutating F290 to another amino acid that would maintain the long side chain, such as isoleucine,
could be sufficient to facilitate virus replication but reduce the DUB activity enabling testing of my hypothesis. Further mutagenesis of this residue is required to resolve this issue.

Even though the first set of mutants did not reveal the residues that would enable separating PLP2 protease and DUB activities, it allowed for the refinement of the MHV PLP2- ubiquitin model (Figure 34). This new, refined model showed new residues on PLP2 that likely interact with ubiquitin molecule. In the second screen of mutants I tested seven new candidates. I generated a double R253A/R257A mutant because previous data showed that both single mutants have reduced DUB activity and that R257A maintains protease activity suggesting that combining those two mutations could lead to a stronger phenotype i.e. loss of DUB activity. Further, I generated the following mutants I249R (changing the non-polar amino acid to a positively charged), R281E (changing the positive charge to negative), and Y320F (changing the polar amino acid to non-polar). I also made three alanine mutants F270A, I304A, and V313A to reduce the size of the amino acid side chain. The analysis showed that the R281E mutation impairs proteolytic activity to the level of the catalytic mutant (Figure 35). However, I found that three mutants R253A/R257A, F270A and I304A exhibit protease activity comparable to wild-type PLP2. Furthermore, they all have reduced DUB activity although to various degrees (Figure 36A). It would be interesting to combine those mutations to generate double and triple mutants to determine if the degree of loss of DUB activity can be improved.

Interestingly, I249R mutation increases all activities of PLP2 (Figures 35 and 36). The increased activity of this mutant could be because positively charged arginine present
at position 249 could enhance catalytic activity of the protein and/or binding with the substrates. It remains to be determined if increased activity of the I249R mutant would have an impact on virus replication and/or pathogenesis *in vivo*. It is possible that too efficient processing could be detrimental to the virus. On the other hand, such a virus could be even more pathogenic than wild-type due to the enhanced ability to inhibit an innate immune response.

When I tested the mutants for their deISGylating activity I found that many of them, including I304A, F270A, and R253A/R257A have completely lost their deISGylating ability, and Y302F and V313A showed partial reduction (Figure 36B). It has been shown that the degree of deISGylating and DUB activities of PLPs vary depending on the virus, for example SARS-CoV PLpro is a better deISGylating than deubiquitinating enzyme (Ratia et al., 2006). MHV PLP2 seems to exhibit the opposite preference and *in vitro* studies showed that this enzyme is a better DUB than deISGylating enzyme (Chen et al., *in preparation*). For this reason I might have seen stronger phenotypes when the mutants were tested in the context of the deISGylating assay. However, I cannot say that I separated MHV PLP2 protease and deISGylating activities for Y302F and V313A because the reduction in deISGylating activity might be due to a small decrease in protease activity and the fact that the deISGylating assay is more sensitive than the DUB assay. Nevertheless, F270A, I304A, and R253A/R257A have completely lost deISGylating activity while maintaining protease activity, thus it would be important to determine the effect of these mutations on virus pathogenesis.
Finally, using the knowledge from the arterivirus study I generated a triple MHV PLP2 mutant with the goal of separating MHV PLP2 protease and DUB activities. Previous studies on the interaction of ubiquitin with various substrates showed that the isoleucine 44 (Ile 44) patch on the ubiquitin molecule was an important site required for the interaction between ubiquitin and DUBs (Hospenthal, Freund, & Komander, 2013). Furthermore, EAV PLP2 co-crystal structure with ubiquitin showed that the Ile 44 patch is the site of interaction between those two molecules (van Kasteren et al., 2013). The Ile 44 patch consists of three residues isoleucine 44, valine 70, and leucine 8. The modeling predicted that the residues on MHV PLP2 that are likely interacting with the Ile 44 patch are F290, I249, and T328 (Figure 38). Considering the information gained from the EAV study I generated a triple mutant where the amino acid changes were not very dramatic. I mutated phenylalanine to tryptophan keeping the size and polarity of the amino acid, but removing the phenyl ring. I mutated isoleucine 249 to valine, also a non-polar amino acid. Finally, I changed threonine 328 to alanine changing the polarity of amino acid. This triple mutant showed protease activity comparable to the wild-type protein; however, the DUB activity of this mutant was maintained as well. It may be informative to generate single mutants of those resides to determine if they independently have any effect on protease and/or DUB activity. Since I know from previous experiments that I249R mutation increases PLP activity it might be similar for the I249V mutant which might be masking a potential effect of the F290W and T328A mutations. Further studies are needed to determine if residues of the Ile 44 patch are required for the interaction between MHV PLP2 and ubiquitin.
During my dissertation research I found several candidate mutants that can be further optimized and tested to separate PLP2 protease and DUB activities. It will be interesting to determine the role of DUB activity on virus pathogenesis and/or replication. The hypothesis is that the DUB activity may be important for inhibition of the innate immune response, which is partially supported by the EAV PLP2 study (van Kasteren et al., 2013). However, it is also possible that PLP2 DUB activity might be important for controlling other processes during virus replication, for example double membrane vesicles formation (DMV) that mediate virus replication, or the packaging of virus particles.

Papain-like protease deubiquitinating activity might also be involved in the formation of replication complexes or double membrane vesicles (DMV) during the virus life cycle. Formation of DMVs is critical for coronavirus replication and formation of DMVs has been observed by multiple groups (Knoops et al., 2008, Snijder et al., 2006). More recently it has been shown that transfection of plasmids encoding full length nonstructural protein 3 (nsp3), nsp4, and nsp6 from SARS-CoV is sufficient to induce the formation of DMV-like structures that resemble DMV present during SARS-CoV infection (Angelini et al., 2013). Further, the authors showed that nsp3 alone is sufficient to induce membrane proliferation, which might be due to either production or expansion of already existing membranes (Angelini et al., 2013). Because SARS-PLpro is encoded within nsp3 it is reasonable to hypothesize that multifunctional PLpro might be involved in the accumulation of membranous structures or DMV formation.
The origin of the membranes that form DMVs during SARS-CoV infection is not known. However; the recent investigation of membrane structures during infection with avian coronavirus, Infectious Bronchitis Virus (IBV), showed that even though DMVs are present, previously undescribed structures are present as well. Upon IBV infection the authors observed novel membranous structure that they termed zippered endoplasmic reticulum and spherules that appeared to be invaginations of zipped ER (Maier et al., 2013). This was the first report suggesting the origin of membrane rearrangements during CoV infection and also showed that the spherules are connected with the cytoplasm by the channel with 4.4nm diameter that could facilitate transport of newly synthetized RNA from the spherule to the cytoplasm for virion assembly (Maier et al., 2013). A PLpro domain is predicted to be present in the IBV genome thus it is possible that it might play a role in the formation of zipped ER spherules. Deubiquitinating activity of PLPs might be directly involved in the formation of membrane rearrangements or it might also facilitate the interaction with host proteins that are responsible for the generation of membranous structures and formation of virus replication complexes. Furthermore, not only PLP DUB activity might be critical for this process but also the above described UBL domain of PLP. UBLs have been shown to have different roles and functions in the context of different DUBs. Ubl domains can impact enzyme activity and specificity, and also be involved in the recruitment of other non-target proteins. For example, the Ubl domain of USP14 is required for targeting of this cellular DUB to the proteasome, where USP14 removes ubiquitin molecules from proteins that are modified with K-48-linked ubiquitin which directs them for proteosomal degradation (Hu et al., 2005). Thus, it is
possible that PLP UBL domain targets it to the replication complexes or recruits other host proteins to form DMVs or spherules. Further studies using electron microscopy and mutants that lack DUB activity could help answer this question.

SUMMARY

In my dissertation I investigated the enzymatic activity of coronavirus papain-like proteases. I determined that PLPs are multifunctional enzymes with protease, deubiquitinase, deISGylating and interferon antagonism activities. I found that PLPs multifunctionality is conserved among various alpha and betacoronaviruses, suggesting that PLPs are valuable targets for the development of antiviral drugs and vaccines. My studies with efficacious inhibitors of SARS-CoV replication that target PLpro support this observation.

Further, I determined, for the first time, that the UBL domain that is located upstream of the MHV PLP2 domain is important for protein stability and virus pathogenesis. The UBL mutants alone, or in conjunction with other known attenuating mutations, might be new vaccine candidates in the future.

Finally, my research enabled generation of MHV PLP2 crystal structure that is a valuable tool in studying MHV PLP2. The knowledge from the crystal structure and modeling facilitated the mutagenesis with the goal of separating PLP2 protease and deubiquitinase activities. I generated three mutants that have reduced DUB activity and maintain protease activity that can be further validated. Separating DUB and protease activities in the context of the virus will facilitate studies on the role of DUB activity in
virus replication and pathogenesis, and can lead to the development of new vaccine candidates for coronaviruses.
APPENDIX I:

SEQUENCES OF CONSTRUCTS USED FOR EXPERIMENTS
1. MERS-CoV PLpro

MERS-CoV PLpro sequence optimized for expression in human cells. The native MERS-PLpro sequence (from amino acids 1485 to 1802) was subjected to codon optimization, removal of putative splice acceptor and splice donor sites, and addition of restriction sites (in bold) for cloning, and in frame V5 epitope tag (in orange). The synthetic sequence was cloned into pCDNA3.1-V5/His-B at the EcoRI and PmeI sites.

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GAATTC
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2. MHV PLP2-A

MHV-PLP2-A sequence optimized for expression in human cells. The native MHV PLP2 sequence (from amino acids 1611 to 1970) was subjected to codon optimization, removal of putative splice acceptor and splice donor sites, and addition of restriction sites (in bold) for cloning, and in frame V5 epitope tag (in orange). The synthetic sequence was cloned into pCAGSS-MCS at the SaeI, and XmaI sites.

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GAGCTC
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[151]
3. MHV PLP2-B

MHV-PLP2-B sequence optimized for expression in human cells. The native MHV PLP2 sequence (from amino acids 1525 to 1911) was subjected to codon optimization, removal of putative splice acceptor and splice donor sites, and addition of restriction sites (in bold) for cloning. The synthetic sequence was cloned into pCAGSS-MCS PLP2-A-Δ7-V5 at the KpnI and MluI sites.
4. HCoV-229E PLP2

HCoV-229E PLP2 sequence optimized for expression in human cells. The native HCoV-229E PLP2 sequence (from amino acids 1693 to 2019) was subjected to codon optimization, removal of putative splice acceptor and splice donor sites, and addition of restriction sites (in bold) for cloning, and in frame V5 epitope tag (in orange). The synthetic sequence was cloned into pCDNA3.1-V5/His-B at the EcoRI and PmeI sites.
5. HCoV-OC43 PLP2

HCoV-OC43 PLP2 sequence optimized for expression in human cells. The native HCoV-OC43 PLP2 sequence (from amino acids 1628 to 1952) was subjected to codon optimization, removal of putative splice acceptor and splice donor sites, and addition of restriction sites (in bold) for cloning, and in frame V5 epitope tag (in orange). The synthetic sequence was cloned into pCDNA3.1-V5/His-B at the EcoRI and PmeI sites.

6. HCoV-HKU1 PLP2

HCoV-HKU1 PLP2 sequence optimized for expression in human cells. The native HCoV-HKU1 PLP2 sequence (from amino acids 1701 to 2025) was subjected to codon optimization, removal of putative splice acceptor and splice donor sites, and addition of restriction sites (in bold) for cloning, and in frame V5 epitope tag (in orange). The synthetic sequence was cloned into pCDNA3.1-V5/His-B at the EcoRI and PmeI sites.
FIPV PLP2 sequence optimized for expression in human cells. The native FIPV PLP2 sequence (from amino acids 1488 to 1811) was subjected to codon optimization, removal of putative splice acceptor and splice donor sites, and addition of restriction sites (in bold) for cloning, and in frame V5 epitope tag (in orange). The synthetic sequence was cloned into pCDNA3.1-V5/His-B at the EcoRI and PmeI sites.
GCACAAGCTGGTGACCTGGTATGCTATGTGATTCTGCCGTGACAGTCACTCATA
CACACGCTTCGAGAAGTGGACATGCAACATAGCCTTACAGGAGACTGAGTG
CGCAGCTCCACTGCTTGGTGCACGGGACAGATGAAATCTGCGTCACGGCTG
CATGTCAACGTGAAGGTCACTTCTCCATTCGAGACAGTGGCTATCATACCTCT
GATTGGACAGCGCTGATAAGGCATATCATTTCGAGAAAAATCTGCTGCAAAT
GACTACCACATCGCACGTAACCTTTTGTGCTTATACACCTGAAGAAGAGATT
ATGCCAACAGACTAGGGCACAAGAATCAAAGATCAAGGACATGCAACCTG
8. SARS-CoV PLpro

SARS-CoV PLpro sequence optimized for expression in human cells. The native SARS-CoV PLpro sequence (from amino acids 1541 to 1855) was subjected to codon optimization, removal of putative splice acceptor and splice donor sites, and addition of restriction sites (in bold) for cloning, and in frame V5 epitope tag (in orange). The synthetic sequence was cloned into pCDNA3.1-V5/His-B at the EcoRI and PmeI sites.

**GAAATTCACCATG**GAGGTCAAGACAATCAAGGTGTTTACTACAGTGGAACAATA
CAAATCTGATCTACAGCTGGTGATATGACATGATTACGCGCAGCAGTCTC
CGGCGCCACCTGCTGAGCGGCGCCGACAGGTGCAAAAAGATGAAAACCA
GCTGCAAAAGACTCAGGCAAAAGAATCAAAAGCCAAAGGCAAGG

**TGA**GTTTAAAC

**GGCAAG**

CCTATTCCATAACCCCTCCTGGCCTGGATTCATCAGCTGCAACCTG

8. SARS-CoV PLpro

SARS-CoV PLpro sequence optimized for expression in human cells. The native SARS-CoV PLpro sequence (from amino acids 1541 to 1855) was subjected to codon optimization, removal of putative splice acceptor and splice donor sites, and addition of restriction sites (in bold) for cloning, and in frame V5 epitope tag (in orange). The synthetic sequence was cloned into pCDNA3.1-V5/His-B at the EcoRI and PmeI sites.

**GAAATTCACCATG**GAGGTCAAGACAATCAAGGTGTTTACTACAGTGGAACAATA
CAAATCTGATCTACAGCTGGTGATATGACATGATTACGCGCAGCAGTCTC
CGGCGCCACCTGCTGAGCGGCGCCGACAGGTGCAAAAAGATGAAAACCA
GCTGCAAAAGACTCAGGCAAAAGAATCAAAAGCCAAAGGCAAGG

**TGA**GTTTAAAC
GTCAAGCCTGGAGGGACCAAGCGGCAAGCCTATTCCTAACCCCTGCTGGGA
CTGGACTCTACCTAAGTTAAAC
APPENDIX II:

REVERSE GENETICS PROTOCOL
Important notes before you start:

1. BHK-R cells are located in box B2 (liquid nitrogen tank). Those cells required selection with G418 (media that contains .8 mg/mL G418 (8 uL stock [100 mg/mL] per 1 mL media used). Those cells look different than regular BKH21 cells. The receptor expression makes them look really funky with appendages all over the place. Also, most of your cells will die upon thawing. Don’t get discouraged. Keep them under selection and they will recover eventually. Then they will grow pretty fast. Split 1:10 every 2 days. Media: DMEM 10% FCS.

2. It’s all about the fragments. If your fragments are ok everything else goes pretty smoothly. Believe me a year sequence them to make sure they are ok, even if your digest pattern is ok. If your digest pattern is not 100% correct, and you have any doubts, DO NOT PROCEED. Never use glycerol stock to store or recover the plasmids.

3. Enzymes: use NEB enzymes, do not switch to Fermentas. I was able to generate wild type virus using Fermentas cut fragments but the efficiency is much lower compared to NEB cut fragments. Also, you save a lot of time using NEB for digest.

Bacterial Cell Culture

It is important to transform your DNA for growth for assembly into the correct E.coli cell type. We have found that the A clone which is propagated in the Topo vector, grows best in DH5 alpha cells. B, C, D, E, F and G (the pSMART vector clones, G is in pMH54 vector) grow best in Top Ten cells (Invitrogen Grow A on a Kanamycin plate; all the rest are on Ampicillin plates.

If you are sure of the integrity of your clones (i.e., got them from the archive and didn’t just make them yourself), you don’t have to screen transformants. BUT it’s better to screen first. Do few mini preps and check digest pattern. Then when you are sure you can start maxi scale in 150-200 mL of LB plus antibiotic. Let this grow at “~30oC” for at least 24 hours. You get better yields the more you let them grow—30 hours is really better. Patience pays off here.

DNA Purification

Note Alpha: Be advised: we have run into contamination of predicted attenuated virus cultures with wild-type virus when wild-type DNA and mutant DNA are prepared in the same sitting. The precise step of contamination is likely the gel box, but it can obviously be anywhere there’s DNA. Because of this, we no longer construct a wild-type control. If you absolutely must construct wild-type or are planning on constructing wild-type-like mutants along with severely attenuated mutants, I advise prepping the DNA up to final extract stage on different days, and cleaning the equipment thoroughly in between.
Next do a maxiprep of your DNA using PureYield Maxiprep System Promega. Follow the manufacturer instructions. Elute with water that is warmed up to 37°C.

Note from original protocol (never tested by myself): about prepping volumes—if you don’t want to have so much DNA for the fragments you’re only going to use once (i.e., if you’re making mutations in the first 5 kb, the A fragment), you can just prep a 20-40 mL culture and do what amounts to 3-6 minipreps. You can do the lysate steps (everything up to and including the high-speed spin where you get the white pellet after ten minutes) in one centrifuge tube—scale up amounts for 3.5-7 minipreps, then divide the cleared lysate at the end of the spin into 3-6 columns each separate fragment and proceed as normal.

Note 1: It is important to test digest a small amount of your prepped DNA to make sure its integrity is intact. A digest can go badly even from screened DNA, probably because of something to do with the purification. I have had this occur with both F and G fragments.

However you get your DNA, you need quite a lot for a round of virus production. The actual amounts you need for each fragment depend on the size of the fragment, but if it’s a backbone fragment (one that’s the same for all, not your mutant fragment) try to get 20 ug each backbone fragment per virus you are constructing. Example: you are making 4 viruses with mutations in A. You need 80 µg each of B, C, D, E, F, and G, and 20 ug each of your mutant As. You hardly ever end up using all of your mutant fragments, though.

Original protocol told you to IPA (isopropanol) precipitate your DNA at this point. You don’t have to do this as long as you use Promega kit.

**Digests**

Again: learn from me and other people in our lab: use NEB enzymes. DO NOT SWITCH TO FERMENTAS. If for some reason you want to or have to follow digestion procedures in my hand-written notebook. It is possible to get wild type virus but again the efficiency is lower than using NEB cut fragments (It took me a while to figure it out).

These are large-scale digests. I have provided the setups I do, but you can adjust buffer/water amounts depending on what final volume you use. Other important notes:

1. When you set up the digest: do not exceed 5% of glycerol (present in your enzyme tube) in your reaction as it will influence digest efficiency.

2. I usually cut at least 40ug of DNA (2 tubes of 20ug). This will give you about 3 rounds for virus, depending on the fragment.
3. I do not recommend doing all of them the same day. Divide into two days. Follow the procedure all the way to gel purification the same day. Do not freeze gel fragments.

4. NEB double digests use different buffers so make sure you have the right one.

5. Digest must be done in PCR machine.

**A Fragment**

- **20ug DNA** x uL
- **Buffer 3** 20 uL
- **Mlu I** x uL*
- **BsmB I** x uL*
- **H2O** to 200 uL

*x uL of enzyme is a function of how much DNA you have. I generally use 2 ul per 10 ug DNA, up to 10 ul enzyme, which works out to a 2-fold excess enzyme concentration for most of these guys, allowing for a cut that can be anywhere between 1 hour (absolute minimum, and I in fact never go below 1.5 h) and overnight if necessary. I do 3hr cut at each temperature.

When I reach 2 hr of incubation I run a little of ample on agarose gel to make sure the cur is correct, so I would be able to gel purify when the digests complete.

Note Beta: Wild-type A runs at 4.8 kb, and vector runs at 3.5 kb. This is a close cut in the best of circumstances when you’re digesting a lot of DNA, and becomes problematic if you’re doing deletions in the A fragment. To circumvent this, you can perform the MluI cut first, then precipitate, and perform a BsmBI/SfiI cut in Buffer 2 + BSA, which will cut the vector into two roughly equal-size pieces and get it out of your way.

Incubate at 37oC for 3 h, then move to 55oC and incubate for 3 h.

**B and C Fragments**

- **DNA 20ug** x uL
- **Buffer 3** 20 uL
- **Bgl I** x uL*
- **BsmB I** x uL*
- **H2O** to 200 uL

Incubate at 37oC for 3 h, then move to 55oC and incubate for 3 h.
D and E fragments

DNA  20ug  x  uL
Buffer  4  20 uL
  Nci I  x  uL*
  BsmB I  x  uL*
  H2O  to 200 uL

Incubate at 37°C for 3 h, then move to 55°C and incubate for 3 h.

F Fragment

DNA  20ug  x  uL
Buffer  3  20 uL
  BsmB I  x  uL*
  H2O  to 200 uL

Incubate at 55°C for 3 h.

G Fragment

DNA  100 uL
Buffer  2  20 uL
  10X BSA  20 uL
  Sfi I  x  uL*
  BsmB I  x  uL*
  H2O  to 200 uL

Incubate at 55°C for 3 h

Note 2: The original published protocol calls for breaking the A and G digests into two stages—doing the Mlu/Sfi digest first, CIP treating, chloroform extracting and precipitating DNA, then doing the BsmB I digest. This was implemented to prevent concatemerization of MHV cDNA, but we have found the step is unnecessary if all other steps are done carefully. If you are having difficulty having a particular mutant “take” or anticipate it being severely attenuated (or if your DNA yield is low and you are digesting <20 ug per virus) I would suggest putting the CIP treatment step back in. Basically, do the Mlu/Sfi digest alone for 1.5-2 h, then add 4 uL CIP and incubate at 37°C for 1 hour, chloroform extract (adjust salt concentration like in IPA precipitation, add 1 volume chloroform, shake about a minute, spin two minutes, draw off DNA-containing top aqueous phase, continue with IPA precipitation after salt step), then do the BsmB I digest.
While these digests are going on, I prepare the gels. We pour 75ml, .8 % Sea Plaque agarose gels set with the 4-tooth combs (for old type agarose box). Use 1.5 uL EtBr per gel. If your final digest volumes are 200 uL, you will use one gel per fragment.

Run your gels at 90-120 mA for however long it takes. C doesn’t separate from its vector pieces very well (it’s 2 kb and the vector pieces are 1.2 and .8 kb but they consistently run a little high), but if you run the DNA as far as it can go, you can tell which band to cut. The advantage of running so much DNA is that it sops up EtBr like a sponge, and you don’t have to worry about your DNA running out of the EtBr front. You can still see it. Use the Dark Reader to cut. Try to minimize agarose as much as possible—you’re going to have plenty as it is. If you ran 200 uL digests in four lanes per fragment, you can cut one lane per tube.

N gene linearization

In our lab N gene is cloned into vector. All you have to do is to linearize the plasmid using AdeI enzyme (in this case I used Fermentas and it worked fine). Then gel purify as other fragments (see below).

Gel Extraction

We use the Promega Wizard Gel/PCR Purification kit. Columns in this kit have a capacity of 40 ug bound DNA, so I use two per fragment. Follow the manufacturer’s instructions, weighing your gel slices in tubes before adding the membrane-binding buffer. If a tube weighs above 700 mg, you’ve got to split it into two tubes, because you add the buffer to wt/vol and you won’t have room in the tube. Incubate gel/buffer in tubes at 55oC for 10 minutes, shaking at 5 minutes. If at the end of 10 minutes you still have some DNA chunks, incubate a little longer.

You may have to apply the melted gel slices to the columns in shifts. That’s okay. Just follow the manufacturer’s protocol. When you elute from the column, elute the same fragments into 1 tube and use water that has been heated to 65oC. Use 50uL/column of nuclease free water to elute.

IMPORTANT STEP YOU SHOULD UNDER NO CIRCUMSTANCES SKIP: You must chloroform extract your eluted DNA. Don’t add any salt—just squirt in a little less than one volume of chloroform, shake for a minute, spin for 2 minutes, and draw off the top DNA-containing aqueous layer, being very careful to avoid any white schmutz (which may or may not be visible) at the interface. This is agarose that came through the column with the DNA and will interfere severely with your seven-part ligation.

My protocol: I have 2 tubes so almost 100uL of eluted DNA. In 500uL tube I add 20uL of water and 100uL of chloroform. Then vortex for 1 min and spin at 14K for 5 min. Take the upper layer for further steps.
Quantitation and Ligation of DNA

This is the most anal-retentive part of the whole process. If you cut corners here, however, you will adversely affect the yield of virus you get out, and this could mean the difference between seeing virus and not seeing it if your bug is attenuated. I have successfully isolated and propagated viruses that popped up from ONE cell that took, but be careful. Quantitate your DNA (at least your backbone pieces) by spectrophotometer. Depending on how careful you’ve been and how much DNA you started with, expect concentrations anywhere from 30 ng/uL to over 100 ng/uL for fragments.

Determining how much of a given fragment should go into the ligation is done by determining rough molar ratios. To do this, you have to divide the concentration the spec gives you (which does not take into account DNA size) by the size of the fragment. Then you find your average molar ratio and determine load volume by a ratio of average/experimental X average load volume (predetermined by elution amount and how many viruses you are making that round). I have included one of my calculations to demonstrate: (these were mutant A viruses, so A is not included)

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Concentration (ng/uL)</th>
<th>L (Conc./length)</th>
<th>Total amount loaded (uL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>52.9</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>B</td>
<td>53.2</td>
<td>12</td>
<td>19.6</td>
</tr>
<tr>
<td>C</td>
<td>48.4</td>
<td>24</td>
<td>9.8</td>
</tr>
<tr>
<td>D</td>
<td>42</td>
<td>28</td>
<td>8.4</td>
</tr>
<tr>
<td>E</td>
<td>191</td>
<td>67</td>
<td>3.5</td>
</tr>
<tr>
<td>F</td>
<td>86</td>
<td>12</td>
<td>19.6</td>
</tr>
<tr>
<td>G</td>
<td>74</td>
<td>8</td>
<td>20</td>
</tr>
</tbody>
</table>

L is determined by dividing concentration by length of fragment: A=4.8; B=4.3; C=2.0; D=1.5; E=2.8; F=7.0; G=8.7

Amount loaded is determined by finding the average of L (in this case, L/5-exclude E because of very high concentration=11.8) and then determining average and maximum load amounts based on how much DNA volume you have and how many viruses you have to split it between. In this case, I had 4 viruses to make and 100 uL of backbone, concentrations were very good, and variation in amounts between fragments was minimal, so in this instance I set average load at 20 uL and max load at 25 uL. Amount loaded is then determined by (Laverage/L.fragment) X (average load), not surpassing max load. Example: for B, 11.8/12 X 20 = 19.6.

If you quantitate your mutant fragments, I don’t recommend figuring them into your load equations, because you only need to make one of each mutant. You may need to stretch your backbone fragments, though. Another thing to consider: if one or two
concentrations of backbone lie well outside a narrow range defined by the other backbone fragments, consider those outliers and don’t figure them into your calculations. Just add your load maximum (or, if it’s superconcentrated DNA, a ballpark minimum) of DNA.

**I set up the ligation as follows:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragments</td>
<td>100.9 (from above—will vary from round to round)</td>
</tr>
<tr>
<td>10X Ligase Buffer (NEB)</td>
<td>20 uL (1/10th a final ligation volume of 200 uL)</td>
</tr>
<tr>
<td>T4 DNA Ligase (NEB)</td>
<td>15 uL</td>
</tr>
<tr>
<td>H2O</td>
<td>to final volume of 200 uL</td>
</tr>
</tbody>
</table>

Mix well, and ligate overnight at 16°C. (water bath in the fridge set to 16OC.

**Getting DBT cells ready**

About 4-5 hours prior to electroporation I prepare DBT cells. You need to seed one T75 per virus being made with a 1:5 dilution of DBT cells, use DMEM 10% FCS media.

Because the virus coming off these flasks is going to be a stock virus, I try to use a minimal volume of media, bringing the flask that’s being split up in 5 (T75) or 10 (T150) and passing 1 mL into 4-5 mL media. Be careful: using 5 mL total or less in a T75 sometimes makes the cells settle thickly around the edges and sparsely in the middle.

This day is all about RNA, so take RNA precautions. Wear clean gloves and use plastics and reagents set aside for RNA work. If you do something else in any interim, change your gloves.

**DNA Extraction**

1. Add 1:5-1:10 volume NaOAc and 1:1 volume chloroform to DNA. I have usually 200uL ligation I use 30uL 3MNaOAc and 230ul chloroform.

2. Shake by hand for 1 minute

3. Spin at 14K rpm for 2 minutes

4. Transfer the aqueous fraction (top layer) to a new, labeled tube. Keep the nonpolar fraction in case the pellet is lost.

5. Add 1:1 volume of isopropanol (usually I recover 170uL of liquid)
6. Mix well and let sit 10 minutes at room temperature. Set a timer as a reminder to put the tubes back in the centrifuge.

7. Spin at 14K rpm for 10 minutes

8. Remove supernatant and place in a labeled tube (in case the pellet is lost). For each of the next few steps, a labeled tube with the supernatant should be kept to prevent loss of the pellet.

9. Look for pellet (about the size of a ball point pen tip, you have to see it. Always make sure pellet is there when you proceed). Decant the supernatant into another tube, leave a small volume (~ 40 µl) so as to not disrupt the pellet.

10. Add 300 µl 70% ethanol and spin for 5 minutes. Decant the supernatant into another tube, leave a small volume (~ 40 µl) so as to not disrupt the pellet.

11. Add 300 µl 95% ethanol and spin for 5 minutes. Extract very carefully all of the supernatant possible.

   NOTE: I extracted about 280 µl and spun several times to get all ethanol out

12. Let sit 10 minutes and add 10 µl nuclease free water.

13. Set up transcription reactions in same tube and label a tube for N-gene (use mMessage mMaschine kit-Ambion). Add in order!

<table>
<thead>
<tr>
<th>Ligated Fragments</th>
<th>N-gene (per virus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP (3 mM-dilute the stock!)</td>
<td>7.5uL</td>
</tr>
<tr>
<td>NF H2O</td>
<td>------</td>
</tr>
<tr>
<td>2X NTP/Cap</td>
<td>25 uL</td>
</tr>
<tr>
<td>DNA</td>
<td>7.5 uL</td>
</tr>
<tr>
<td>10X Rxn Buffer</td>
<td>5 uL</td>
</tr>
<tr>
<td>T7 RNA Pol Mix</td>
<td>5 uL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>50 uL</strong></td>
</tr>
<tr>
<td></td>
<td>25 uL / virus</td>
</tr>
</tbody>
</table>

14. Run *in vitro* reaction in PCR machine:

   40.5°C 25 min
   37.5°C 50 min
   40.5°C 25 min
Resuspending BHK-R cells from T150s (About 0.5 hr from transcription completion)

Towards the end of the transcription reaction, you need to get your BHK-R cells ready. BHK-R cells were made from nonpermissive BHK cells by stably transfecting them with the receptor CEACAM-1. To maintain the receptor expression, these cells must be passaged in media that contains .8 mg/mL G418 (8 uL stock [100 mg/mL] per 1 mL media used). However, after electroporation, these cells will be laid on a permissive seed layer of DBTs, so selection in the mixed population flask doesn’t need to be maintained.

15. Wash flask with TC PBs (+CaCl2, MgCl2)
16. Add 3 ml trypsin and wait for cell monolayer to come off
17. Add 7 ml DMEM and resuspend
18. Add the 10 ml cell suspension to a 15 ml conical
19. Spin 750rpm for 5 min, remove media.
20. Add 10 ml cold PBS (no salts), resuspend cells
21. Spin conicals at 750rpm for 5 min
22. Aspirate out PBS and resuspend cells well in 10 ml cold PBS.
23. Take one 0.5 ml Eppendorf tubes and pipette 18 µl PBS
24. Put coverslip on hemocytometer
25. Take 2 µl cells and add to the 0.5 Eppendorf tube to make a 1:10 dilution
26. Pipette out 10 µl of diluted cells and load onto hemocytometer
27. Spin conicals at 750rpm for 5 min
28. Count cells in each of 4 squares (only along 2 of the sides)
Ex. 42 cells from 4 squares
42:4=10.5 x 10^5 x 10 (ml in PBS) = 1.05 x 10^7 cells
1.05 x 10^7 = 1.05 ml OptiMEM
1 x 10^7

NOTE: this is plenty b/c I only need 600 µl for each virus
29. Aspirate out PBS and add calculated amount (1.05 ml) OptiMEM to cells

**Electroporation of Cells**

30. Bring RNA (ligated A-G, N) on ice, labeled 4 mm gap cuvettes (one for each virus), IMPORTANT Keep cuvette in refrigerator prior to electroporation

31. In each cuvette to be electroporated, add, in order:

   1) 22 µl N gene
   2) 45 µl ligated RNA
   3) 600 µl BHK-R cells

32. Pipette up and down several times to mix well

33. Tap cuvette on counter to ensure that the mixture is evenly distributed along the inside gap

34. Wipe down cuvette (metal part) with a kimwipe and secure in place inside the pulse pod

35. Use Dr. T. Gallagher lab gene pulser. Conditions 850V, 25uF. Your time constant should be at least 0.6ms for each pulse.

36. Press button 3 times total, waiting a good 5 seconds between each press

   NOTE: look for froth to ensure that cells were electroporated thoroughly

37. Keep the cuvettes in room temperature and until 10 minutes after the last electroporation

38. Retrieve pre-prepared DBT-9s in T75s from 37°C incubator

39. Under hood, put as much of the electroporated cells as possible in the designated T75 by tipping cuvette while pipetting out. I also add some media to wash all of the contents of the cuvette and add it to the flask.

40. Incubate at the appropriate temperature (37°C for garden-variety viruses, 33°C or 30°C for TS mutants).

41. Look for the virus the next day.

It takes at least 12 hours for anything to appear, I’ve noticed. The later in the day you electroporate, the later the next day you should expect to see anything, unless you’re shocking wild-type. Depending on the success of your ligation (which is amounts- and proportions-dependent and a little lucky besides), the amount of raw material you started with, the number of viruses you divided the backbone into, and the electroporation itself, your flask(s) may produce anything from a robust response (starting from little 2-3 cell
syncytia all over the place that are easy to pick out) to a sparse response (as little as ONE plaque per flask, which I have had before, and which are difficult to catch until they actually form multinucleate plaques of about 20 nuclei). You can sometimes see stuff starting to happen if you hold the flask up to the light to contrast the monolayer. Plaques appear as tiny holes.

If your virus is slow, you can expand the flask into a T150. Don’t put any selective antibiotic onto the cells—I don’t trust the D9s to handle it and the BHK-Rs won’t lose their receptor that quickly.
REFERENCES


VITA

The author, Anna Maria Mielech was born in Gdynia, Poland on August 6, 1984 to Wladyslaw and Romulada Bajda. In June 2008, she received a Master’s of Science degree in Molecular and Pharmaceutical Biotechnology from Gdansk University of Technology, Poland. Her thesis project focused on developing a new PCR-based method for Candida strains differentiation. Following, she was accepted to Ph. D. program in the Department of Microbiology at Gdansk University of Technology, Poland where her research focused on the development of novel tools for molecular diagnostics of uropathogenic *E. coli* (UPEC). During this time, Anna was awarded Fulbright Graduate Student Award and in July 2009 she joined the Integrated Program in Biomedical Sciences at Loyola University Chicago.

In 2010, Anna joined Dr. Susan Baker’s laboratory and the Department of Microbiology and Immunology, Loyola University Chicago. Her research focused on coronavirus multifunctional proteases as targets for the development of antiviral drugs and vaccines. While at Loyola, Anna received multiple travel awards to present her research at national and international meetings. She also revived the Arthur J. Schmitt Dissertation Fellowship and was awarded in the 2013 St. Albert’s Day Graduate Student Oral Competition at Loyola University Chicago. She was also involved in mentoring of many undergraduate summer students and rotation graduate students. After completing her Ph. D., Anna will continue research as a post-doctoral fellow in Dr. Susan Baker lab.