Structural and biochemical characterization of endoribonuclease Nsp15 encoded by Middle East respiratory syndrome coronavirus

Running title: Crystal structure of MERS-CoV Nsp15

Lianqi Zhang[1‡], Lei Li[2‡], Liming Yan[1], Zhenhua Ming[3], Zhihui Jia[1], Zhiyong Lou[1] and Zihe Rao[1,4,5*]

[1]Laboratory of Structural Biology, School of Medicine, Tsinghua University, Beijing 100084, China.

[2]State Key Laboratory of Biotherapy/Collaborative Innovation Center for Biotherapy, West China Hospital, Sichuan University, Chengdu, Sichuan, China

[3]State Key Laboratory of Conservation and Utilization of Subtropical Agro-Bioresources, College of Life Science and Technology, Guangxi University, Nanning, China

[4]National Laboratory of Macromolecules, Institute of Biophysics, Chinese Academy of Science, Beijing 100101, China

[5]College of Life Sciences, Nankai University, Tianjin 300071, China

[*]**Corresponding author**: Zihe Rao, raozh@mail.tsinghua.edu.cn

[‡] Lianqi Zhang and Lei Li contributed equally to this work.

Key words: MERS-CoV, endoribonuclease, crystal structure, oligomerization

Word counts: Abstract: 233; Importance: 102; Main text: 5596 (excluding references and legends)

ABSTRACT

Non-structural protein 15 (Nsp15) encoded by coronavirus (CoV) is a uridylate specific endoribonuclease (NendoU) that plays an essential role in the life cycle of the virus. Structural information of this crucial protein from the Middle East respiratory syndrome (MERS) CoV, which is lethally pathogenic and has caused severe respiratory diseases worldwide, is lacking. Here, we report the crystal structure of MERS-CoV Nsp15 at a 2.7 Å resolution and perform the relevant biochemical assays to study how NendoU activity is regulated. Although the overall structure is conserved, MERS-CoV Nsp15 shows unique and novel features compared to its homologs. Serine substitution of residue F285, which harbors an aromatic side chain that disturbs RNA binding compared with other homologs, increases catalytic activity. Mutations of residues residing on the oligomerization interfaces that distort hexamerization, namely, N38A, Y58A and N157A, decrease thermostability, decrease binding affinity with RNA and reduce the NendoU activity of Nsp15. In contrast, mutant D39A exhibits increased activity and a higher substrate binding capacity. Importantly, Nsp8 is found to interact with both monomeric and hexameric Nsp15. The Nsp7/Nsp8 complex displays a higher binding affinity for

Nsp15. Furthermore, Nsp8 and the Nsp7/Nsp8 complex also enhance the NendoU activity of hexameric Nsp15 *in vitro*. Taken together, this work first provides evidence on how the activity of Nsp15 may be functionally mediated by catalytic residues, oligomeric assembly, RNA binding efficiency or the possible association with other non-structural proteins.

IMPORTANCE

The lethally pathogenic Middle East respiratory syndrome coronavirus (MERS-CoV) and the severe acute respiratory syndrome coronavirus (SARS-CoV) pose serious threats to humans. Endoribonuclease Nsp15 encoded by coronavirus plays an important role in viral infection and pathogenesis. This study determines the structure of MERS-CoV Nsp15 and demonstrates how the catalytic activity of this protein is potentially mediated, thereby providing structural and functional evidence for developing antiviral drugs. We also hypothesize that the primase-like protein Nsp8 and Nsp7/Nsp8 complex may interact with Nsp15 and affect enzymatic activity. This contributes to the understanding of the association of Nsp15 with the viral replication and transcription machinery.

INTRODUCTION

A decade after the severe acute respiratory syndrome coronavirus (SARS-CoV) epidemic, a zoonotic coronavirus called the Middle East respiratory syndrome coronavirus (MERS-CoV) circulated throughout the human population (1, 2). The lack of CoV-specific anti-viral drugs or an effective vaccine has severely hampered efforts to combat the spread of this virus. It is therefore important to study the life cycle of the virus and the role viral proteins play in its propagation so that they can be targeted for the development of anti-viral therapeutics.

CoVs are enveloped, single-stranded positive RNA viruses (3). The genomes of CoVs are the largest among RNA viruses and range between 26 and 32 kb (4). Almost two-thirds of the genome encompasses two large open reading frames (ORFs), ORF 1a and ORF 1b, which encode 16 non-structural proteins (Nsps) that play essential roles in coronavirus RNA replication and transcription. A unique feature shared by all CoVs is that the ribosome undergoes a -1 frameshift following the translation of Nsp10. This results in the production of a large polypeptide, 1ab, that is then proteolytically processed to produce the 16 viral Nsps (5, 6). In addition to these Nsps, several structural and accessory proteins are synthesized from ORFs located at the 3' end of the viral genome. These ORFs are transcribed into a nested set of subgenomic RNAs that are ultimately translated into structural proteins (7, 8).

Non-structural protein 15 (Nsp15) is a nidoviral uridylate specific endoribonuclease (NendoU) (9). It was reported to preferentially cleave 3' of uridylates over cytidylates and generate a 2',3'-cyclic phosphate and 5'-OH ends (10). The activities of SARS-CoV Nsp15 and mouse hepatitis virus (MHV) Nsp15 were reported to be significantly stimulated by Mn^{2+} (11-12). Previous studies have demonstrated that the recombinant

89 SARS-CoV Nsp15 and MHV Nsp15 both existed in a monomer-trimer-hexamer
90 equilibrium in solution, with the hexamer possessing endoribonuclease activity (10,
91 12-14). Crystal structures of Nsp15 from SARS-CoV and MHV reveal that
92 hexamerization of the protein lends structural support to maintain integrity of the active
93 site. Two loops in the catalytic domain (residues 234-249 and 276-295 in SARS-CoV
94 Nsp15) are packed against each other and are stabilized by intimate inter-monomer
95 interactions (12-14). N-terminal truncation of SARS-CoV Nsp15 resulted in an inactive
96 monomeric state, with the catalytic loop containing two catalytic residues, H234 and
97 H249, falling into the active site cleft (15), which provides structural evidence to support
98 that the hexamer is the active form.
99 Several *in vivo* studies have also been conducted to evaluate the function of Nsp15.
100 Loss-of-function mutations in the catalytic sites of MHV Nsp15 reduced subgenomic
101 RNA accumulation and profoundly attenuated virus infection, and similar results were
102 also observed with SARS-CoV and Arterivirus (16, 19, 21). More recently, Nsp15 from
103 SARS-CoV was found to be an inhibitor of mitochondrial antiviral signaling adaptor
104 (MAVS) inducing apoptosis (17). In addition, Nsp15 from both MHV and SARS-CoV
105 can interact with retinoblastoma tumor suppressor protein (pRb), thus affecting cell
106 cycle-associated gene expressions (18). It is therefore likely that Nsp15 impacts not only
107 the viral life cycle but also the metabolic status and immune response of the host cells.
108 Moreover, MHV Nsp15 has been demonstrated to colocalize and interact with the viral
109 primase Nsp8 and polymerase Nsp12 *in vivo* (20), suggesting its possible involvement in
110 RNA replication and transcription.
111 Here, we first describe the crystal structure of Nsp15 from MERS-CoV refined to a 2.7 Å
112 resolution. Crucial residues within the active site pocket and interprotomer interaction

surfaces played essential roles in the enzymatic function of the protein by directly regulating catalysis or imparting oligomeric arrangement and stability, thus modulating the substrate-RNA binding process. Furthermore, we demonstrated that primase-like Nsp8 and the Nsp7/Nsp8 complex could interact with MERS-Nsp15 and affect NendoU activity, indicating a possible association of Nsp15 with other important non-structural proteins that are involved in RNA replication and transcription.

RESULTS

Overall structure of MERS-CoV Nsp15

The recombinant full-length MERS-CoV Nsp15 was expressed, purified to homogeneity, crystallized in the *H3* space group and diffracted to a 2.7 Å resolution. The final coordinates consist of protein residues 1-341 with good crystallographic quality (Table 1). For simplicity, MERS-Nsp15 and SARS-Nsp15 are used to represent MERS-CoV Nsp15 and SARS-CoV Nsp15, respectively, in this paper.

The overall structure of MERS-Nsp15 consists of three distinct domains (Fig. 1A) and shares homology with the reported SARS-Nsp15 and MHV Nsp15 (12-14). Residues 1 to 60 are folded into a small N-terminal domain, in which three anti-parallel β-strands (β1-β3) are observed forming a curved β-sheet with two short α-helices (α1, α2) right beneath it. The N-terminal domain is followed by a middle domain that contains a central β-sheet (β4, β7, β8, β11) flanked by two small α-helices (α3, α4) on either side. Two short β-strands (β9-β10) arranged in a β-hairpin are located at the interface of the central domain and the C-terminal domain. Residues I190 to R341 within the C-terminal domain pack into two β-sheets consisting of β-strands β13-β15 and β-strands β16-β18, which

137 constitute the catalytic site cleft located at one side of the C-terminal domain. A group of
138 five small α-helices (α5-α9) packed at the other side of the domain face the concave
139 surface of the β-sheets.

140 Crystal packing of MERS-Nsp15 is suggestive of a hexamer model. A dimer of trimers
141 that constitutes a hexameric architecture with the crystallographic three-fold axis passing
142 through the center of the hexameric assembly (Fig. 1B-C). The N-terminal domains of
143 the protomers within the two trimers pack back-to-back into a hexamer, placing the
144 C-terminal domains that harbour the active site at the apexes of the cloverleaf-like
145 symmetry (Fig. 1C). Within the trimer assembly, the N-terminal domain of one protomer
146 packs with a cleft between the central domain and the C-terminal domain of an adjacent
147 protomer (Fig. 1C).

148 The structure of Nsp15 is conserved, with the root mean square deviations (r.m.s.d.) of
149 Cα atoms at 1.23 Å and 1.17 Å between monomeric MERS-Nsp15 and SARS-Nsp15 as
150 well as MHV Nsp15. The r.m.s.d. for comparison of the trimeric MERS-Nsp15 with
151 SARS-Nsp15 and MHV Nsp15 is 2.49 Å and 3.02 Å, respectively (Fig. 2A). Three
152 domains within the monomer are also conserved, with r.m.s.d. of Cα atoms of aligned
153 residues ranging from 0.71-1.25 Å (Fig. 2B). Additionally, many of the conserved
154 residues that may contribute to the function of the protein (key residues that make up the
155 catalytic site within the C-terminal domain and polar residues forming hydrogen bond
156 networks) may be essential for protein function (Fig. 3).

157

158 **Key residues within the catalytic site**

159 To better uncover the structural information within the catalytic site, the structures of
160 MERS-Nsp15 and SARS-Nsp15 were superimposed (Fig. 4A). Three highly conserved

residues clustering in a positively charged groove are known to drive NendoU-mediated catalysis: the two catalytic histidines, H231 and H246, are located on a long, convoluted loop wedged between two adjacent β-sheets, and the third catalytic residue, K286, resides on strand-β15. The spatial arrangements reveal that residues S290 and Y339 in MERS-Nsp15 correspond to residues S293 and Y342 in SARS-Nsp15, which are postulated to interact with substrate and confer uridylate specificity (14), suggesting that there is conserved recognition for uridylate. However, not all residues within the active site are conserved among coronaviruses. Several notable differences include the phenylalanine (F285) in MERS-Nsp15 that is located on strand-β15 within one of the 2 β-sheets at one end of the catalytic site. The aromatic side chain of F285 protrudes outward at a roughly perpendicular angle to the β-strand plane, compared to the corresponding serine (S288) in SARS-Nsp15. A threonine (T241), which is spatially located next to F285 and H246, occupies the position of Q244 in SARS-Nsp15, whereas an isoleucine is located in the corresponding position in MHV-Nsp15 (Fig. 3, Fig. 4A). In addition, an arginine (R341) in MERS-Nsp15 replaces a lysine (K344) in SARS-Nsp15 (Fig. 3). Thus, residues that are not conserved revealed unique features in MERS-Nsp15.

Functional characterization of MERS-Nsp15 and its active site mutants

To explore the functional importance of residues within the catalytic site, the residues listed in Fig. 4A were substituted by alanine. Gel filtration revealed that all mutants shared similar elution profiles to that of the wild-type Nsp15. The elution profiles exhibited a dominant peak corresponding to a hexamer (data not shown). To further investigate the thermostability of these mutants, we conducted differential scanning fluorimetry (DSF) assay, which has been used to assess interactions among protein

185 subunits (22). The denaturation profile of wild-type Nsp15 and its mutants in Fig. 4A
186 revealed that they all exhibited a major transition at the melting temperature (Tm) of 46
187 °C, which is suggestive of a native fold (Fig. 4C). Taken together, the mutation of key
188 residues in the active site had no effect on the oligomeric assembly and stability of
189 MERS-Nsp15.

190 We subsequently investigated the NendoU activity of these active-site mutants using
191 fluorescent resonance energy transfer (FRET) assays, in which a substrate containing a
192 nucleotide rU was used. Alanine substitution of the three highly conserved residues
193 (H231, H246 and K286), as well as the residues located in the immediate surrounding
194 regions (Y339, T241 and R341), decreased ribonuclease activity to background level
195 (Fig. 4D-E). Notably, when T241 was substituted with the corresponding residue
196 glutamine in SARS-Nsp15, T241Q exhibited wild-type activity. Mutating R341 to lysine,
197 the corresponding residue in SARS-Nsp15, decreased NendoU activity to approximately
198 50 % of wild-type protein activity (Fig. 4E). Interestingly, the catalytic rate of Nsp15
199 slightly increased to 1.34 ± 0.06 nM s^{-1} when F285 was substituted by alanine, which is
200 found in the equivalent position in EAV (equine arteritis virus) (Fig. 4). We also replaced
201 F285 with a serine, a residue that is present in all other coronaviruses at this position.
202 Serine substitution resulted in an increase in catalytic activity to 1.63 ± 0.07 nM s^{-1} (Fig.
203 4E). Moreover, alanine substitution of S290, for which the corresponding residue was
204 postulated to confer uridylate specificity, abolished the activity to 67 % of the wild-type
205 Nsp15 activity (Fig. 4D).

206 How does the disruption of active-site residues affect NendoU activity? We next used
207 fluorescence polarization (FP) assay to assess the RNA binding ability of these mutants.

208 Three different RNAs (R1-R3) are designed to identify the one that binds the strongest to
209 Nsp15: R1 contains 20 rU; R2 is derived from the conserved transcriptional regulatory
210 sequence (TRS) of the viral genome; and R3 is a double-stranded RNA annealed by R2
211 and its complementary strand. Among the three oligomers, Nsp15 exhibited the highest
212 binding affinity to RNA R1 (Fig. 4B). RNA R1 also had the highest inhibitory effect on
213 NendoU activity in the FRET-based assays, suggesting that it possesses the strongest
214 binding ability, and was thus used in all FP assays to assess the RNA-Nsp15 interactions
215 in this paper (Fig. 4B). Mutants K286A, H246A, H231A, Y339A, T241A and R341A,
216 which exhibited no activity, were all able to bind RNA, indicating that their decreased
217 activity was not caused by substrate binding, but possibly by the directly catalytic
218 function of these residues (Table 2). Of note, the F285A and F285S mutants exhibited
219 stronger binding compared to the wild-type protein, which may explain why the NendoU
220 activity of these two mutants increased (Fig. 4E, Table 2). Consistently, mutant S290A,
221 which exhibited partly diminished activity, conferred a moderate decrease in RNA
222 binding ability. In conclusion, these observations corroborated that mutation of critical
223 residues within the active site, through either directly disrupting catalysis or impacting
224 RNA binding affinity, may influence NendoU activity.
225
226 **Oligomeric assembly affects RNA binding and NendoU activity**
227 To gain insight into the correlation between NendoU activity and the distinct oligomeric
228 forms, we examined the interprotomer surfaces and disrupted several key residues that
229 contribute to the hydrogen bond networks: 1) the $\delta 1$ −oxygen atom of N38 on strand-β3
230 in the N-terminal domain bonds with the backbone nitrogen atom of G95 on the loop

connecting strand-β6 with strand-β7; 2) the $\delta 2$ −oxygen atom of D39 on strand-β3 bonds with the $\gamma 1$ −oxygen atom of T48 on the loop connecting strand-β3 with helix-α2 in the middle domain of another protomer; 3) the nitrogen atom of $\delta 2$ −N157 on the turn connecting strand-β10 with strand-β11 stacks against the $\gamma 1$ −oxygen atom of T278 on β14; and 4) the η −oxygen atom of Y58 on helix-α2 stacks against $\varepsilon 2$ −oxygen atom of E263 on strand-β13 in the C-terminal domain (Fig. 5). Within the interface of the two trimers, L2 and E3 on helix-α1 as well as V26 on strand-β1 of protomer B pack face to face against E3 and L2 on helix-α1 as well as N52 on helix-α2 of its counterpart protomer A, respectively. Moreover, the N-terminus of subunit C is close to monomer A, with the closest atom-to-atom distance being 3.2 Å between residues A112 in protomer C and N110 in protomer A, suggesting that this close proximity may assist in the hexamer assembly process (Fig. 5).

To validate the significance of the interfaces, analytical ultracentrifugation (AUC) analysis was conducted to assess the oligomeric form of soluble and stable variants. Mutant D39A primarily existed as a hexamer, while mutant E263A distributed equally between the monomer and hexamer states. By comparison, mutants N38A, Y58A and N157A existed predominately as monomers (Fig. 6A). DSF assays revealed that the denaturation profiles of the hexameric proteins (wild type and D39A Nsp15) indicated a melting temperature (Tm) of 46 °C. E263A denatured at 43 °C, while the Tm shifted to approximately 37-38 °C for the monomeric mutants N38A, N157A and Y58A (Fig. 6B). Taken together, we inferred that the hexameric form rendered the protein more stable and may thus impact protein function.

In an effort to evaluate the functional state and demonstrate how oligomeric assembly

affects NendoU activity, the RNA binding ability and the NendoU activity of these variants were further assessed. The results of the FP and FRET-based assays showed that the hexameric mutant D39A bound to RNA R1 with a binding affinity (626.22 $\pm$ 24.80 nM) higher than that of the wild-type protein, which may explain why mutant D39A exhibited greater enzymatic activity than the wild-type Nsp15. Mutant E263A, which displayed an equal distribution between the monomer and hexamer states, retained an impaired binding affinity (1013.43 $\pm$ 77.38 nM), matching its moderately inhibited activity. Consistently, associations between the monomeric variants (N38A, Y58A and N157A) and RNA R1 were barely detectable, and the activities of the monomeric mutants were abolished (Fig. 6C-D). Altogether, the correlation between RNA binding affinity and NendoU activity showed that RNA binds only to the hexameric state, indicating that oligomeric assembly may regulate NendoU activity by impacting its RNA binding ability (Table 3).

Effects of Mn^{2+} and Mg^{2+} on MERS-Nsp15

We further investigated the effect of Mn^{2+} on the activity of MERS-Nsp15. A significant increase in activity was observed as the concentration of Mn^{2+} was increased from 0 to 15 mM, with a 3-fold increase in activity under 10 mM Mn^{2+} (Fig. 7A). Fluorescence polarization (FP) assays were then conducted to examine the Nsp15-RNA binding affinity with increasing Mn^{2+} concentration. The Nsp15-RNA binding affinity increased with the addition of Mn^{2+}, revealing that the function of Mn^{2+} may be a result of the increasing substrate binding ability of Nsp15 (Fig. 7B).

To determine whether Mg^{2+} can also affect the activity of MERS-Nsp15, we performed FRET-based assays and FP assays in which Mg^{2+} concentrations were increased. The catalytic activity of Nsp15 increased slightly in the presence of 15 mM Mg^{2+} (Fig. 7C). Consistently, the Nsp15-RNA binding ability was similar in the presence or absence of Mg^{2+} (Fig. 7D), indicating that the effect of Mg^{2+} *in vitro* was minor compared to the effect of Mn^{2+}.

Effects of CoV RTC-associated protein Nsp8 and the Nsp7/Nsp8 complex on Nsp15

In agreement with previous evidence that MHV Nsp15 strongly colocalizes with primase Nsp8 as well as polymerase Nsp12, immunoprecipitation (IP) experiments also confirmed the binding of MHV Nsp15 to MHV Nsp12 as well as to MHV Nsp8 (20). Pull-down assays were further performed, and the direct interaction between Nsp15 and Nsp8 was monitored, but it was not monitored between Nsp15 and Nsp12 (Fig. 8A). To ascertain whether the interaction between Nsp15 and Nsp8 is dependent on the hexameric state of Nsp15, his-tagged Nsp8 was copurified with the flag-tagged Nsp15 mutants (N38A, D39A, E263A and N157A) harboring distinct oligomeric states and detected by pull-down assays. Nsp8 was shown to interact with each of the Nsp15 variants, indicating that both monomeric Nsp15 and hexameric Nsp15 interact with Nsp8 (Fig. 8A).

Nsp8 was cocrystallized with Nsp7 as a hexadecameric complex that displayed primase-like RdRp activity and dramatically increased the polymerase activity of Nsp12 (23, 25). We therefore examined whether Nsp15 binds to Nsp8 in the context of an Nsp7/Nsp8 complex and affects the endoribonuclease activity of Nsp15. To obtain the Nsp7/Nsp8 complex, purified Nsp7 and Nsp8 were incubated in a 1:1 molar ratio, and the

299 elution peak representing the Nsp7/Nsp8 complex was isolated via gel filtration and
300 corroborated by SDS-PAGE. We next detected and quantified the interaction of Nsp15
301 with Nsp8 and the Nsp7/Nsp8 complex by microscale thermophoresis (MST) assay, a
302 sensitive method that can be used to monitor and quantify binding affinities of complex
303 formation between proteins (24). MST results revealed binding between Nsp15 and Nsp8
304 with a Kd value of 16.33± 3.21 μM (Fig. 8B). Notably, relatively enhanced affinity
305 (6.48 ± 1.47 μM) was observed for the Nsp7/Nsp8 complex titrated with fluorescently
306 labeled Nsp15 (Fig. 8C). As a control, Nsp7 alone displayed no binding to Nsp15 by
307 MST (Fig. 8C). However, we have been unable to detect binding between Nsp12 and the
308 fluorescently labeled Nsp15 by the MST assay (Fig. 8B).
309 To test whether Nsp8 or Nsp7/Nsp8 impact the NendoU activity of Nsp15, we
310 preincubated Nsp15 with Nsp8 and the Nsp7/Nsp8 complex at a 1:1 molar ratio before
311 performing the FRET-based assays. The catalytic rate increased from 1.25 ± 0.06 nM s^{-1}
312 to 1.88 ± 0.09 nM s^{-1} in the presence of Nsp8, while the Nsp7/Nsp8 complex increased
313 the NendoU activity to 1.89 ± 0.04 nM s^{-1} (Fig. 8D). To further demonstrate whether this
314 enzymatic enhancement is related to the oligomeric state of Nsp15, we measured the
315 catalytic rate of the variants D39A, E263A and N157A in the presence of Nsp8 or the
316 Nsp7/Nsp8 complex. Nsp8 enhanced the catalytic rate of the hexameric D39A protein by
317 58 %, while the Nsp7/Nsp8 complex increased the catalytic rate by 54 % (Fig. 8E). For
318 E263A, the activity increased by 33 % in the presence of Nsp8 and increased by 40 % in
319 the presence of Nsp7/Nsp8 (Fig. 8E). However, the activity of the monomeric N157A
320 protein was similar in the presence of Nsp8 or Nsp7/Nsp8, demonstrating that this
321 enhancement effect is highly dependent on the hexameric state of Nsp15.

322

323

324 **DISCUSSION**

325 In this study, we described the crystal structure of MERS-Nsp15 refined to a 2.7 Å
326 resolution. Structural examination of the active site and subunit interaction surfaces,
327 together with biochemical characterization of the critical mutants, revealed that NendoU
328 activity was mediated by 1) catalytic residues within the active site pocket that were
329 indispensable for NendoU activity; 2) residues directly interacting with RNA; and 3)
330 residues providing structural support for hexameric assembly, which is the functionally
331 active state that is responsible for its RNA binding ability.

332 Notably, several different features were identified in MERS-Nsp15 compared to its
333 homologs. For example, the endoribonuclease activity of the variant F285A was slightly
334 increased and the activity of F285S increased about 31% compared to that of the
335 wild-type protein. Examination of the neighboring structure shows that substitution of
336 F285 with alanine or serine likely offsets the steric obstruction of the aromatic side chain,
337 which may spatially interfere with substrate binding in the pocket, and thus, likely widens
338 the active site cavity and facilitates access to the substrate. FP assays confirmed that
339 F285A and F285S displayed increased binding affinity for RNA. In particular, the effect
340 of serine substitution on activity enhancement was more dramatic than that of alanine
341 substitution, possibly due to the fact that serine is hydrophilic, whereas alanine is
342 hydrophobic. More importantly, sequence alignment demonstrated that at the equivalent
343 position of F285, a serine is conserved among other coronaviruses as well as most
344 arteriviruses, and an alanine is present at the equivalent position in EAV (equine arteritis
345 virus) (Fig. 4). Thus, considering that MERS-Nsp15 is the only exception, it is plausible

346 that F285 may exist in MERS-Nsp15 to mediate the function of this protein.

347 Another example is the hexameric mutant D39A, which exhibited higher activity than the
348 wild-type protein. The corresponding mutant D39A from SARS-Nsp15 primarily existed
349 as a monomer and displayed no activity (14). It is likely that apart from interacting with
350 T48, D39 from SARS-Nsp15 also forms an ionic bond with R90, which contributes more
351 to the interprotomer interactions and may explain why mutant D39A exists in different
352 oligomeric states in SARS-Nsp15 and MERS-Nsp15. Remarkably, mutant D39A from
353 MERS-Nsp15 exhibited a higher RNA binding affinity compared to wild-type protein,
354 which may explain its higher activity.

355 Previous studies have demonstrated that the activity of Nsp15 from both SARS-CoV and
356 MHV can be stimulated by Mn^{2+} (11-14). The activity of MERS-Nsp15 increased with
357 the addition of Mn^{2+}, with enhanced RNA binding affinity (Fig. 7A-B). However, Mg^{2+}
358 only slightly enhanced NendoU activity and did not influence RNA binding (Fig. 7C-D).
359 It is plausible that NendoU activity could be regulated by RNA binding via Mn^{2+}. In
360 contrast, structures of MERS-Nsp15 and its orthologs all show no obvious metal binding
361 sites, arguing against the direct involvement of Mn^{2+}. We speculate that Mn^{2+} may serve
362 as a cofactor to stabilize the RNA-Nsp15 structure and thus enhance NendoU activity.
363 Moreover, given that the intracellular concentration of these divalent metal ions is
364 estimated to be within the micromolar range, these biochemical observations from *in*
365 *vitro* studies may not be applicable to the *in vivo* environment. Taken together, more
366 information is needed to elucidate the roles that Mn^{2+} and Mg^{2+} play in the catalytic
367 process and the possible mechanism.

368 If the hexamer assembles in such a way that three monomers first constitute a trimer and
369 two trimers pack back-to-back into a hexamer as proposed, then the trimer state should be

possible. Nonetheless, the trimer could not be isolated and biochemically characterized in our work nor in previous studies, as the mutation of any of these residues (L2, E3, V26, N52 and N110), which are located at the preconceived trimer-trimer interfaces, only yielded proteins that were predominantly monomeric in solution (14). One possible explanation of this discrepancy may be that the trimer is highly unstable and is transient in nature.

Nsp15 interacted with Nsp8 during both the pull-down and MST assays. Remarkably, Nsp15 binds to the Nsp7/Nsp8 complex with a stronger binding affinity compared to Nsp8. In contrast to previous IP results (20), both the pull-down and MST assays could not detect an interaction between Nsp15 and Nsp12, possibly due to the extremely weak interaction. Moreover, given that Nsp12 can complex with Nsp8 and Nsp7 (20), another possible explanation might be that the detected interaction between Nsp12 and Nsp15 by IP was a result from the indirect binding of Nsp12 with Nsp8 instead of the direct binding with Nsp15.

In addition, NendoU activity may be increased by both Nsp8 and the Nsp7/Nsp8 complex, with this increase only being observed for the hexameric state of Nsp15, further strengthening the hypothesis that the hexameric state is the functional form. However, the mechanism through which NendoU activity is enhanced by Nsp8 and the Nsp7/Nsp8 complex remains unclear. It is likely that the interaction between Nsp8 and Nsp15 may provide a possible explanation (*ie.*, the direct interaction might induce a conformational change in Nsp15). Indeed, the SARS-CoV Nsp7/Nsp8 complex may bind RNA and has been shown to confer RNA binding ability to SARS-CoV Nsp12 (23, 25). We speculate, therefore, that Nsp8 or the Nsp7/Nsp8 complex may increase NendoU activity by enhancing the RNA binding ability of Nsp15. Further studies are required to elucidate

mechanisms for the involvement of Nsp15 in the Nsp7/Nsp8/Nsp15 complex and the role Nsp15 may play in viral replication/transcription complex machinery and coronavirus pathogenesis.

In conclusion, we provide the first structure of MERS-Nsp15, and our structure-function studies demonstrate crucial features and provide important novel insights into how NendoU activity is possibly mediated. Moreover, given that inhibitors targeting the catalytic sites may potentially interfere with endoribonuclease within host cells, this work also provides new insights on how drugs designed to disrupt interprotomer interaction surfaces may be applied.

MATERIALS AND METHODS

Construct design and molecular cloning. The sequence encoding the MERS-Nsp15 protein was optimized for expression in *E. coli* and was synthesized by Genscript. A hexahistidine tag was inserted at the N-terminal end via PCR with the forward primer (5'-CATGCCATGGGCCACCACCACCACCACCACGGCCTGGAAAACATTGCGTTTAATG-3') and the reverse primer (5'-CCGCTCGAGTTATTGCAGGCGCGGATAGAAGGTTTGCAC-3'). The PCR product was then cloned into the pRSF-Duet1 vector between the *NcoI* and *XhoI* sites. Point mutations were introduced into the wild-type Nsp15 plasmid using the Fast Mutagenesis System (Transgene Biotech). Nsp15 and Nsp15 mutants (N38A, D39A, N157A and E263A) possessing a flag tag at the N-terminus were constructed in a similar procedure.

418 **Expression and purification of MERS-Nsp15.** The sequence-verified MERS-Nsp15
419 plasmid was transformed into *E. coli* strain BL21 (DE3) cells. The cells were cultured in
420 LB medium containing kanamycin (100 mg liter^{-1}) until the optical density (OD_{600nm})
421 reached 0.6-0.8. The cell culture was then cooled to 16 °C and induced with 0.4 mM
422 isopropyl-β-D-1-thiogalactopyranoside (IPTG). After 14-16 hours of induction, the cells
423 were harvested and lysed in buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl
424 and 70 mM imidazole. The lysate was centrifuged at 25,000 × g for 40 min, and the
425 soluble supernatant was purified by immobilized metal ion affinity chromatography. The
426 eluate was fractionated via a Superdex-200 10/300 (GE Healthcare) column with buffer
427 containing 10 mM Tris-HCl, pH 7.5, and 50 mM NaCl. The protein was concentrated to 8
428 mg/ml for crystal screening or stored at -80 °C for further use. The MERS-Nsp15
429 mutants described in this paper were expressed and purified following a similar protocol
430 as described above.
431 Recombinant MERS-CoV Nsp8 containing both GST and hexahistidine tags and
432 MERS-CoV Nsp7 with a GST tag were expressed and purified as previously reported for
433 SARS-CoV Nsp7 and Nsp8 (25). To obtain Nsp7/Nsp8 complex, purified Nsp7 and Nsp8
434 were incubated in a 1:1 molar ratio at 4°C for 1 hour and then the proteins were separated
435 by Superdex-200 10/300 column. The elution peak representing Nsp7/Nsp8 complex was
436 further corroborated by SDS-PAGE, and the oligomeric state was evaluated by analytical
437 ultracentrifugation (AUC) analysis. MERS-CoV Nsp12 carrying both the N-terminal
438 GST tag and the C-terminal hexahistidine tag was expressed in the baculovirus

expression system (Bac-to-Bac system, Invitrogen) with the Sf9 insect cell line. The GST-fused Nsp12 was primarily purified via a glutathione affinity column and was then digested by thrombin protease overnight to release the GST tag. Then, Nsp12 was further purified using a Hitrap Q (GE Healthcare) column and a Superdex-200 10/300 column (GE Healthcare) to over 95% purity.

Crystallization and structure determination. MERS-Nsp15 crystals were grown at 289 K by the hanging drop vapor diffusion method. Crystals were grown overnight in a mixture of 1 µl protein and 1 µl reservoir solution (4 % v/v tacsimate, pH 4.0, and 12 % w/v polyethylene glycol 3350). Crystals were transferred to a cryoprotected buffer (reservoir solution and 25 % glycerol) and flash frozen in liquid nitrogen.

The data set for Nsp15 was collected at BL19 beamline in the Shanghai Synchrotron Radiation Facility (SSRF), and the data were processed and scaled using the XDS program suite (26). The native Nsp15 structure was solved by molecular replacement with the program Phaser of the CCP4 using MHV Nsp15 (PDB code: 2GTH) as the search model, and the structure refinement was carried out with PHENIX (12, 27-29). Final refinement statistics are summarized in Table 1. Structural figures were drawn with PyMOL (30).

Analytical ultracentrifugation analysis. Purified MERS-Nsp15 and mutant proteins were subjected to analytical ultracentrifugation (AUC) analysis in a buffer containing 20 mM Tris, pH 7.5, and 50 mM NaCl. Sedimentation rate experiments were conducted at 4 °C in a proteomeLab XL-1 Protein Characterization System (Beckman Coulter). The data

were collected at 45,000 rpm using an An-60 Ti rotor (Beckman Coulter) and processed according to a c (S) distribution model.

Differential scanning fluorimetry assay. A differential scanning fluorimetry (DSF) assay was carried out in a real-time PCR machine (LightCycler480II). Each 20-μl reaction mixture contained 0.5 mg/ml purified protein. SYPRO orange was diluted to a 5× final concentration. The temperature was held for 10s and increased from 25 °C to 95 °C at a rate of 1 °C/min. The average fluorescence intensity and melting temperature (Tm) of three measurements were determined and used.

RNA oligonucleotides synthesis. RNA oligonucleotides (R1-R3) were synthesized and purified by Genewiz to over 95% purity. RNA R1 contains 20 rU (5'-rUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrU-3'). RNA R2 (5'-rUrUrArArCrGrArArCrU-3') is derived from the conserved sequence of the transcriptional regulatory sequence (TRS) of the MERS-CoV genome, and RNA R3 is a double-stranded RNA annealed by R2 and its complementary strand (5'-rArGrUrUrCrGrUrUrArA-3').

Fluorescence polarization assay. RNA oligonucleotides used for the fluorescence polarization (FP) assays were labeled at the 3' end with Cy3 and diluted to a final concentration of 100 nM. Purified Nsp15 was diluted with buffer containing 20 mM Tris, pH 7.5, and 50 mM NaCl. The effects of divalent ions were detected in the presence of 0 mM, 2mM, 5 mM, 10 mM or 15 mM $MnCl_2$ or $MgCl_2$. Reaction mixtures with a total

485 volume of 100 µl were prepared, and FP assays were performed using a Perkin-Elmer
486 Envision with the excitation wavelength of 555 nm and the emission wavelength of 595
487 nm. All Nsp15 proteins used for the FP assays in this paper harbored the K286A mutation
488 that abolishes NendoU activity to avoid substrate digestion. The anisotropy values
489 reported were the average of three replicates, and the means ± SD results were shown.
490 Data were further analyzed in Origin 8.0 (OriginLab) using the Hill equation. The
491 calculated dissociation constant (Kd) and the Hill coefficient are listed in Table 2.
492
493 **Endoribonuclease assay.** Real-time endoribonuclease assays were performed using
494 fluorescent resonance energy transfer (FRET) as described previously (12). Substrate
495 (5'-6-FAM-dArUdAdA-6-TAMRA-3') was purchased from TAKARA. The substrate had
496 a carboxyfluorescein (FAM) at the 5' end and a tetramethylrhodamine (TAMRA) at the 3'
497 end, which quenches the FAM fluorescent emission at 518 nm. The cleavage of the
498 substrate leads to increasing fluorescence emission at 518 nm. The cleavage reaction was
499 performed at room temperature and contained 0.2 µM protein and 1.2 µM RNA substrate
500 in a final volume of 100 µl. The fluorescence intensity over time was monitored by the
501 EnSpire Multimode Plate Reader system (PerkinElmer) with the excitation wavelength of
502 498 nm and the emission wavelength of 518 nm.
503 The endoribonuclease assays of wild-type Nsp15 and its mutants were performed in
504 buffer containing 50 mM Tris, pH 7.5, and 50 mM KCl. Nsp8, the Nsp7/Nsp8 complex,
505 Nsp12 and the spike protein (spike protein of MERS-CoV) were adjusted to a 1:1 molar
506 ratio with Nsp15 at a final concentration of 0.2 µM. Nsp15 was preincubated with Nsp8,
507 Nsp7/Nsp8 complex, Nsp12 and S protein for 30 min, respectively, before RNA substrate

was introduced. Wild-type Nsp15 and mutants D39A, E263A and N157A were preincubated with 0.2 μM Nsp8 or 0.2 μM Nsp7/Nsp8 complex, respectively. The effects of divalent ions were detected in the presence of 0 mM, 2mM, 5 mM, 10 mM or 15 mM $MnCl_2$ or $MgCl_2$. The enzymatic activity data were analyzed in Origin 8.0 (OriginLab). All assays were repeated three times, and the means ± SD results were shown in this paper.

Binding affinity quantifications by microscale thermophoresis. Binding affinity was detected by microscale thermophoresis (MST) using Monolith NT.115 (Nanotemper Technologies) as previously reported (24). Purified MERS-Nsp15 was labeled and centrifuged at 14000 rpm for 10 min to eliminate precipitates. A serial dilution of recombinant Nsp7, Nsp8, Nsp12 and the Nsp7/Nsp8 complex was applied within buffer containing 20 mM Tris, pH 7.0, 50 mM NaCl and 0.05% Tween-20. Affinity measurements were conducted in hydrophilic capillaries (Nanotemper, K002 Monolith™ NT.115 Hydrophobic Capillaries). Each measurement was repeated at least three times. The sigmoidal curves were normalized with the mean ± SD of each data point, and Kd values were calculated.

Pull-down and western blot. Purified flag-tagged Nsp15 was incubated with his-tagged Nsp8, his-tagged Nsp8/Nsp7 (Nsp7 has no tag) complex and his-tagged Nsp12 at 4 °C overnight and then applied to anti-flag beads. Nsp15 mutants N38A, D39A, N157A and E263A were bound to anti-flag beads and incubated with his-tagged Nsp8, with his-tagged Nsp12 or without protein. Beads were washed with PBS buffer and

resuspended in electrophoresis sample buffer for SDS-PAGE. Polyacrylamide gel was transferred to polyvinylidene difluoride (PVDE) membranes and blocked by 5 % milk in TBST buffer. Anti-his antibodies were applied for 1 hour at room temperature and then washed with TBST buffer. After incubation with the second antibody, blots were washed, detected and documented on X-ray film.

Accession number. The coordinates and structure factors for MERS-Nsp15 have been deposited in the Protein Data Bank (PDB) under the accession code 5YVD.

ACKNOWLEDGMENTS

We are grateful to the staff at the Shanghai Synchrotron Radiation Facility (China) for their assistance with X-ray diffraction data collection. This work is supported by the National Major Project Grants 2017YFC0840300 and the National Natural Science Foundation of China (81330036, 31570717, 81621005, and 81520108019).

REFERENCES

1. Hilgenfeld R, Peiris M. 2013. From SARS to MERS: 10 years of research on highly pathogenic human coronaviruses. Antiviral Research 100:286-95.
2. Fehr AR, Channappanavar R, Perlman S. 2016. Middle East Respiratory Syndrome: Emergence of a Pathogenic Human Coronavirus. Annual Review of Medicine 68.

553 3. Cavanagh D. 1997. Nidovirales: a new order comprising Coronaviridae and
554 Arteriviridae. Archives of Virology 142:629-633.
555 4. Sawicki SG, Sawicki DL. 1995. Coronaviruses use discontinuous extension for
556 synthesis of subgenome-length negative strands. Oxygen Transport to Tissue
557 XXXIII 380:499-506.
558 5. Prentice E, Mcauliffe J, Lu X, Subbarao K, Denison MR. 2004. Identification and
559 characterization of severe acute respiratory syndrome coronavirus replicase proteins.
560 Journal of Virology 78:9977.
561 6. Gao F, Ou HY, Chen LL, Zheng WX, Zhang CT. 2003. Prediction of proteinase
562 cleavage sites in polyproteins of coronaviruses and its applications in analyzing
563 SARS-CoV genomes. Febs Letters 553:451-456.
564 7. Pasternak AO, Spaan WJ, Snijder EJ. 2006. Nidovirus transcription: how to make
565 sense...? Journal of General Virology 87:1403-1421.
566 8. Sawicki SG, Sawicki DL. 2005. Coronavirus Transcription: A Perspective. Current
567 Topics in Microbiology & Immunology 287:31.
568 9. Ivanov KA, Hertzig T, Rozanov M, Bayer S, Thiel V, Gorbalenya AE, Ziebuhr J.
569 2004. Major genetic marker of nidoviruses encodes a replicative endoribonuclease.
570 Proceedings of the National Academy of Sciences of the United States of America
571 101:12694.
572 10. Bhardwaj K, Palaninathan S, Alcantara JM, Yi LL, Guarino L, Sacchettini JC, Kao
573 CC. 2008. Structural and functional analyses of the severe acute respiratory

syndrome coronavirus endoribonuclease Nsp15. Journal of Biological Chemistry 283:3655.

11. Bhardwaj K, Guarino L, Kao CC. 2004. The severe acute respiratory syndrome coronavirus Nsp15 protein is an endoribonuclease that prefers manganese as a cofactor. Journal of Virology 78:12218.

12. Xu X, Zhai Y, Sun F, Lou Z, Su D, Xu Y, Zhang R, Joachimiak A, Zhang XC, Bartlam M. 2006. New antiviral target revealed by the hexameric structure of mouse hepatitis virus nonstructural protein nsp15. Journal of Virology 80:7909-17.

13. Ricagno S, Egloff MP, Ulferts R, Coutard B, Nurizzo D, Campanacci V, Cambillau C, Ziebuhr J, Canard B. 2006. Crystal structure and mechanistic determinants of SARS coronavirus nonstructural protein 15 define an endoribonuclease family. Proceedings of the National Academy of Sciences of the United States of America 103:11892-11897.

14. Bhardwaj K, Palaninathan S, Alcantara JM, Yi LL, Guarino L, Sacchettini JC, Kao CC. 2008. Structural and functional analyses of the severe acute respiratory syndrome coronavirus endoribonuclease Nsp15. Journal of Biological Chemistry 283:3655.

15. Joseph JS, Saikatendu KS, Subramanian V, Neuman BW, Buchmeier MJ, Stevens RC, Kuhn P. 2007. Crystal Structure of a Monomeric Form of Severe Acute Respiratory Syndrome Coronavirus Endonuclease nsp15 Suggests a Role for Hexamerization as an Allosteric Switch. Journal of Virology 81:6700-8.

16. Kang H, Bhardwaj K, Li Y, Palaninathan S, Sacchettini J, Guarino L, Leibowitz JL, Kao CC. 2007. Biochemical and genetic analyses of murine hepatitis virus Nsp15 endoribonuclease. Journal of Virology 81:13587-13597.

17. Lei Y, Moore CB, Liesman RM, O'Connor BP, Bergstralh DT, Chen ZJ, Pickles RJ, Ting JPY. 2009. MAVS-Mediated Apoptosis and Its Inhibition by Viral Proteins. Plos One 4:e5466.

18. Bhardwaj K, Liu P, Leibowitz JL, Kao CC. 2012. The Coronavirus Endoribonuclease Nsp15 Interacts with Retinoblastoma Tumor Suppressor Protein. Journal of Virology 86:4294.

19. Deng X, Hackbart M, Mettelman RC, O'Brien A, Mielech AM, Yi G, Kao CC, Baker SC. 2017. Coronavirus nonstructural protein 15 mediates evasion of dsRNA sensors and limits apoptosis in macrophages. Proceedings of the National Academy of Sciences of the United States of America 114:E4251.

20. Athmer J, Fehr AR, Grunewald M, Smith EC, Denison MR, Perlman S. 2017. *In Situ* Tagged nsp15 Reveals Interactions with Coronavirus Replication/Transcription Complex-Associated Proteins. Mbio 8:e02320-16.

21. Posthuma CC, Nedialkova DD, Zevenhoven-Dobbe JC, Blokhuis JH, Gorbalenya AE, Snijder EJ. 2006. Site-directed mutagenesis of the Nidovirus replicative endoribonuclease NendoU exerts pleiotropic effects on the arterivirus life cycle. Journal of Virology 80:1653-1661.

615 22. Hoover HS, Wang CY, Middleton S, Ni P, Zlotnick A, Vaughan RC, Kao CC. 2016.
616 Phosphorylation of the Brome Mosaic Virus Capsid Regulates the Timing of Viral
617 Infection. Journal of Virology 90:7748.
618 23. Subissi L, Posthuma CC, Collet A, Zevenhoven-Dobbe JC, Gorbalenya AE, Decroly
619 E, Snijder EJ, Canard B, Imbert I. 2014. One severe acute respiratory syndrome
620 coronavirus protein complex integrates processive RNA polymerase and
621 exonuclease activities. Proceedings of the National Academy of Sciences of the
622 United States of America 111:3900-9.
623 24. Seidel SAI, Dijkman PM, Lea WA, Bogaart GVD, Jerabek-Willemsen M, Lazic A,
624 Joseph JS, Srinivasan P, Baaske P, Simeonov A. 2013. Microscale thermophoresis
625 quantifies biomolecular interactions under previously challenging conditions.
626 Methods 59:301-315.
627 25. Zhai Y, Sun F, Li X, Pang H, Xu X, Bartlam M, Rao Z. 2005. Insights into
628 SARS-CoV transcription and replication from the structure of the nsp7–nsp8
629 hexadecamer. Nature Structural & Molecular Biology 12:980.
630 26. Kabsch W. 2010. XDS. Acta Crystallogr D Biol Crystallogr 66:125–132.
631 27. Mccoy AJ, Grossekunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. 2007.
632 Phaser crystallographic software. Journal of Applied Crystallography 40:658-674.
633 28. Potterton E, Mcnicholas S, Krissinel E, Cowtan K, Noble M. 2010. The CCP4
634 molecular-graphics project. Acta Crystallogr D Biol Crystallogr 58:1955-1957.

635 29. Sauter NK. 2010. PHENIX: building new software for automated crystallographic
636 structure determination. Acta Crystallographica 58:1948-1954.
637 30. Delano WL. 2002. The PyMOL Molecular Graphics System (2002). My
638 Publications 30:442-454.
639 31. Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the
640 sensitivity of progressive multiple sequence alignment through sequence weighting,
641 position-specific gap penalties and weight matrix choice. *Nucleic Acids Res,*
642 22:4673– 4680.
643 32. Gouet P, Jones JD, Hoffman NE, Nussaume L. ESPript: analysis of multiple
644 sequence alignments in PostScript. *Bioinformatics,* 15:305–308.
645
646
647 **FIGURE LEGENDS**
648 **FIG 1 Overall structure of MERS-Nsp15.** (A) Cartoon representation and schematic
649 diagram of the overall structure of a protomer. A schematic diagram of the domain
650 boundaries in the amino-acid sequence is shown above the cartoon. The N-terminal
651 domain, middle domain and C-terminal domain are colored red, green and blue,
652 respectively. The surface transparency is set to 20 %. (B) Side view of the cartoon
653 representation of the hexamer. Three protomers within a trimer are colored green, yellow
654 and blue, with the other trimer colored gray. (C) Side view and top view of the
655 distribution of the N-terminal domain, middle domain and C-terminal domain within the

656 hexamer by surface representation. Six protomers are colored differently. The N-terminal
657 domain, middle domain and C-terminal domain within one protomer are colored red,
658 green and blue and are labeled N, M and C, respectively. Six protomers form a hexamer
659 through the N- to N-terminal interaction.
660
661 **FIG 2 Comparison of MERS-Nsp15 with SARS-Nsp15 and MHV Nsp15.** (A) The
662 structures of MERS-Nsp15, SARS-Nsp15 (PDB code: 2H85) and MHV (PDB code:
663 2GTH) Nsp15 are superposed together. Monomers and trimers are shown separately, and
664 r.m.s.d. of Cα atoms are listed. (B) Three domains of the monomer for MERS-Nsp15,
665 SARS-Nsp15 and MHV Nsp15 are aligned, and r.m.s.d. of Cα atoms are listed.
666
667 **FIG 3 Sequence alignment of MERS-Nsp15 with coronaviruses and arteriviruses.**
668 Key residues within the catalytic center are marked by red arrowheads and residues in the
669 subunit interfaces are marked by blue circles. Strictly conserved residues are depicted in
670 white characters on a black background. Secondary structure elements are shown on top
671 of the alignment (helices are represented by squiggles, β-strands by arrows and turns by
672 the letters TT). Sequences are aligned using ClustalW (31), and the figure is drawn by
673 ESPript (32). NCBI accession numbers are as follow: YP_009047226 for Middle East
674 respiratory syndrome-related coronavirus (MERS-CoV); AGT21317 for SARS
675 coronavirus (SARS-CoV); NP_740619 for murine hepatitis virus strain A59 (MHV);
676 AGT21366 for human coronavirus 229E (HCoV-229E); AIM47753 for porcine epidemic
677 diarrhea virus (PEDV); AGZ84515 for feline infectious peritonitis virus (FIPV);

678 ABG89333 for transmissible gastroenteritis virus virulent Purdue (TGEV); and
679 NP_705592 for equine arteritis virus (EAV).
680
681 **FIG 4 Identification and characterization of residues within the catalytic center of**
682 **MERS-Nsp15.** (A) Structural superposition of MERS-Nsp15 and SARS-Nsp15 (PDB
683 code: 2H85) (13). The structure of MERS-Nsp15 is colored magenta, and the structure of
684 SARS-Nsp15 is colored yellow. The catalytic center of MERS-Nsp15 superimposed with
685 SARS-Nsp15 is enlarged in panel A (the cartoon transparency is set to 20 %). Residues
686 discussed in this paper are labeled with stick representations (magenta, MERS-Nsp15;
687 yellow, SARS-CoV Nsp15). Equivalent residues located in the catalytic site of
688 SARS-Nsp15 are listed below. (B) Protein-RNA binding profiles and the inhibitory
689 effects of the cleavage of the fluorescent substrate by RNA R1, R2 and R3. R1 contains
690 20 rU and exhibits a Kd value of 818.28 $\pm$ 50.39 nM. R2 binds to Nsp15 with a Kd
691 value of 1190.52 $\pm$ 137.91 nM. R3 exhibits a Kd value of 932.49 $\pm$ 49.40 nM. The
692 activity of the wild-type Nsp15 in the absence of R1, R2 or R3 is set to 100%.
693 Fluorescent intensity is measured at each RNA (R1-R3) concentration, and the values
694 shown are the average of three measurements. (C) DSF profile of wild-type Nsp15. All
695 mutants listed in panel A share similar melting temperatures and DSF profiles as
696 wild-type Nsp15. (D) NendoU activity profile for the mutants with alanine substitution of
697 residues located within the catalytic site. FRET-based assays for different mutants are
698 conducted, and the reaction rate is calculated. (E) NendoU activity profile for active-site
699 mutants with alanine substitutions and the corresponding residue in SARS-Nsp15.
700

FIG 5 Stick representation of residues involved in the interprotomer interaction of MERS-Nsp15. A side view of the surface representation of the interactions within three protomers is shown. Protomers A, B and C are colored blue, yellow and magenta, respectively. Residues involved in the subunits interaction are labeled with stick representations (blue, subunit A; yellow, subunit B; and magenta, subunit C; the cartoon transparency is set to 20 %). Four contact regions are boxed and enlarged. The atomic distances are measured by PyMOL.

FIG 6 Biochemical characterization of mutants critical for the oligomeric assembly of MERS-Nsp15. (A) Analytical ultracentrifuge (AUC) analysis of mutants containing mutations located at interaction surfaces. AUC profiles of wild-type protein, D39A, E263A, N38A, Y58A and N157A mutants are shown. The first peak at approximately 40 kD represents the position of the monomer, and the second peak at approximately 240 kD represents the position of the hexamer. (B) DSF profiles of wild-type Nsp15 and mutants D39A, E263A, N38A, Y58A and N157A. (C) Protein-RNA binding profiles of the Nsp15 mutants by fluorescence polarization (FP). Alanine substitution is performed at K286 in the wild type and mutants (D39A, E263A, N38A, Y58A and N157A) for this assay to prevent substrate digestion during the experiment. (D) NendoU activity profiles for the mutants related to oligomeric assembly.

FIG 7 Effects of Mn^{2+} and Mg^{2+} on MERS-Nsp15. (A) Catalytic rate profile for the effects of Mn^{2+} on the activity of MERS-Nsp15. (B) Nsp15 (K286A)-RNA binding profiles for Nsp15 with increasing Mn^{2+} concentration determined via fluorescence polarization. (C) Catalytic rate profile for the effects of Mg^{2+} on the activity of

MERS-Nsp15. (D) Nsp15 (K286A)-RNA binding profiles for Nsp15 with increasing Mg^{2+} concentration determined via fluorescence polarization.

FIG 8 Influence of MERS-CoV Nsp8 and the Nsp7/Nsp8 complex on MERS-Nsp15. (A) Pull-down assays detecting the interaction of the Nsp12/Nsp15, Nsp8/Nsp15 or Nsp8/Nsp15 mutants. (B) MST binding curves for the titration of fluorescently labeled Nsp15 into Nsp8 (green) and Nsp12 (orange). Error bars showing SDs are calculated from triplicate experiments. (C) Determination of the binding affinities of Nsp15 with Nsp7 (black) and the Nsp7/Nsp8 complex (blue) via MST assays. (D) Effects of Nsp8 and the Nsp7/Nsp8 complex on the endoribonuclease activity of MERS-Nsp15. (E) Effects of Nsp8 and the Nsp7/Nsp8 complex on the catalytic rate of Nsp15 mutants.

749 **TABLES**

750 **Table 1 Data collection and refinement statistics.**

Parameters (Data collection statistics)	MERS-Nsp15
Data collection statistics	
Cell parameters	
a (Å)	145.6
b (Å)	145.6
c (Å)	96.7
α, β, γ (°)	90.0, 90.0, 120.0
Space group	*H*3
Wavelength used (Å)	0.9798
Resolution (Å)	50.0–2.70 (2.75-2.70)[c]
No. of all reflections	222,454 (9,905)
No. of unique reflections	21,016 (1,065)
Completeness (%)	100.0 (100.0)
Average I/σ(I)	10.6 (2.33)
$CC_{1/2}$	0.952(0.796)
R_{merge}[a] (%)	10.4 (69.9)
Refinement statistics	
No. of reflections used (σ(F) > 0)	19,908
R_{work}[b] (%)	17.5
R_{free}[b] (%)	24.7
r.m.s.d. bond distance (Å)	0.011
r.m.s.d. bond angle (°)	1.453
Average B-value (Å^2)	44.62
No. of protein atoms	5,484
No. of ligand atoms	17
No. of solvent atoms	252
Ramachandran plot	
res. in favored regions (%)	93.66
res. in allowed regions (%)	6.19
res. in outlier regions (%)	0.15

751 [a] $R_{merge} = \Sigma_h\Sigma_l \mid I_{ih} - I_h \mid / \Sigma_h\Sigma_l I_h$ where I_h is the mean of observations I_{ih} of reflection h.

752 [b] $R_{work} = \Sigma(\mid\mid F_p(obs)\mid - \mid F_p(calc)\mid\mid) / \Sigma \mid F_p(obs)\mid$. R_{free} is an *R* factor for a pre-selected subset

753 (5%) of reflections that was excluded in the refinement.

754 [c] Numbers in parentheses are corresponding values for the highest resolution shell.

Table 2 Catalytic activity and RNA binding properties of wild-type Nsp15 and its active site mutants.

MERS-Nsp15	Catalytic rate (nM/s)	Kd value (nM)[a]	Hill coefficient[b], n
WT	1.25 ± 0.06		
K286A	ND[c]	818.28 ± 50.39	2.24 ± 0.24
H246A	ND	916.47 ± 102.13	2.07 ± 0.53
Y339A	Very low	1029.04 ± 102.73	2.16 ± 0.44
H231A	ND	811.01 ± 51.00	3.11 ± 0.64
S290A	0.83 ± 0.02	1156.49 ± 94.12	2.38 ± 0.37
F285A	1.34 ± 0.06	678.63 ± 36.75	2.72 ± 0.43
F285S	1.63 ± 0.07	616.28 ± 33.09	2.39 ± 0.34
T241A	ND	997.11 ± 121.47	2.30 ± 0.64
T241Q	1.17 ± 0.04	798.96 ± 53.70	2.44 ± 0.47
R341A	ND	1037.28 ± 72.79	2.57 ± 0.44
R341K	0.61 ± 0.06	807.61 ± 51.92	2.65 ± 0.51

[a,b] All Nsp15 mutants for the FP assays harbored the K286A mutation to avoid the possible digestion of the RNA substrate. The Kd value and Hill coefficient of the wild-type protein were determined and are represented by mutant K286A. The means ± SDs of the Kd and Hill coefficient values shown here were obtained by fitting the data to the Hill equation.

[c] Not detected.

768

769

770 **Table 3 Catalytic activity and RNA binding properties of wild-type Nsp15 and its**

771 **subunit interface mutants.**

MERS-Nsp15	Catalytic rate (nM/s)	Kd value (nM)[a]	Hill coefficient[b], n
WT	1.25 ± 0.06		
K286A	ND[c]	818.28 ± 50.39	2.24 ± 0.24
N38A	0.38 ± 0.04	ND	ND
D39A	1.37 ± 0.04	626.22 ± 24.80	2.15 ± 0.17
N157A	ND	ND	ND
Y58A	0.25 ± 0.03	ND	ND
E263A	0.89 ± 0.06	1013.43 ± 77.38	2.09 ± 0.20

772 [a,b] All Nsp15 mutants for the FP assays harbored K286A mutation to avoid digestion of

773 the RNA substrate. The Kd value and Hill coefficient of the wild-type protein were

774 measured and are represented by mutant K286A. The means ± SDs of the Kd and Hill

775 coefficient values shown here were obtained by fitting the data to the Hill equation.

776 [c] Not detected.