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Catalytic Function and Substrate Specificity of the PLpro Domain of nsp3 from the Middle East Respiratory Syndrome Coronavirus (MERS-CoV)

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Running Head: MERS-CoV papain-like protease substrate specificity

Abstract

The papain-like protease (PLpro) domain from the deadly Middle East Respiratory Syndrome coronavirus (MERS-CoV) was over-expressed and purified. MERS-CoV PLpro constructs with or without the putative ubiquitin-like (UBL) domain at the N-terminus were found to possess protease, deubiquitinating, deISGylating, and interferon antagonism activities in transfected HEK293T cells. The quaternary structure and substrate preferences of MERS-CoV PLpro were determined and compared to those of SARS-CoV PLpro, revealing prominent differences between these closely related enzymes. Steady-state kinetic analyses of purified MERS-CoV and SARS-CoV PLpros uncover significant differences in their rates of hydrolysis of 5-aminomethyl coumarin (AMC) from C-terminally labeled peptide, ubiquitin and ISG15 substrates, as well as in their rates of isopeptide bond cleavage of K48- and K63-linked polyubiquitin chains. MERS-CoV PLpro was found to have an 8-fold and 3,500-fold higher catalytic efficiency for hydrolysis of the ISG15-AMC over the Ub-AMC and Z-RLRGG-AMC substrates respectively. A similar trend is observed for SARS-CoV PLpro although it is much more efficient than MERS-CoV PLpro towards ISG15-AMC and peptide-AMC substrates. MERS-CoV PLpro was found to process K48- and K63-linked polyubiquitin chains with similar rates and debranching patterns producing monoubiquitin species. However, SARS-CoV PLpro much prefers K48-linked polyubiquitin chains to K63-linked chains, and it rapidly produces di-ubiquitin molecules from K48-linked chains. Finally, potent inhibitors of SARS-CoV PLpro were found to have no effect on MERS-CoV PLpro. A homology

model of MERS-CoV PLpro structure was generated and compared to the X-ray structure of SARS-CoV PLpro to provide plausible explanations for differences in substrate and inhibitor recognition.

Importance

Unlocking the secrets of how coronavirus (CoV) papain-like proteases (PLpros) perform their multifunctional roles during viral replication entails a complete mechanistic understanding of their substrate recognition and enzymatic activities. We show that the PLpro domains from the MERS and SARS coronaviruses can recognize and process the same substrates but with different catalytic efficiencies. The differences in substrate recognition between these closely related PLpros suggest that neither enzyme can be used as a generalized model to explain the kinetic behavior of all CoV PLpros. As a consequence, decoding the mechanisms of PLpro-mediated antagonism of the host innate immune response and the development of anit-CoV PLpro enzyme inhibitors will be a challenging undertaking. The results from this study provide valuable information for understanding how MERS-CoV PLpro-mediated antagonism of the host innate immune response is orchestrated and insight into the design of inhibitors against MERS-CoV PLpro.

Introduction/Background

Coronaviruses (CoV) can infect and cause diseases in a wide range of vertebrates including humans and a variety of livestock, poultry, and domestic

animals. Diseases caused by coronaviruses range from respiratory, enteric, hepatic and neurological, and they have variable incidence and clinical severity (1, 2). Until 2012, five human coronaviruses (HCoV) were known. The first two human coronaviruses were discovered in the mid 60s, HCoV-229E and HCoV-OC43, as the causative agents of mild respiratory infections (3, 4). In 2003, a new human coronavirus was identified as the causative agent of the first global pandemic of the new millennium. This new human coronavirus was named severe acute respiratory syndrome (SARS-CoV) as it caused a pathogenic respiratory infection in over 8,000 humans in nearly 30 countries and exhibited a case-fatality rate of nearly 10% (5-8). This event prompted interest in the coronavirus research, resulting in the discovery of two additional human coronaviruses (HCoV-NL63 in 2004 (9) and HCoV-HKU1 in 2005 (10)). However, because of the lack of effective diagnostic methods, it was not until recently that human coronaviruses, with the exception of SARS-CoV, were found to be circulating in the human population and they are now implicated as contributing a significant percent of known human respiratory tract infections (11). Most recently, nearly 10 years after the discovery of SARS-CoV, a new human coronavirus was discovered in the Middle East and thus far it has a significantly higher case-fatality rate (~30%) than SARS-CoV (12, 13). The new human coronavirus was named MERS-CoV for Middle East respiratory syndrome, (formerly HCoV-EMC/2012 for Eramus Medical Center) and is associated with severe acute respiratory infection (SARI) often combined with kidney failure (14). So far, there are 837 laboratory-confirmed cases of MERS-CoV infection in 20

countries, with the first case in the United State, Indiana, recently reported in May 2, 2014 (15). The reminiscence of MERS-CoV to the initial stages of SARS-CoV pandemic has raised important public health concerns and research interest (16). As a result, the complete genome sequence has been obtained, animal models are being developed, and phylogenic, evolutionary, receptor interaction and tissue tropism analyses are now becoming available (14, 17-19).

As with all coronaviruses, MERS-CoV is an enveloped, positive-sense RNA virus with a genome of nearly 30 kb (14). Similar to SARS-CoV, MERS-CoV belongs to the virus genus *Betacoronavirus* but constitutes a sister species in the Group C (14). The complete genomic analysis suggests that MERS-CoV is phylogenetically related to bat coronaviruses HKU4 and HKU5, previously found in Lesser Bamboo bats and Japanese Pipistrelle bats from Hong Kong, respectively (14, 16). As observed previously with the zoonotic acquisition of HCoV-OC43 and SARS-CoV, the close genomic relationship of MERS-CoV PLpro to bat coronavirus HKU4 and HKU5 suggests a zoonotic origin from bat coronaviruses (17). Recently, a number of animals, including dromedary camels and Egyptian cave bats, have been considered as potential intermediate host animals for the animal-to-human transmission of MERS-CoV, however more research is necessary for confirmation (18-21). Alarmingly, human-to-human transmission has now been reported with higher prevalence in immunocompromised patients or patients with underlying diseases (22-24).

The host immune response to viral infection has been directly linked to MERS-CoV outcome in patients (25). As a mechanism to promote viral replication, coronaviruses encode for proteins that can actively antagonize cellular signaling pathways, which leads to the host establishment of an antiviral state (26). The coronavirus nsp3 multifunctional protein contains numerous domains including the interferon antagonist papain-like protease (PLpro) domain. PLpro is a multifunctional cysteine protease that hydrolyzes peptide and isopeptide bonds in viral and cellular substrates, essential functions for coronavirus replication. In SARS-CoV, the main roles of PLpro enzymatic activity involve processing of the replicase polyprotein at the N-terminus of pp1a, releasing the nonstructural proteins (nsp) nsp1, nsp2 and nsp3 (27). Importantly, because of the essentiality of these events, inhibition of PLpro enzymatic activity is an ongoing approach for the development of anticoronaviral drugs (28-38). Other enzymatic activities involve the removal of the cellular substrates ubiquitin (Ub), termed deubiquitination (DUB), and the interferon stimulated gene 15 (ISG15), termed deISGylation, from host cell proteins (reviewed in (39)). Processing of the replicase polyprotein (40, 41) and cellular DUB/deISGylation activities (41, 42) have also been recently characterized for the PLpro domain from MERS-CoV. The DUB and deISGylating activities of PLpro have important implications during the PLpro-mediated interferon (IFN) antagonism of the host innate immune response. We recently demonstrated that the PLpro domain from MERS-CoV exhibits both DUB and deISGylating activity in host cells and that these activities block the production of interferon β (IFNβ) in transfected cells (42). Similarly,

Yang et al. showed that MERS-CoV PLpro blocks the signaling pathway that leads to the activation of the IFN regulatory factor 3 (IRF3) (41).

Most of the findings involving the cellular functions of PLpro were initially elucidated with the PLpro domain from SARS-CoV and later with HCoV-NL63 and MHV (43-48). However, the exact mechanism by which coronavirus PLpros performs their multifunctional roles via the recognition and catalysis of viral and cellular substrates remains elusive. The relatively low amino acid conservation among HCoV PLpro domains suggests that there are unique mechanistic aspects to each enzyme. Therefore, in order to better understand the mechanism behind CoV PLpro-mediated antagonism of the innate immune response and to develop anti-coronaviral inhibitors, further research must emphasize the enzymatic characterization of the PLpro domain from newly discovered human coronaviruses. Here we report the purification, biochemical and kinetic characterization, and substrate specificity of the PLpro domain from MERS-CoV nsp3. A detailed comparison between MERS-CoV PLpro and SARS-CoV PLpro steady-state kinetic parameters, substrate preferences and inhibition is also presented and sheds light on the convergent and divergent functional roles of these two enzymes.

MATERIALS AND METHODS

Expression and enzymatic activity of MERS-CoV PLpro N-terminal deletion constructs in HEK293T cells.

Cells and transfections. HEK293T cells and BHK-21 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 cells in cell culture plates (Corning) using Lipofectamine 2000 for BHK-21 cells or cell bind plates (Corning) for HEK293T cells using *Trans*IT-LT1 Reagent (Mirus) according to manufacturer's protocols.

Expression constructs. The MERS-CoV PLpro (pcDNA-MERS-PLpro) expression plasmid and generation of the catalytic mutant were described previously (40). The 20, 40, and 60 N-terminal deletions of MERS-CoV PLpro ubiquitin-like domain (UBL, designated N20, N40, and N60) with in frame C-terminal V5 tag were generated by PCR amplification from pcDNA-MERS-PLpro using a forward primer (N20-Fwd: AGTGAATTCACCATGAAAAATACTTATCGGTCTC; N40-Fwd: AGTGAATTCACCATGGATACTATTCCCGACGAG; or N60-Fwd: AGTGAATTCACCATGGATGAGACTAAGGCCCTG) and a reverse primer PLpro-Rev: CGGGTTTAAACTCATGTTGAATCCAATC, and ligated into pcDNA3.1-V5/His-B vector (Invitrogen). For the *trans*-cleavage assay, the nsp2/3-GFP substrate construct was kindly provided by Ralph Baric (University of North Carolina) (44). For the luciferase assay experiments, we used IFNβ-Luc provided by John Hiscott (Jewish General Hospital, Montreal, Canada) and the *Renilla*-luciferase expression plasmid pRL-TK (Promega) as previously described (45). The pEF-BOS MDA5 (Addgene #27225) expression plasmid was a gift

from Kate Fitzgerald (University of Massachusetts Medical School). The epitope tagged constructs for the DUB and de-ISGylation assays including pcDNA3.1-Flag-Ub (provided by Dr. Adriano Marchese, Loyola University Medical Center), pcDNA3-myc6-mISG15 (a gift from Dr. Min-Jung Kim, Pohang University of Science and Technology, Pohang, Republic of Korea), and the E1, E2 and E3 ISG15 conjugating enzymes expressed by pcDNA3-Ube1L, pcDNA3-UbcH8, and pcDNA-Herc5 (provided by Dr. Robert M. Krug, University of Texas) were used as described below.

DeISGylating and DUB Activity Assays. For deISGylating assay, BHK-21 cells in 24-well plates were co-transfected with 200 ng of MERS-CoV PLpro plasmids, 250 ng pISG15-myc, 125 ng pUbcH8, 125 ng pUbe1L, and 125 ng pHerc5. For DUB assay, HEK293T cells were transfected with 300 ng Flag-Ub plasmid and 1 µg MERS-CoV PLpro plasmids. At 18 hours post-transfection, cells were lysed with lysis buffer A (4% SDS, 3% dithiothreitol (DTT), and 65 mM Tris, pH 6.8). Proteins were separated by SDS-PAGE, and transferred to PVDF membrane. Following transfer, the membrane was blocked using 5% dried skim milk in TBST buffer (0.9% NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% Tween 20) for 2 hours at ambient temperature. For deISGylating assay, the membrane was incubated with mouse anti-myc antibody (MBL) at the dilution of 1:2,500 overnight at 4°C. For DUB assay, the membrane was incubated with mouse anti-Flag M2 antibody (Sigma) at the dilution of 1:2,000 for 1 hour at ambient temperature. The membrane was washed 3 times for 10 minutes in TBST buffer. The membrane was then incubated with secondary goat-anti-mouse-HRP antibody at the dilution

1:10,000 (Amersham) for 1 hour at ambient temperature. After washing in TBST buffer the detection was performed using Western Lighting Chemiluminescence Reagent Plus (PerkinElmer) and visualized using ProteinSimple FluorChem® E system. The membrane was probed with mouse anti-V5 antibody (Invitrogen) at the dilution 1:5,000 to verify the expression of PLpro.

Luciferase Assay. HEK293T cells in 24-well plates were transfected with 50 ng Renilla-luciferase, 100 ng IFN-β-luc, and increasing doses of MERS-CoV PLpro UBL-deleted mutants (25, 50, and 100 ng), or 100 ng wild-type or catalytic mutant MERS-CoV PLpro expression plasmids. As a stimulation, the cells were transfected with 150 ng pEF-BOS MDA5. At 16 hours-post transfection cells were lysed using 1X Passive Lysis buffer (Promega). The *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 and analyzed by SDS-PAGE for the detection of PLpro expression as described above.

Construction of the MERS-CoV PLpro expression plasmid. The PLpro catalytic domain of nsp3 (1484–1802aa) from MERS-CoV was synthesized with codon optimization for *E.coli* expression by Bio Basic Inc. The gene was inserted into Bio Basic's standard vector pUC57. The MERS-CoV PLpro coding region (1484–1802aa) was amplified using forward and reverse primers containing

complementary sequences to an expression plasmid, pEV-L8, and PLpro at the -5' and -3', respectively. The PCR amplicon was then inserted into the pEV-L8 vector by ligation-independent recombinant cloning using the linearized pEV-L8 vector digested by *Ssp*I and XL1-Blue supercompetent cells. The resulting MERS-CoV pEV-L8-PLpro expression plasmid was transformed into *E. coli* BL21(DE3) for protein expression.

Expression and purification of MERS-CoV PLpro. Four liters of *E. coli* BL21(DE3) cells containing MERS-CoV pEV-L8-PLpro (1484–1802aa) were grown for 24 hours at 25°C in media containing 3 g KH_2PO_4, 6 g Na_2HPO_4, 20 g Tryptone, 5 g Yeast Extract, 5 g NaCl, pH 7.2, supplemented with 0.2% lactose, 0.6% glycerol, 0.05% glucose and 50 µg/ml kanamycin. Approximately, 29 g of cells were harvested by centrifugation (18,500 x g for 30 minutes at 4°C) and resuspended in 125 ml of buffer A (20 mM Tris, pH 7.5, 500 mM NaCl, 10 mM imidazole, 10 mM 2-mercaptoethanol (βME) and 10% glycerol) containing lysozyme and *DNase*I. The resuspended cells were lysed on ice via sonication, and the cells debris was pelleted by centrifugation. The clarified lysate was loaded at 2 ml/min onto a 5 ml HisTrap™ FF column (GE Healthcare) pre-charged with Ni^{2+}. Unbound proteins were washed with 5 column volumes (CV) of buffer A. Bound proteins were eluted using a linear gradient of 0% to 100% buffer B (20 mM Tris, pH 7.5, 500 mM NaCl, 500 mM imidazole, 10 mM βME and 10% glycerol), at 2 ml/min, followed by a 5 x CV 100% buffer B wash. Fractions containing MERS-CoV PLpro were concentrated and buffer exchanged into

buffer C (20 mM Tris, pH 7.5, 10 mM βME and 10% glycerol) and loaded onto a Mono Q 10/100 GL (GE Healthcare) column equilibrated with buffer C. MERS-CoV PLpro was eluted with a linear gradient of 0% to 60 % Buffer D (20 mM Tris, pH 7.5, 500 mM NaCl, 10 mM βME and 10% glycerol). Fractions containing MERS-CoV PLpro were concentrated to 500 µl and loaded onto a HiLoad 26/60 Superdex 75 (GE Healthcare) equilibrated with final buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 10 mM DTT and 5% glycerol). For enzyme kinetic studies, the $(his)_8$-tag was removed via TEV protease cleavage prior to the MonoQ chromatography step. Aliquots of 100 µl at 10 mg/ml were flash-frozen in liquid nitrogen in buffer containing 10 mM Tris, pH 7.5, 150 mM NaCl, 10 mM DTT and 20 % glycerol.

Expression and Purification of SARS-CoV PLpro. The PLpro catalytic domain of nsp3 from SARS-CoV was expressed and purified according to our previously published methods (28).

Size-Exclusion chromatography & multi-angle light scattering (SEC-MALS). A total of 100 µl of MERS-CoV PLpro at concentrations of 4.2 mg/ml, 2.1 mg/ml and 1.0 mg/ml in buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM TCEP and 5% glycerol) were used for analytical size-exclusion chromatography (SEC) coupled with multi-angle light scattering (MALS) analyses. The SEC was perform using a GE Healthcare Superdex™ 75 analytical gel filtration column at a flow rate of 0.5 ml/min and was coupled to a DAWN HELEOS™ MALS instrument (Wyatt

Technology) and an Optilab™ rEX (Wyatt Technology). The on-line measurement of the intensity of the Rayleigh scattering as a function of the angle as well as the differential refractive index of the eluting peak in SEC was used to determine the weight average molar mass ($\overline{M}$w) of eluted oligomers and protein complexes using the ASTRA™ (Wyatt Technologies) software. The number average molar mass ($\overline{M}_{\mathrm{n}}$) was also determined to calculate the polydispersity index ($\overline{M}_{\mathrm{w}}/\overline{M}_{\mathrm{n}}$) of each peak.

Steady-state kinetic studies. The enzymatic rates of MERS-CoV and SARS-CoV PLpros catalyzed reactions were determined using a modified version of our previously described methods (29, 49). The rate of hydrolysis of a peptide substrate, Z-RLRGG-AMC (Bachem), that contains the C-terminal sequence (RLRGG) of ubiquitin (Ub), and of the full-length Ub and ISG15 substrates, Ub-AMC (LifeSensors, Inc.) and ISG15-AMC (Boston Biochem/R&D Systems), were determined by monitoring the increase in fluorescence of the AMC group released (excitation λ= 340 nm; emission λ= 460 nm) as a function of time. The assays were conducted at 25°C and the fluorescence was monitored using an EnVision® multi-mode plate reader from PerkinElmer. The initial slope of the reaction in units of fluorescence intensity per unit time (AFU/min) was converted into the amount of hydrolyzed substrate per unit of time (μM/min) using a standard curve generated from the fluorescence measurements of well-defined substrate concentrations after complete hydrolysis of the peptide-substrates by PLpro to liberate all AMC. All enzymatic reactions were carried out in triplicate.

Assays were initiated by the addition of PLpro in assay buffer (50 mM HEPES, pH 7.5, 0.1 mg/ml BSA, 150 mM NaCl and 2.5 mM DTT). For the Z-RLRGG-AMC assays, the 100 µl reaction was conducted in a 96-well black microplate containing varying substrate concentrations (50 µM to 1.6 µM). The reactions were initiated by the addition of 140 nM of SARS-CoV PLpro, or 1.6 µM of MERS-CoV PLpro. For Ub-AMC and ISG15-AMC assays, the 30 µl reactions were carried out in half area, 96-well black microplates from Corning. The Ub-AMC assay contained substrate concentrations varying from 25 µM to 0.08 µM. The reactions were initiated with enzymes to yield a final concentration of 32 nM for SARS-CoV PLpro and 80 nM MERS-CoV PLpro. The ISG15-AMC assay contained varying substrate concentrations from 16 µM to 0.03 µM, and the reactions were initiated with enzymes to yield a final concentration of 1 nM for SARS-CoV PLpro and 6.3 nM MERS-CoV PLpro. The initial rates of the reactions as a function of substrate concentration were fit to the Michaelis-Menten equation using the Enzyme Kinetics Module of SigmaPlot (v11 Systat Software Inc.). The resulting steady-state kinetic parameters (k_{cat} and K_M) and their associated errors (Δk_{cat} and ΔK_M) from the fits were then used to calculate k_{cat}/K_M values. The associated error in k_{cat}/K_M values $\Delta(k_{cat}/K_M)$ was calculated from the following equation; $\Delta(k_{cat}/K_M) = (k_{cat}/K_M)((\Delta k_{cat}/k_{cat})^2+(\Delta K_M/K_M)^2)^{1/2}$. When the response of PLpro catalytic activity to increasing substrate concentrations was linear over the substrate concentration range investigated, i.e. when the enzyme could not be saturated with substrate, the apparent k_{cat}/K_M

values were determined by fitting the initial velocity data as a function of substrate concentration using linear regression.

Enzyme specific activity. To determine enzyme purity and yields during the purification procedure, the specific activity of MERS-CoV PLpro was measured using 250 nM of Ub-AMC in assay buffer containing 50 mM HEPES, pH 7.5, 0.1 mg/ml BSA, 150 mM NaCl and 2.5 mM DTT at 25°C using the same procedure as described above.

Protein concentration. The protein concentration during the purification was determined using the cuvette-based Bio-Rad Bradford Protein Assay.

Inhibition assays and IC_{50} value (K_i Value) determination. Inhibition of MERS-CoV PLpro by free ubiquitin, free ISG15 and chemical compounds was determined using 30 µl assays containing 250 nM Ub-AMC as substrate and were performed in triplicate using half area, 96-well black microplates from Corning. The final enzyme concentrations were 32 nM for SARS-CoV PLpro and 80 nM MERS-CoV PLpro. The assays were performed at 25°C with increasing concentrations of either free Ub or ISG15 over a range of 55 µM to 0.11 µM. Inhibition assays with compounds were performed at fixed compound concentrations of 100 µM for known SARS-CoV PLpro inhibitors (28) or 10 µM for E64. Inhibition assays for HCoV-NL63 PLP2 were performed as described previously (28). Initial rate measurements were determined as described above.

336 IC_{50} values for free ubiquitin and ISG15 were determined by plotting the percent
337 inhibition value versus concentration of inhibitor and then fitting the data using
338 non-linear regression using the equation, $\%I = \%I_{max}/(1+(IC_{50}/[I]))$ and the
339 Enzyme Kinetics module in the software SigmaPlot (v11 Systat Software Inc).
340 The resulting IC_{50} values under these experimental conditions are within 5% of
341 the calculated K_i values, which is within experimental error, assuming competitive
342 inhibition (50).
343
344 **Polyubiquitin chain-processing assays.** The ability of MERS-CoV and SARS-
345 CoV PLpros to process polyubiquitin chains was determined using assays
346 containing 50 mM HEPES, pH 7.5, 0.01 mg/ml BSA, 100 mM NaCl and 2 mM
347 DTT. For SARS-CoV PLpro, a total of 20 nM of enzyme was incubated at room
348 temperature with 12 µg of different ubiquitin chain substrates including K48-
349 linked $Ub_{(4)}$, K48-linked $Ub_{(5)}$, K63-linked $Ub_{(6)}$ and linear $Ub_{(4)}$. For MERS-CoV
350 PLpro, a total of 30 nM of enzyme was incubated with 6 µg of K48-linked $Ub_{(5)}$,
351 K63-linked $Ub_{(6)}$ and linear $Ub_{(4)}$. Reaction aliquots of 10 µl were quenched at
352 different time points after the start of the reaction using NuPAGE® sample buffer
353 (Life Techonologies™) to a final concentration of 1X. Identification of the cleaved
354 products was performed on a NuPAGE® Bis-Tris gel (Life Techonologies™) and
355 visualized after staining with Coomassie blue. Each gel was then photographed
356 using a ProteinSimple FluorChem® E system. All substrates were purchased
357 from BostonBiochem.
358

Generation of a MERS-CoV PLpro Structural Model. Homology models of MERS-CoV PLpro and HCoV-NL63 PLP2 were generated using the structure of SARS-CoV PLpro in complex with ubiquitin aldehyde (PDB: 4MM3) as the template and the automated web-based homology modeling server 3D-JIGSAW (Bimolecular Modeling Laboratory, Cancer Research UK, England). Further model refinement was performed using the programs Phenix (51) and Coot (52)

Results

The UBL domain of MERS-CoV nsp3 is not required for the proteolytic, deubiquitinating or deISGylating activities of PLpro. We previously described the construction of an expression plasmid for a region of nsp3 (residues 1485-1802) in MERS-CoV that produced an active form of PLpro, capable of catalyzing the *trans*-cleavage of an nsp2/3-GFP substrate in HEK293T cells (40), and deubiquitination and deISGylation of host cell proteins (42). This expression construct contains both the PLpro catalytic and UBL domain (also known as the UB2 domain (53)) of MERS-CoV nsp3 with the addition of 2 amino acids at the N-terminus (methionine and alanine) to allow efficient translation and a V5 epitope tag on the C-terminus for V5 antibody detection (Figure 1A). To probe the necessity of the UBL domain to the catalytic function of the PLpro domain in MERS-CoV, we truncated the N-terminus by 20, 40 and 60 amino acids (Figure 1A) within the UBL domain and evaluated the effects of these truncations on the MERS-CoV PLpro protease activity in cell culture. HEK293T cells were transfected with each of the UBL-deleted mutants along with plasmid DNA

382 expressing the SARS-CoV nsp2/3-GFP substrate (40). Efficient catalytic
383 processing of the nsp2/3-GFP substrate is observed for full length wild-type (WT)
384 and all UBL-deleted mutants (N20, N40 and N60) (Figure 1B). In contast, the
385 MERS-CoV PLpro catalytic cysteine 1594 mutant (CA), as shown previously, is
386 unable to process the substrate (40). These results strongly suggest that the
387 UBL domain of MERS-CoV PLpro is not required for proteolytic processing.
388
389 We next tested whether the UBL domain was required for deubiquitination and
390 deISGylation of host cell proteins in cell culture (42). To determine the
391 deISGylating activity of MERS-CoV PLpro constructs, we transfected HEK293T
392 cells with a c-myc-ISG15 plasmid, ISG15 conjugation machinery, and either
393 MERS-CoV PLpro WT, catalytic mutant or UBL-deleted mutants. We harvested
394 cell lysates at 18 hours post-transfection to evaluate the presence of ISGylated
395 proteins (Figure 1C). We found that PLpro WT and UBL-deleted mutants can
396 deconjugate ISG15 from multiple cellular substrates and that the catalytic
397 cysteine is required for the deconjugation of ISG15. To assess the requirement
398 of MERS-CoV UBL domain for deubiquitinating activity (DUB) of MERS-CoV
399 PLpro, we transfected HEK293T cells with plasmid expressing Flag-Ub, and
400 either MERS-CoV PLpro WT, catalytic mutant or UBL-deleted mutants. We
401 determined that both PLpro WT and UBL-deleted mutants can deubiquitinate
402 multiple cellular substrates, and that PLpro catalytic activity is required for DUB
403 activity (Figure 1D). Together, these data indicate that the UBL domain is not
404 required for the deISGylating and DUB activities of MERS-CoV PLpro.

405 **The UBL domain of MERS-CoV PLpro is not required for its IFN antagonism**
406 **activity.** The observation that the UBL domain of MERS-CoV PLpro is not
407 required for its catalytic activities is consistent with previous studies where it was
408 shown that deletion of the PLpro UBL domain from nsp3 of SARS-CoV did not
409 alter intrinsic proteolytic and DUB activities (44). However, the role of the UBL
410 domain in interferon antagonism is controversial (44, 45). Therefore, we
411 investigated whether MERS-CoV PLpro without its UBL domain can inhibit
412 MDA5-mediated induction of IFNβ. MDA5 has been implicated in recognition of
413 coronaviruses during virus infection (54) and we showed previously that MERS-
414 CoV PLpro with an intact UBL functions through this pathway (42). We
415 transfected HEK293T cells with plasmids expressing IFN-β-luciferase, *Renilla*
416 luciferase, pEF-BOS-MDA5 (55) and either MERS-CoV PLpro WT or catalytic
417 mutant at a single concentration, or increasing concentrations of UBL-deletion
418 mutants N20, N40 or N60. At 16 hours post-transfection we assessed luciferase
419 reporter activity. We determined that MERS-CoV PLpro without its UBL domain
420 can potently inhibit MDA5-mediated induction of IFNβ in a dose-dependent
421 manner and that catalytic activity is required for IFNβ antagonism (Figure 2).
422
423 **Expression and Purification of the MERS-CoV PLpro and UBL domains of**
424 **nsp3.** The results of the UBL-deletion analysis of MERS-CoV PLpro suggest that
425 we could potentially express and purify a version of MERS-CoV PLpro without its
426 UBL domain. However, we previously attempted to express and purify a version
427 of SARS-CoV PLpro without its UBL domain and found it to be inherently

unstable during purification as it lost catalytic activity over time (56). Therefore, we decided to overexpress and purify MERS-CoV PLpro with its UBL domain intact (residues 1485-1802, herein called PLpro) so that we could make a direct comparison with the enzymatic activity of purified SARS-CoV PLpro.

The PLpro domain from MERS-CoV was overexpressed in *E. coli* and purified via three chromatographic steps: 1) Ni^{2+}-charged affinity chromatography followed by removal of the $(his)_8$-tag via TEV protease cleavage, 2) Mono-Q strong anion-exchange and 3) Superdex 75 size-exclusion. A summary of the purification procedure including the enzyme activity yields, fold-purification and resulting specific activity is presented in Table 1. An SDS-page analysis of purified PLpro compared to its expression level in crude lysate is shown in Figure 3A. The final purified MERS-CoV PLpro enzyme is judged to be over 98% pure. A total yield of 20 mg per liter of cell culture can be obtained by this method. Further experimentation revealed that the addition of reducing agent (10 mM βME) and 5% – 10% glycerol is required to avoid protein aggregation during purification and final concentration. The concentrated enzyme was stored at -80°C.

Quaternary Structure of MERS-CoV PLpro. We used size-exclusion chromatography coupled with multi-angle light-scattering (SEC-MALS) to determine the oligomeric state of MERS-CoV PLpro as well as any potential for aggregation. SEC-MALS analysis revealed an excellent monodispersity of > 90% at the three tested PLpro concentrations (Figure 3B), in which each sample eluted at the same retention time. For each peak the calculated molecular

weight (M_w) is 38.4 ± 3.3 kDa, which is consistent with both the apparent M_w (37 kDa) on SDS-PAGE and the expected M_w for a monomer (38.1 kDa). These results indicate that MERS-CoV PLpro exists almost exclusively as a monomer in solution with no detectable higher order oligomers or aggregates. The unliganded form of SARS-CoV PLpro on the other hand tends to form a trimer at higher protein concentrations and it was this form that crystallized with a trimer in the asymmetric unit (49, 56).

Kinetics of hydrolysis of Z-RLRGG-AMC, Ub-AMC and ISG15-AMC substrates by MERS-CoV and SARS-CoV PLpros. The rates of MERS-CoV PLpro and SARS-CoV PLpro catalyzed reactions were examined using three fluorescence-based substrates including the peptide Z-RLRGG-AMC, which consists of the Ub and ISG15 C-termini sequence, Ub-AMC and ISG15-AMC. The kinetic parameters for each coronavirus PLpro and each substrate were determined under the same assay conditions on the same day so that side-by-side experiments could be made for the most direct comparisons. Due to limitations from inner filter effects produced from the AMC fluorophore at high concentrations of substrate, the assays with Z-RLRGG-AMC were performed at no higher than 50 μM substrate concentration. The kinetic responses of MERS-CoV and SARS-CoV PLpros to increasing concentrations of the 3 substrates are shown in Figure 4, and the resulting kinetic parameters are summarized in Table 2. As previously observed for SARS-CoV PLpro (57-59), MERS-CoV PLpro exhibited a linear response to increasing substrate concentration with the peptide

substrate Z-RLRGG-AMC (Figure 4A). Since both enzymes were unable to be saturated with the Z-RLRGG-AMC substrate, we calculated the apparent $(k_{cat}/K_M)_{apparent}$ values from the slope of the line in Figure 4A in order to compare their catalytic efficiencies (Table 2). Surprisingly, the activity of MERS-CoV PLpro with the Z-RLRGG-AMC peptide substrate is significantly lower (~100-fold) compared to SARS-CoV PLpro (k_{cat}/K_M = 0.003 ± 0.0001 μM^{-1} min^{-1} for MERS-CoV PLpro versus 0.3 ± 0.1 μM^{-1} min^{-1} for SARS-CoV PLpro). This result suggests that there are significant differences between the enzyme's active sites in terms of recognition and catalysis of the peptide substrate.

In contrast to the Z-RLRGG-AMC peptide substrate, the response of both PLpro enzymes from MERS-CoV and SARS-CoV to increasing concentrations of the ISG15-AMC substrate is hyperbolic over a concentration range of 0.03 µM to 16 µM (Figure 4C). The kinetic response of MERS-CoV PLpro to increasing concentrations of the substrate Ub-AMC is also clearly hyperbolic over a substrate concentration range of 0.08 µM to 25 µM (Figure 4B). Therefore, the kinetic responses of both MERS-CoV and SARS-CoV PLpros to increasing substrate concentrations were fit to the Michaelis-Menten equation to derive the V_{max} and K_M values and these values are given in Table 2.

Over a concentration range of 0.08 µM to 25 µM, SARS-CoV PLpro exhibits a curvilinear response to increasing concentrations of Ub-AMC (Figure 4B). The downward curvature becomes apparent after a concentration of 5 µM suggesting

498 that the response of SARS-CoV PLpro to Ub-AMC follows Michaelis-Menten
499 kinetics but that the enzyme is still undersaturated at a concentration of 25 µM.
500 Since the initial rate data were obtained in triplicate, and the error associated with
501 each measurement was small, we decided to fit the kinetic data to the Michaelis-
502 Menten equation to derive estimates of the kinetic parameters V_{max} and K_M with
503 the expectation that the error in these fitted parameters would be higher than the
504 other values reported in Table 2. However, the errors in the fitted parameters for
505 SARS-CoV PLpro with Ub-AMC are within the errors associated with V_{max} and K_M
506 for the response of MERS-CoV PLpro with ISG15-AMC and Ub-AMC (Table 2).
507
508 The turnover number, k_{cat}, and the catalytic efficiency, k_{cat}/K_M, were calculated for
509 each enzyme (Table 2). Based upon the k_{cat} values, SARS-CoV PLpro catalyzes
510 the turnover of the Ub-AMC and ISG15-AMC substrates approximately 4-fold
511 (75.9 min^{-1} versus 18.8 min^{-1}) and 14-fold (436 min^{-1} versus 32.6 min^{-1}) faster
512 than MERS-CoV PLpro. SARS-CoV PLpro is also 3-times more efficient than
513 MERS-CoV PLpro in hydrolyzing the ISG15-AMC substrate (k_{cat}/K_M = 29 μM^{-1}
514 min^{-1} versus 9.9 μM^{-1} min^{-1}). However, MERS-CoV and SARS-CoV PLpros are
515 equally efficient in hydrolyzing Ub-AMC as a substrate since their k_{cat}/K_M values
516 are each about 1.3 μM^{-1} min^{-1}, due to a ~4-fold equivalent difference between the
517 K_M and k_{cat} values between each enzyme.
518
519 In agreement with previous studies using these three substrates (57-59), SARS-
520 CoV PLpro has a significantly higher catalytic efficiency for hydrolysis of the

ISG15-AMC substrate over the Ub-AMC (~20-fold) and Z-RLRGG-AMC (~100-fold) substrates. A similar pattern in substrate preference is also observed for MERS-CoV PLpro as it hydrolyzes the ISG15-AMC (k_{cat}/K_M value of 9.9 μM^{-1} min^{-1}) substrate approximately 8-times more efficiently than the Ub-AMC substrate (k_{cat}/K_M = 1.3 μM^{-1} min^{-1}) and 3,300-times more efficiently than the Z-RLRGG-AMC substrate. Although MERS-CoV and SARS-CoV PLpros exhibit different kinetic parameters for each substrate, they still each prefer a substrate containing ISG15 over Ub.

The most striking kinetic differences between MERS-CoV and SARS-CoV PLpros appear to be in the efficiencies of hydrolysis of the Z-RLRGG-AMC and ISG15-AMC substrates. The origins of the differences for the Z-RLRGG-AMC substrate cannot be ascribed to either k_{cat} or K_M since we cannot determine these individual kinetic parameters for this substrate. However, the higher activity of SARS-CoV PLpro for ISG15-AMC stems from the more significant differences in the k_{cat} values (436 min^{-1} for SARS versus 32.6 min^{-1} for MERS) than the K_M values (15.1 µM for SARS versus 3.3 µM for MERS). Interestingly, if one assumes that the K_M values reflect the relative affinity of the enzymes for the substrate, i.e. $K_M = K_d$, then both ISG15-AMC and Ub-AMC appear to interact more strongly with MERS-CoV PLpro than to the SARS-CoV PLpro enzyme.

Since K_M values often do not represent the K_d values in enzyme catalyzed reactions as a result of kinetic complexity, i.e. $K_M \neq k_{-1}/k_1$, we determined the

affinities of free ISG15 and Ub for MERS-CoV and SARS-CoV PLpros via steady-state kinetic inhibition studies. Under the experimental conditions utilized and assuming competitive inhibition, the IC_{50} values determined for ISG15 and Ub are close to the actual K_i values (50). The IC_{50} value for free Ub and ISG15 were therefore determined from a dose-response assay (Figures 4D and 4E). The affinity of free Ub for MERS-CoV PLpro (IC_{50} = 21.3 ± 4.0 μM) is substantially higher than for SARS-CoV PLpro since no inhibition is observed up to a concentration of 60 μM. In contrast, the affinity of free ISG15 for SARS-CoV PLpro (IC_{50} = 18.4 ± 12.2 μM) is significantly higher than for MERS-CoV PLpro (IC_{50} = 54.4 ± 17.7 μM) (Table 2). The differences in IC_{50} values suggest that MERS-CoV PLpro binds Ub significantly tighter than SARS-CoV PLpro and that SARS-CoV PLpro binds ISG15 tighter than MERS-CoV PLpro. Together, the steady-state kinetic studies suggest that MERS-CoV and SARS-CoV PLpros differ in their abilities to recognize and hydrolyze ubiquitinated and ISGylated substrates.

Recognition and Processing of Ubiquitin Chains by MERS-CoV and SARS-CoV PLpros. Recent X-ray structural and kinetic studies have revealed the complexity behind SARS-CoV PLpro substrate specificity towards polyubiquitin and ISG15 substrates (60). SARS-CoV PLpro was shown to be significantly more active towards K48-linked Ub chains compared to K63-linked Ub chains as a result of the enzyme possessing a unique bivalent binding site for K48-linked di-Ub chains. Since the molecular structure of ISG15 resembles that of di-Ub, the

preference of SARS-CoV PLpro for ISG15 over Ub is presumed to result from this similarity (60). Therefore, we next examined whether any conservation exists in the abilities of MERS-CoV PLpro and SARS-CoV PLpro to recognize and process K48-linked, K63-linked or linear polyubiquitin chains.

MERS-CoV and SARS-CoV PLpros, at concentrations of 1.6 nM, were first incubated overnight with 1 µg each of the Ub-based substrates; K48-linked $Ub_{(5)}$, K63-linked $Ub_{(6)}$ and linear Met1-$Ub_{(4)}$. Analysis of the reaction products by SDS-PAGE indicated that only SARS-CoV PLpro was capable of processing the K48-linked $Ub_{(5)}$ and K63-linked $Ub_{(6)}$ substrates under these conditions as little to no reaction products were observed with the MERS-CoV PLpro reactions (data not shown). The low activity of MERS-CoV PLpro was the first indication that the enzyme has poorer catalytic activity towards polyubiquitin chains than the SARS-CoV PLpro enzyme. Therefore, in order to detect any patterns in the cleaved products by MERS-CoV PLpro, the PLpro enzyme concentration was increased to 5 nM and the reaction products were analyzed over a period of 18 hours by SDS-PAGE (Figure 5). Over the first 1 hour of the reaction of MERS-CoV PLpro with both K48-linked $Ub_{(5)}$ and K63-linked $Ub_{(6)}$ substrates, the accumulation of lower molecular weight ubiquitin-chain products was apparent (Figures 5A and 5B). We observed no significant differences in the debranching patterns or processing rates of K48- vs. K63-linked substrates by MERS-CoV PLpro over a 1 hour time period, and after 18 hours the reactions were almost complete. Neither

589 MERS-CoV or SARS-CoV PLpro enzymes are able to hydrolyze linear $Ub_{(4)}$
590 (Figure 5C).
591
592 The processing of K48-linked $Ub_{(5)}$ and K63-linked $Ub_{(6)}$ substrates by MERS-
593 CoV PLpro ultimately resulted in the formation of a mono-Ub species after 18
594 hours. SARS-CoV PLpro, on the other hand, hydrolyzed K48-linked $Ub_{(5)}$ (Figure
595 5D) significantly faster than K63-linked $Ub_{(6)}$ (Figure 5E). Moreover, SARS-CoV
596 hydrolysis of K48-linked $Ub_{(5)}$ led to the accumulation di-Ub products over time
597 (Figures 5D and 5F), whereas hydrolysis of the K63-linked $Ub_{(6)}$ substrate was
598 much slower and did not lead to the accumulation of di-Ub species. Because
599 SARS-CoV PLpro has a higher affinity for K48-linked di-Ub molecules (60), the
600 accumulation of K48-linked di-Ub in the SUb2 and SUb1 binding subsites leads
601 to product inhibition by slowing down the rate of debranching of the longer K48-
602 linked Ub chains or the further cleavage of di-Ub into mono-Ub. This
603 phenomenon is better observed during the processing of polyubiquitin chains
604 with an even number of ubiquitins such as K48-linked $Ub_{(4)}$. With this substrate,
605 little to no mono-Ub is produced during the course of the reaction (Figure 5D),
606 whereas cleavage of K48-linked $Ub_{(5)}$ produces $Ub_{(4)}$, $Ub_{(3)}$, $Ub_{(2)}$, and mono-Ub
607 over time (Figure 5F). However, for MERS-CoV PLpro, debranching of K48-
608 linked polyubiquitin chains with an even or odd number of ubiquitins results in an
609 increase of mono-Ub. These results support a model whereby MERS-CoV PLpro
610 does not interact with K48-linked polyubiquitin chains via a bivalent recognition
611 mechanism, as does SARS-CoV PLpro (60). Therefore, recognition of

polyubiquitin chains by MERS-CoV PLpro occurs primarily through a monovalent Ub interaction presumably within the zinc finger and palm regions of the enzyme.

Inhibitors of SARS-CoV PLpro and HCoV-NL63 PLP2 do not inhibit MERS-CoV. Our most recent effort towards the development of SARS-CoV PLpro inhibitors generated a new series of competitive inhibitors with significant improvements towards the development of anti-SARS drugs (28). These newer inhibitors have improved inhibitory potency and SARS-CoV antiviral activity, better metabolic stability and lower cytotoxicity than our previous generations of inhibitors. Furthermore, none of the compounds show off-target inhibitory activity towards a number of human DUBs or cysteine proteases. Interestingly, a number of the compounds also show inhibitory activity against the PLP2 catalytic domain of nsp3 from HCoV-NL63, providing a basis for the potential development of broader-spectrum inhibitors against various CoV PLpro domains. Therefore, we tested whether any of these compounds have the ability to inhibit the enzymatic activity of MERS-CoV PLpro. The inhibitory activity of 28 compounds was tested against MERS-CoV PLpro, SARS-CoV PLpro and HCoV-NL63 PLP2 and the data are shown as percent inhibition in Figure 6. Surprisingly, even though both SARS-CoV PLpro and MERS-CoV PLpro belong to Group 2 coronaviruses and share significantly higher amino-acid sequence homology (~50% homology), no significant inhibition of MERS-CoV PLpro was observed for any of the compounds at a concentration of 100 μM. In contrast, HCoV-NL63 PLP2 is from the more distantly related Group 1 coronavirus and

635 shares only about 30% homology with SARS-CoV PLpro, yet it is inhibited by
636 over half of the compounds and 10 of them produce greater than 50% inhibition.
637 These results suggest that a low level of sequence conservation may exist
638 between the inhibitor-binding site that is not necessarily related to the
639 coronavirus group specification and that subtle structural differences may be
640 significant determinants when attempting to develop broad-spectrum inhibitors
641 against CoV PLpro enzymes. In support of this hypothesis, we found that E64, a
642 cysteine-protease inhibitor that reacts covalently with the active site cysteine of
643 proteases, exclusively inhibited HCoV-NL63 PLP2 but not MERS-CoV or SARS-
644 CoV PLpros suggesting that the binding site near the active site cysteine is not
645 highly conserved among these PLpros.
646
647 **Homology model of MERS-CoV PLpro.** To gain insight into the structural
648 differences between MERS-CoV and SARS-CoV PLpros that may elicit the
649 differences in their substrate and inhibitor specificity, we generated an energy-
650 minimized molecular model of MERS-CoV PLpro based on the available
651 structures of SARS-CoV PLpro (Figure 7). The homology model was built and
652 refined against the electron density of SARS-CoV PLpro in complex with Ub
653 aldehyde (PDB:4MM3) (60). The resulting structural model of MERS-CoV PLpro
654 was analyzed by overlaying it with the structures of SARS-CoV PLpro in complex
655 with Ub and inhibitor **3k** (28). The domains of SARS-CoV PLpro (aa 1541-1884)
656 and MERS-CoV PLpro (aa 1484-1802) share 52% overall homology. During
657 model refinement, we examined the substrate/inhibitor-binding domain at the

enzyme subsites in the palm domain, the oxyanion hole and the ridge region (60) of the thumb domain (Figure 7A). The resulting and refined homology model was then compared to the recently reported X-ray crystal structure of unliganded MERS-CoV PLpro (61). The structures were found to be very similar with the exception of the active site loop that is missing in the X-ray structure as a result of no observable electron density. More details of the comparison, especially around the active site loop can be found in Figure 8. Since our homology structure coincided closely with the X-ray structure and since our structure contains an energy minimized model of the active site loop, we continued our analysis with the homology model and indicate any major differences with the X-ray structure which were few in the structural regions of interest.

The X-ray crystal structure of SARS-CoV PLpro in complex with Ub-aldehyde revealed that the majority of PLpro-Ub interactions occur between PLpro and the five C-terminal (RLRGG) residues of Ub (60, 62). Therefore, we examined the amino acid conservation at the enzyme subsites of MERS-CoV PLpro. We predict that only 8 out of 12 hydrogen bonds (H-bonds) are likely to be conserved in the MERS-CoV PLpro-Ub C-termini interactions, of which 5 H-bonds occur between Ub and the backbone of PLpro (Figure 7B). The loss of 4 H-bonds is due to the non-conserved replacements of E168, Y265 and W107 from SARS-CoV PLpro to R170, F271 and L108 in MERS-CoV PLpro, respectively. These predictions are in agreement with the kinetics studies, which show that SARS-CoV PLpro is 100-fold more active than MERS-CoV PLpro with the peptide

substrate Z-RLRGG-AMC (Table 2). Therefore, unlike SARS-CoV PLpro in which the Ub C-terminus provides a significant energetic contribution of binding, for MERS-CoV PLpro, greater binding energy is likely provided by interactions outside the Ub C-terminal RLRGG residues.

Other potential amino acid differences within the enzyme subsites could also explain the lack of inhibition by compounds designed to be inhibitors of SARS-CoV PLpro. A structural alignment of the MERS-CoV PLpro homology model with the X-ray structure of SARS-CoV PLpro in complex with inhibitor **3k** (28), depicting the amino-acid residues involved in SARS-CoV PLpro-inhibitor binding, is shown in Figure 7C. Because SARS-CoV PLpro inhibitors can also inhibit the PLP2 domain from HCoV-NL63, a homology model of HCoV-NL63 PLP2, constructed via the same approach used for MERS-CoV PLpro, is included for comparison in Figure 7C. From these two structural models, we predict that a number of amino acid differences between the enzymes occur within the hydrophobic pocket comprising P248 – P249, and at the flexible *β*-turn/loop (BL2 loop or Gly267-Gly272) known to participate in an induced-fit-mechanism of inhibitor association (28). Modeling of the *β*-turn/loop of MERS-CoV PLpro was significantly challenging due to the presence of an additional amino acid and therefore rendering a longer loop with absolutely no amino acid conservation to SARS-CoV PLpro. On the other hand, more conserved substitutions are predicted for HCoV-NL63 PLP2 in which Y269 and Q270, both important for binding of compound **3k** (28), are replaced by F255 and D265, respectively.

Another important difference is observed at the entrance of the active site in which L163 in SARS-CoV PLpro acts as a gatekeeper, blocking the access to the catalytic triad (56). Upon inhibitor binding, L163 folds backwards accommodating the substituted benzylamides groups of the inhibitors (28-30). For HCoV-NL63, this amino acid is replaced by K152 but yet in MERS-CoV PLpro, the less conserved replacement by P165 at this position could render the entrance to the active site much more rigid and therefore unable to accommodate inhibitor substituents.

Since bulky or rigid amino-acid residues at the S-sites hinder the access to the active site and catalytic cysteine, we then examined the oxyanion hole of HCoV-NL63 PLP2 as possible inhibitor-binding site for the covalent cysteine protease inhibitor E64 (Figure 7D). We found that the oxyanion hole of HCoV-NL63 PLpro is occupied by the small amino acid T96, compared to the bulky oxyanion hole residues W107 and L108 found in SARS-CoV PLpro and MERS-CoV PLpro, respectively. The presence of a smaller amino-acid residue in the oxyanion hole of HCoV-NL63 PLP2 could render a larger cavity at the S'-sites of the enzyme and thus explaining why E64 can only form a covalent adduct onto the catalytic cysteine of HCoV-NL63 PLP2.

We have shown that MERS-CoV PLpro does not share SARS-CoV PLpro substrate specificity at the SUb2 site for distal Ub molecules. Therefore, we examined the amino acid conservation at the ridge region of the thumb domain,

727 which is the site in SARS-CoV PLpro responsible for the SUb2-Ub interaction
728 (60). In our homology model we find very low amino acid conservation at the
729 ridge of the thumb domain. Moreover, the model suggests that a longer helix α2
730 (56) may exist at the SUb2 site (Figure 7E). Therefore, the lack of conservation
731 between MERS-CoV PLpro and SARS-CoV PLpro ridge region of the thumb
732 domain can explain why MERS-CoV PLpro cannot interact with Ub/UBL
733 modifiers with a bivalent mechanism of binding.
734
735 **Discussion**
736 The papain-like protease (PLpro) domains of coronavirus nsp3's are monomeric
737 enzymes that perform multiple cellular functions to facilitate viral replication
738 (reviewed in (39)). Among these functions is the essential role of recognizing
739 and processing the viral replicase polyprotein at the boundaries of nsp1/2, nsp2/3
740 and nsp3/4 (27, 40, 41, 63). Other physiological roles of CoV PLpros are less
741 understood but involve the removal of Ub (deubiquitination) and the ubiquitin-like
742 modifier ISG15 (deISGylation) from cellular proteins. The global removal of
743 ISG15 and ubiquitin from numerous host cell proteins has been shown to
744 interfere with the production of Type 1 interferon (IFN), which facilitates viral
745 evasion from the host's antiviral defenses (64). So far, the multifunctionality of
746 PLpro domains within nsp3 appears to be a conserved feature among CoVs as
747 at least one of the encoded two PLpro domains, typically PLP2, has isopeptidase
748 activity (43-45, 48, 57). However, SARS-CoV and MERS-CoV, which belong to
749 the *Betacoronavirus* group 2, encode only one PLpro domain within nsp3, which
750 is an ortholog to the PLP2 domain from other CoVs encoding two PLpro domains.

751 Although CoV PLpros catalyze the same chemical reaction, hydrolysis of peptide
752 and isopeptide bonds, recent structural and kinetic studies on the substrate
753 specificities of SARS-CoV PLpro demonstrate the uniqueness of SARS-CoV
754 PLpro among other CoV PLpros studied so far in terms of recognizing and
755 processing ubiquitin chains (60, 62). Those studies and the ones reported here
756 for MERS-CoV PLpro suggest that even the most closely related orthologs can
757 differ significantly in terms of substrate recognition, enzymatic activity and
758 inhibition by small molecule compounds. Such differences emphasize the
759 importance of investigating in detail the biochemical reaction mechanisms in
760 conjunction with *in cellular* activities to gain a better understanding of how CoV
761 PLpros conduct their multifunctional roles.
762
763 The steady-state kinetic characterization of MERS-CoV PLpro and SARS-CoV
764 PLpro reveals differences among their substrate preferences. Recent X-ray
765 structural analyses of SARS-CoV PLpro in complex with Ub show that the C-
766 terminal amino acids, RLRGG, of ubiquitin occupy the S4-S1 enzyme subsites of
767 SARS-CoV PLpro (60, 62). These interactions appear to provide a significant
768 amount of the total binding energy for stabilization of the PLpro-Ub complex by
769 formation of 12 intermolecular H-bonds that result from substrate-induced
770 conformational rearrangement of the flexible *β*-turn/loop (60, 62), also called the
771 BL2 loop (56) or the β14 - β15 loop (62). The S4-S2 subsites are also the
772 binding sites for SARS-CoV PLpro competitive inhibitors and similarly to
773 substrate binding, the flexible *β*-turn/loop adopts a conformational change to

allow for optimal inhibitor interactions (28-30). In contrast, we find that MERS-CoV PLpro behaves significantly different to SARS-CoV PLpro in terms of recognition and hydrolysis of the Ub/ISG15 C-termini-based substrates, Z-RLRGG-AMC, and inhibition by SARS-CoV PLpro inhibitors. The activity of MERS-CoV PLpro towards the Z-RLRGG-AMC substrate is 100-fold lower than with SARS-CoV PLpro (Figure 4A, Table 2), suggesting that the enzymes differ in substrate recognition at the subsites. Analysis of the amino acid conservation in the predicted S4-S1 subsites of MERS-CoV PLpro indicates low sequence conservation, which could lower the available number of intermolecular H-bonds between the MERS-CoV PLpro active site and the Ub C-terminal residues (Figure 7B). The net effect of these sequence differences could perhaps reduce the affinity of the Z-RLRGG-AMC substrate with MERS-CoV and/or lower the catalytic activity.

Additional support for differences in molecular recognition between SARS-CoV and MERS-CoV PLpros comes from the fact that the numerous SARS-CoV PLpro inhibitors tested here do not inhibit MERS-CoV PLpro (Figure 6). The lack of inhibition of MERS-CoV PLpro by these inhibitors most likely stems from the structural differences between the S4-S1 subsites, which are revealed via comparison of the MERS-CoV PLpro homology model and SARS-CoV X-ray structures (Figure 7). Noteworthy structural differences are observed at the flexible β-turn/loop, which in MERS-CoV PLpro is one residue longer than SARS-CoV (Figure 7C). A comparison of the amino acids within the β-turns/loops

(between the flanking glycine residues) among the different human and animal CoVs indicates little to no conservation (Figure 9). One notable exception is HCoV-NL63 PLP2, which is moderately inhibited by SARS-CoV PLpro inhibitors (Figure 6) (28). HCoV-NL63 has the same number of residues within the *β*-turn/loop and also has a phenylalanine (F255) in an equivalent position to the tyrosine residue (Y269) in SARS-CoV PLpro that interacts with inhibitors (Figure 7C). Based on the low amino acid conservation within the *β*-turns/loop among the PLpros, we predict that this series of inhibitors is unlikely to be effective against the other clinically relevant HCoVs including: 229E-CoV, which has the same number of amino acids; MERS-CoV, which has an extra amino acid and lastly; HKU1 and OC43, which have shorter *β*-turns/loops by one amino acid (Figure 9). Similarly, these predictions apply to CoVs from animals such as Bovine CoV (BCoV), Porcine Hemagglutinating Encephalomyelitis Virus (PHEV), Porcine Respiratory Corona Virus (PRCV), Transmissible Gastroenteritis virus (TGEV), and Feline/Canine CoVs (FCoV/CCoV).

The Ub and UBL modifier specificity of many viral and human deubiquitinating enzymes (DUBs) depends strongly on the type of polyubiquitin linkage, the chain length, and the number of Ub-interacting domains encoded in the structure of the enzyme (65-68). Moreover, it is well established that the great topological diversity postulated by 8 different types of polyubiquitin chains provides additional regulatory elements of Ub recognition by DUBs (65). We show through the studies reported here that the MERS-CoV PLpro substrate specificity

for Ub/UBL modifiers differs from SARS-CoV PLpro. MERS-CoV PLpro can interact more strongly with mono-Ub substrates than SARS-CoV PLpro, but its polyubiquitin chain debranching activities towards K48-linked and K63-linked polyubiquitin substrates are less robust than SARS-CoV PLpro. MERS-CoV PLpro is able to process both K48- and K63-linked substrates equally well, converting both substrates into mono-Ub species over time (Figure 5A and B). SARS-CoV PLpro, on the other hand, has reduced activity towards K63-linked polyubiquitin chains compared to K48-linked polyubiquitin chains (Figure 5), and its activity towards ISG15-linked substrates is higher than any DUB or deISGylating enzyme studied to date (59, 60).

Unlike MERS-CoV PLpro, SARS-CoV PLpro loses its ability to rapidly cleave K48-linked polyubiquitin chains over time due to the accumulation of di-ubiquitin (di-Ub) reaction products (Figure 5). We recently demonstrated that this phenomenon of product inhibition stems from the fact that SARS-CoV PLpro prefers to bind K48-linked di-Ub molecules chains via a bivalent interaction with the enzyme's zinc finger domain and ridge region of the thumb domain (Figure 10). The two Ub-interacting sites are designated SUb1 at the zinc finger and SUb2 at the ridge region of the thumb domain. These two 'distal' Ub/UBL subsites are capable of interacting simultaneously with K48-linked di-Ub and ISG15 but not K63-linked polyubiquitin chains, which are topologically different. Due to the greater affinity of K48-linked di-Ub for the SARS-CoV PLpro enzyme, the accumulation of the di-Ub reaction product during chain processing results in

product inhibition (60). With an even number of K48-linked ubiquitins in the polyubiquitin chain, e.g. with tetra-ubiquitin (Ub_4), we observe even a greater accumulation of the di-Ub species over time with SARS-CoV PLpro (Figure 5D) compared to K48-linked polyubiquitin chains with an odd number of Ub that produce both mono-Ub and di-Ub (Figure 5F). In contrast, MERS-CoV PLpro does not show a build-up of di-Ub in its processing of any polyubiquitin chain suggesting that it does not contain a SUb2 site on the MERS-CoV PLpro enzyme surface.

The lack of amino acid conservation at the predicted SUb2 site (Figure 7E and Figure 9) may be the reason for the polyubiquitin chain processing differences between MERS-CoV and SARS-CoV PLpros. Analysis of the amino acid sequence conservation at the ridge region of the thumb domain among all CoV PLpros shows very little conservation suggesting that the bivalent recognition of K48-linked $Ub_{(2)}$ may be a unique feature of SARS-CoV PLpro (Figure 9). However, since the majority of CoV PLpros have not yet been fully characterized in terms of their polyubiquitin chain recognition and processing activities, more research is required to better understand the potential implications of different polyubiquitin recognition patterns during the PLpro-mediated antagonism of the innate immune response and how differences in recognition can affect the pathogenicity of these human coronaviruses.

We propose in Figure 10 a general model describing the mechanisms of chain processing of K48-linked $Ub_{(4)}$ by SARS-CoV PLpro and MERS-CoV PLpro. For SARS-CoV PLpro, processing begins with the bivalent recognition and interaction of two Ub molecules (Ub1 and Ub2) in a K48-linked polyubiquitin chain at the SUb1 in the zinc finger, and SUb2 in the ridge region of the thumb domain (Figure 10B and D). The endo-trimming of the isopeptide bond between Ub1 bound at the SUb1 subsite and Ub1' bound at the SUb1' subsite results in the overall production of a di-Ub molecule and a single Ub molecule from a Ub_3-chain, a second di-Ub molecule from a Ub_4-chain, and a Ub_3-chain from a Ub_5-chain, which can be further processed to di-Ub and mono-Ub molecules. In order for SARS-CoV PLpro to further cleave the di-Ub molecules to mono-Ub, di-Ub has to be released from the enzyme (product release), which appears to be the slow step in the kinetic mechanism of K48-polyubiquitin chain processing. For MERS-CoV PLpro; however, since there is no detectable accumulation of reaction products over time (Figure 5A and B) and because mono-Ub has moderate affinity for the enzyme (Figure 4D and Table 2), processing occurs in a stepwise manner with equal opportunity for endo- and exo-trimming of the chain (Figure 10C and E). As a result, by trimming polyubiquitin chains via its SUb1 subsite, there is no substantial difference in the rate of processing different lengths of K48-linked chains.

So far, few studies have been reported on the specificity of SARS-CoV PLpro beyond the P1' position of the substrate. It has only been demonstrated that

888 SARS-CoV PLpro is able to cleave peptide substrates containing the P1' amino-
889 acid residues Ala, Gly, Asp, and Lys (69). Surprisingly, even though CoV PLpros
890 can cleave the peptide bonds within the polyprotein cleavage sites and hydrolyze
891 AMC from Ub-AMC and ISG15-AMC, neither MERS-CoV or SARS-CoV PLpro
892 enzymes are able to hydrolyze the peptide bond from Met1-linked linear $Ub_{(4)}$
893 (Figure 5C). The cleavage site for linear ubiquitin would be R-L-R-G-G|M-Q-I-F-V.
894 The lack of cleavage activity with Met1-linked polyubiquitin chain indicates that
895 either the S1' subsites of PLpros cannot accommodate the bulky side chain of
896 Met at the P1' position, or that the amino acids Q, I, F and V at the P2', P3', P4'
897 and P5' may prevent cleavage. It is clear that PLpro enzymes do not have
898 specificity for linear polyubiquitin chains.
899
900 In summary, the substrate, inhibitor and ubiquitin chain recognition patterns of
901 PLpro from MERS-CoV and SARS-CoV are similar, with SARS-CoV PLpro
902 having more robust catalytic activity towards most substrates and exhibiting a
903 unique bivalent recognition mechanism towards polyubiquitin substrates. Both
904 enzymes are capable of recognizing and hydrolyzing fluorophores from the C-
905 terminus of RLRGG peptide, Ub and ISG15 substrates, yet the kinetic
906 parameters associated with these reactions are different. Neither enzyme is
907 capable of cleaving the peptide bond between two Ub molecules within a Met1-
908 linked polyubiquitin chain, but both enzymes are capable of recognizing and
909 cleaving K48-linked and K63-linked polyubiquitin chains. Our detailed analysis
910 revealed that MERS-CoV PLpro prefers to recognize and bind a single Ub

molecule within its SUb1 subsite allowing it to perform either endo- or exo-trimming of K48- and K63-linked polyubiquitin chains, whereas SARS-CoV PLpro performs such trimming only on K63-linked chains and does so slowly. We also found that SARS-CoV PLpro utilizes a unique bivalent recognition mechanism for K48-linked polyubiquitin chains whereby it binds two ubiquitin molecules in the SUb1 and SUb2 subsites and performs mainly endo-trimming reactions releasing di-Ub. The ramifications of these ubiquitin chain preferences on the innate immune response during coronavirus infection should be explored. Indeed, using structure-guided mutagenesis we diminished the ability of SARS-CoV PLpro to preferentially bind di-Ub and ISG15 over mono-Ub, which caused a significant decrease in the ability to stimulate the NFκ-B pathway (60). These results suggest that subtle differences in polyubiquitin chain cleavage specificity may have functional ramifications in viral pathogenesis.

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Figure Legends

Figure 1: MERS-CoV PLpro constructs, expression and enzymatic activities in HEK293T cells.

A) MERS-CoV PLpro constructs: wild type$_{aa1485-1802}$ (WT), catalytic cysteine mutant (Cys1594/A$_{aa1485-1802}$, CA) and three UBL-deleted mutants (N20$_{aa1505-1802}$, N40$_{aa1524-1802}$ and N60$_{aa1545-1802}$) are fused to a V5 epitope tag on the C-terminus for V5 antibody detection. B) *Trans*-cleavage activity of MERS-CoV PLpro in HEK293T cells expressing SARS-CoV nsp2/3-GFP. Lysates were harvested at 24 hours post-transfection, and protein expression was analyzed by western blotting. DeISGylating (C) and deubiquitinating (D) activities of MERS-CoV PLpro constructs. HEK293T cells were transfected with MERS-CoV PLpro expression plasmids WT, CA and UBL-deleted mutants (N20, N40 or N60), along with myc-ISG15, E1, E2, E3 ISGylating machinery plasmids to test the deISGylating (C) activity, or with Flag-Ub expression plasmid to test the deubiquitinating (D) activity of each PLpro construct. Cells were lysed at 18

hours post-transfection and analyzed by Western blotting. The strong bands indicate ISGylated (C) and ubiquitinated (D) proteins. Figure shows representative data from at least two independent experiments.

Figure 2. Interferon antagonism activity of MERS-CoV PLpro. HEK293T cells were transfected with plasmids expressing either wild type (WT) PLpro, catalytic mutant PLpro (CA) or UBL-deleted PLpro mutants (N20, N40 or N60). Cells were also transfected with plasmids expressing IFN-luc, Renilla-luc, and the stimulator Mda5 (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 of the mean.

Figure 3: Purification of MERS-CoV PLpro$_{1484\text{-}1802}$. A) SDS-PAGE analysis of whole cell lysate and purified MERS-CoV PLpro, which runs at the expected molecular weight of 37 kDa. The molecular marker is shown with M. (B) SEC-MALS traces of MERS-CoV PLpro at different protein concentrations. MERS-CoV PLpro at 4.2 mg/ml, 2.1 mg/ml and 1.0 mg/ml eluted at the same retention time from a SEC column. The M_w determined from the molecular mass from the MALS, correspond to a monomer for the peak of each concentration. All analyzed peak areas were monodisperse ($\overline{M}_w/\overline{M}_n$ < 1.01) as shown by the horizontal traces.

Figure 4: MERS-CoV and SARS-CoV PLpro activities with three ubiquitin-based substrates. The activities of MERS-CoV PLpro (gray circles) and SARS-

1228 CoV PLpro (black circles) with each substrate are shown in plots (A), (B) and (C).
1229 Dose response curve of the inhibition by free Ub and ISG15 are shown in plots
1230 (D) and (E). Data were fit to the Michaelis-Menten equation unless the catalytic
1231 activity exhibited a linear response to substrate concentration. In such a case,
1232 data were fit to the equation $v/[E]=k_{cat}/K_M \bullet [S]$, where [E] and [S] are the
1233 concentrations of enzyme and substrate, respectively. The error bars represent
1234 the standard deviation between a minimum of triplicate samples.
1235
1236 **Figure 5. Ubiquitin chain specificity of MERS-CoV and SARS-CoV PLpros.**
1237 The *in vitro* cleavage of K48-linked $Ub_{(5)}$ (A) or $Ub_{(4)}$ (D) by MERS-CoV PLpro
1238 and SARS-CoV PLpro, respectively, and K63-linked $Ub_{(6)}$ by MERS-CoV PLpro
1239 (B) and by SARS-CoV PLpro (E). Cleavage of linear $Ub_{(4)}$ is shown in (C). (F)
1240 Analysis of $Ub_{(2)}$ accumulation during SARS-CoV PLpro-mediated processing of
1241 K48-linked substrates. Processing of the substrates is shown by a production of
1242 lower molecular weight bands at progressive time points in minutes (') or hours
1243 (h). The locations of the different Ub species are shown. The molecular weight
1244 marker is shown with an M.
1245
1246 **Figure 6. MERS-CoV PLpro and HCoV-NL63 inhibition by a series of SARS-**
1247 **CoV PLpro inhibitors.** The percent inhibition of SARS-CoV PLpro, HCoV-NL63
1248 PLP2 and MERS-CoV PLpro activity in the presence of SARS-CoV PLpro
1249 inhibitors. Percent inhibition was calculated from two independent assays at a
1250 fixed concentration of 100 µM compound and are shown as the inhibition mean.
1251 Error bars representing the positive and negative deviation from the average

values were removed for clarity in the figure. The difference between each independent measurement were less than 10% for the entire set of data. Highlighted in bold are the best SARS-CoV PLpro inhibitor candidates including compound **3k** (chemical structure as insets) also shown in Figure 7C.

Figure 7. Analysis of MERS-CoV PLpro subsites, active site and ridge region of the thumb domain. (A) The homology model of MERS-CoV PLpro (gray surface, yellow cartoon) displaying the canonical right-hand architecture with thumb, palm and zinc finger domain with an additional UBL domain at the N-terminus. Modeled Ub (pink) positioned onto the Ub-binding domain in the zinc finger with its C-terminus extending into the active site. Highlighted with boxes are the regions of the thumb domain and palm domain predicted to be responsible for MERS-CoV PLpro divergence from SARS-CoV PLpro substrate and inhibitor specificity. (B) The enzyme subsites displaying the predicted intermolecular interactions with Ub C-terminus. Green dashed lines indicate the H-bonds between SARS-CoV PLpro (blue cartoon) and Ub C-terminus that are predicted to be conserved in MERS-CoV PLpro. The black dashed lines indicate H-bonds or salt bridges that are predicted to be lost in MERS-CoV PLpro-Ub C-terminus interaction. Amino acids involved in SARS-CoV PLpro-Ub C-terminus interactions are shown in blue font and the predicted corresponding amino acids in MERS-CoV PLpro are shown in black font. Highlighted in bold are the non-conserved amino acid substitutions in MERS-CoV PLpro. (C) SARS-CoV PLpro in complex with compound **3k** (orange ball and sticks, PDB: 4OW0) overlay to MERS-CoV PLpro and a homology model of HCoV-NL63 PLP2 (green). The

amino-acid residues important for SARS-CoV PLpro-inhibitor interactions are shown (blue font) along with the predicted corresponding amino acids in HCoV-NL63 PLP2 (green font) and MERS-CoV PLpro (black font). Highlighted in bold are the non-conserved substitutions in MERS-CoV PLpro. At the bottom of panel C is a comparison between SARS-CoV, HCoV-NL63 and MERS-CoV PLpro's amino acid composition of the *β*-turn/loop (highlighted with an arrow) known to be important for the inhibitor-induced-fit mechanism of association of compound **3k** and SARS-CoV PLpro. (D) Comparison of the active site and oxyanion hole showing the corresponding amino acids in SARS-CoV, HCoV-NL63 and MERS-CoV PLpros. (E) An overlay of SARS-CoV PLpro and MERS-CoV PLpro ridge region of the thumb domain. Amino acid numbering (aa #) are defined as follow: for SARS-CoV PLpro aa #1 corresponds to aa #1540 in the polyprotein; for HCoV-NL63 PLP2 aa #1 correspond to aa #1578 in the polyprotein; and for MERS-CoV PLpro amino acid #1 correspond to amino acid #1480 in the polyprotein.

Figure 8: Comparison between MERS-CoV PLpro β-turn region and enzyme subsites identified via molecular modeling and the recently reported X-ray crystal structure. A structural superposition between the refined homology model of MERS-CoV PLpro (yellow cartoon) and the recently reported X-ray crystal structure (PDB: 4P16, green cartoon), which was reported during the review of this manuscript, yields a Cα RSMD value of 2.1 Å for 268 atoms aligned. The $2F_o - F_c$ electron density map from 4P16 is contoured at 1σ (shown

as gray mesh), and confirms the presence and location of the amino acid predicted at the enzyme subsites by structural model (labeled amino acids, shown as sticks). The residues comprising the β-turn in 4P16 are missing in the X-ray structure due to the lack of associated electron density. The refined homology model contains this loop region and therefore serves as a useful structural model for understanding the interactions between the loop and substrates or inhibitors. The striking similarity between the X-ray crystal structure and our energy-minimized structural model demonstrate the high quality of our computational analyses, and makes it a good model to predict a potential conformation for the β-turn of MERS-CoV PLpro.

Figure 9. Multiple sequence alignment generated with ESpript presenting the secondary structure elements on top: α-helices (squiggles), β-strands (black arrows) and turn (TT). Highlighted are the highly conserved areas (blue outlined boxes) containing the conserved residues (red filled boxes), homologous residues (red font), and divergent residues (black font). The structural elements were generated using the X-ray crystal structure of apo SARS-CoV PLpro (pdb: 2FE8). MERS-CoV PLpro UBL truncation sites N20, N40 and N60 are marked in purple and the catalytic triad residues are highlighted with an asterisk. The α-helix 2 (highlighted with a green box), containing the amino-acid residues important for SARS-CoV PLpro interaction with K48-Ub_2 and ISG15, is highly divergent among CoV PLpros. The amino-acid residues important for interactions with SARS-CoV PLpro inhibitors are highlighted with a blue filled box. The *β*-

1322 turn/loop at the inhibitor binding-site (highlighted with a black outlined box) is
1323 highly divergent among CoV PLpros. Accession numbers are as follow: SARS-
1324 CoV (AAP13442.1) $PLpro_{21541-1854}$; HCoV-NL63 (YP_003766.2) $PLP_{21578-1876}$;
1325 MERS-CoV (AFS88944.1) $PLpro_{1484-1802}$; HCoV-HKU1 (YP_173236) $PLP2_{1648-}$
1326 $_{1955}$; HCoV-OC43 (CAA49377.1) $PLP2_{1562-1870}$; HCoV-229E (CAA49377.1)
1327 $PLP2_{1599-1905;}$ PHEV-CoV (YP_459949.1) $PLP2_{1561-1871}$; PRCV-CoV (DQ811787)
1328 $PLP2_{1484-1780}$; TGEV-CoV (CAA83979.1) $PLP2_{1487-1783}$; Feline-CoV (AAY32595)
1329 $PLP2_{1441-1920}$; Canine-CoV (AFX81090) $PLP2_{1441-1920}$; BCoV (NP_150073)
1330 $PLP2_{21562-1870}$; MHV-A59 (NP_068668.2) $PLP2_{1606-1915}$.
1331
1332 **Figure 10. Model for the processing of K48-linked $Ub_{(4)}$ by SARS-CoV**
1333 **PLpro and MERS-CoV PLpro.** A schematic diagram showing two mechanisms
1334 for the recognition of distal Ub (B and C) from a K48-linked $Ub_{(4)}$ (A). The distal
1335 Ub-interacting subsites SUb1 and SUb2 are shown for a bivalent mode of
1336 recognition (B) with one Ub-subsite at the zinc finger and a second Ub-subsite at
1337 the ridge region of the thumb domain, respectively. The monovalent mechanism
1338 of distal Ub recognition only has the SUb1 site at the zinc finger (C). The position
1339 of the substrate's scissile bond in the active site is indicated with a red arrow and
1340 the reaction progress is shown as product accumulation 1, 2 and 3. (D) SARS-
1341 CoV PLpro has a bivalent mode of recognition towards K48-linked polyubiquitin
1342 chains (mechanism 1) and has high affinity for K48-linked di-Ub molecules. In the
1343 case of K48-linked $Ub_{(4)}$, the first cleavage event occurs through the bivalent
1344 interaction of SARS-CoV PLpro zinc finger and ridge region of the thumb domain
1345 with di-Ub, producing two di-Ub cleavage products. Subsequent cleavage events

occur much more slowly due to the less favorable binding of mono-Ub over di-Ub molecules. (E) MERS-CoV PLpro interacts with K48-linked polyubiquitin chains via a monovalent mode of recognition (mechanism 2) and has moderate affinity for mono-Ub molecules. Cleavage of K48-linked $Ub_{(4)}$ occurs through the monovalent interaction of MERS-CoV PLpro zinc finger with mono-Ub, with no significant differences in the rate of processing tetra-, tri- or di-Ub. Other possible cleavage routes are shown with a blue arrow.

Table 1: Purification table of MERS-CoV PLpro from *E. coli* BL21(DE3).

Sample	Total Protein (mg)	$Units_{Total}$ (μM/min)	Specific Activity (μM/mg)	Fold Purification	Yield (%)
Lysate	2625	1,506,335	574	1	100
HisTrap Pool	130	1,492,985	11,529	20	99
Mono-Q Pool	67	1,095,933	16,309	28	73
Superdex-75 pool	63	1,059,747	16,821	29	70

Table 2: Comparison of the kinetic parameters and inhibition of the PLpro domain from SARS-CoV, and MERS-CoV with different substrates.

		Substrate		
		RLRGG-AMC	Ub-AMC	ISG15-AMC
MERS PLpro				
k_{cat}/K_M	(μM^{-1} min^{-1})	[a]0.003 ± 0.0001	1.3 ± 0.2	9.9 ± 1.6
k_{cat}	(min^{-1})	–	18.8 ± 1.2	32.6 ± 1.8
K_M	(μM)	–	14.3 ± 2.0	3.3 ± 0.5
[b]IC_{50}	(μM)	–	21.3 ± 4.0	54.4 ± 17.7
SARS PLpro				
k_{cat}/K_M	(μM^{-1} min^{-1})	[a]0.3 ± 0.1	1.5 ± 0.3	29 ± 5.3
k_{cat}	(min^{-1})	–	75.9 ± 8.1	436 ± 40
K_M	(μM)	–	50.6 ± 7.4	15.1 ± 2.4
[b]IC_{50}	(μM)	–	NI	18.4 ± 12.2

[a]Apparent k_{cat}/K_M values derived from the best-fit slope of the data presented in Figure 4A. [b]IC_{50} values for the inhibition of Ub-AMC hydrolysis by free Ub and free ISG15. Values are reported as mean ± standard deviation based on a minimum of triplicate measurements. –, not determined. NI, no inhibition.