

1 for publication in Journal of Virology

2

3 **The enzymatic activity of the nsp14 exoribonuclease is critical for replication of**

4 **Middle East respiratory syndrome-coronavirus**

5

6

7

8

9 Natacha S. Ogando, Jessika C. Zevenhoven-Dobbe, Clara C. Posthuma^{§#} & Eric J.

10 Snijder^{§*}

11

12

13 Department of Medical Microbiology, Leiden University Medical Center,

14 Leiden, The Netherlands

15

16 [§] These authors contributed equally.

17 * Address correspondence to Eric J. Snijder; E.J.Snijder@lumc.nl

18 # Present address: Netherlands Commission on Genetic Modification, Bilthoven, The

19 Netherlands

20

21 **Running title:** MERS-CoV ExoN activity is critical for virus replication

22 **Keywords:** nidovirus, replicase, nonstructural protein, RNA synthesis,

23 proofreading, reverse genetics, N7-methyltransferase

24

25 **Abstract: 250 words**

26 **Importance: 150 words**

27

28

29 **Figures: 10**

30

31

32 **ABSTRACT**

33 Coronaviruses (CoVs) stand out for their large RNA genome and complex RNA-
34 synthesizing machinery comprising 16 nonstructural proteins (nsps). The bifunctional
35 nsp14 contains an N-terminal 3'-to-5' exoribonuclease (ExoN) and a C-terminal N7-
36 methyltransferase (N7-MTase) domain. While the latter presumably operates during
37 viral mRNA capping, ExoN is thought to mediate proofreading during genome
38 replication. In line with such a role, ExoN-knockout mutants of mouse hepatitis virus
39 (MHV) and severe acute respiratory syndrome coronavirus (SARS-CoV) were
40 previously found to have a crippled but viable hypermutation phenotype. Remarkably,
41 using an identical reverse genetics approach, an extensive mutagenesis study
42 revealed the corresponding ExoN-knockout mutants of another betacoronavirus,
43 Middle East respiratory syndrome coronavirus (MERS-CoV), to be non-viable. This is
44 in agreement with observations previously made for alpha- and gammacoronaviruses.
45 Only a single MERS-CoV ExoN active site mutant could be recovered, likely because
46 the introduced D191E substitution is highly conservative in nature. For 11 other MERS-
47 CoV ExoN active site mutants, not a trace of RNA synthesis could be detected, unless
48 – in some cases – reversion had first occurred. Subsequently, we expressed and
49 purified recombinant MERS-CoV nsp14 and established *in vitro* assays for both its
50 ExoN and N7-MTase activities. All ExoN knockout mutations that were lethal when
51 tested via reverse genetics were found to severely decrease ExoN activity, while not
52 affecting N7-MTase activity. Our study thus reveals an additional function for MERS-
53 CoV nsp14 ExoN, which apparently is critical for primary viral RNA synthesis, thus
54 differentiating it from the proofreading activity thought to boost long-term replication
55 fidelity in MHV and SARS-CoV.

56

57 **IMPORTANCE**

58 The bifunctional nsp14 subunit of the coronavirus replicase contains 3'-to-5'
59 exoribonuclease (ExoN) and N7-methyltransferase (N7-MTase) domains. For the
60 betacoronaviruses MHV and SARS-CoV, the ExoN domain was reported to promote
61 the fidelity of genome replication, presumably by mediating some form of proofreading.
62 For these viruses, ExoN knockout mutants are alive while displaying an increased
63 mutation frequency. Strikingly, we now established that the equivalent knockout
64 mutants of MERS-CoV ExoN are non-viable and completely deficient in RNA
65 synthesis, thus revealing an additional and more critical function of ExoN in coronavirus
66 replication. Both enzymatic activities of (recombinant) MERS-CoV nsp14 were
67 evaluated using newly developed *in vitro* assays that can be used to characterize these
68 key replicative enzymes in more detail and explore their potential as target for antiviral
69 drug development.

70

71

72 **INTRODUCTION**

73 RNA viruses commonly exhibit high mutation rates, a feature attributed to the relatively
74 poor fidelity of their RNA-dependent RNA polymerase (RdRp) and the fact that
75 nucleotide incorporation errors go uncorrected. This lack of proofreading contributes
76 to the generation of 'quasispecies' populations, clouds of genome sequence variants
77 that are subject to continuous natural selection (1-3). On the one hand, their genetic
78 heterogeneity allows RNA viruses to rapidly adapt to changing circumstances, in order
79 to overcome environmental challenges such as host switching, antiviral drug treatment,
80 or host immune responses (4, 5). On the other hand, the accumulation of an excessive
81 number of deleterious mutations can result in 'error catastrophe' and, consequently, in
82 the extinction of a viral species (6-8). In order to balance these opposing principles,
83 RNA viruses are thought to operate close to their so-called 'error threshold', while
84 balancing the interdependent parameters of replication fidelity, genome size, and
85 genome complexity (9, 10). This interplay is thought to have restricted the expansion
86 of RNA virus genome size, which is below 15 kilobases (kb) for most RNA virus families
87 (10-12).

88 The largest RNA virus genomes currently known are found in the order *Nidovirales*,
89 which includes the coronavirus (CoV) family and also the recently discovered planarian
90 secretory cell nidovirus (PSCNV; (12, 13)), which has the largest RNA genome
91 identified thus far (41.1 kb). One of the molecular mechanisms potentially driving the
92 unprecedented expansion of nidovirus genomes emerged about 17 years ago, during
93 the in-depth bioinformatics analysis of the genome and proteome of the severe acute
94 respiratory syndrome coronavirus (SARS-CoV). During this analysis, Alexander
95 Gorbalenya and colleagues identified a putative 3'-to-5' exoribonuclease (ExoN)
96 signature sequence in the N-terminal domain of nonstructural protein 14 (nsp14), a

97 subunit of the large replicase polyprotein encoded by CoVs and related large-genome
98 nidoviruses. Strikingly, this ExoN domain was found to be lacking in the replicases of
99 nidoviruses with small(er) genomes (specifically, arteriviruses), and therefore it was
100 proposed that the enzyme may provide a form of ‘proofreading activity’ that could have
101 promoted the expansion of large nidoviral genomes to their current size (10-12, 14).
102 Comparative sequence analysis with cellular homologs classified the nidoviral/CoV
103 ExoN domain as a member of the superfamily of DEDDh exonucleases, which also
104 includes the proofreading domains of many DNA polymerases as well as other
105 eukaryotic and prokaryotic exonucleases (15). These enzymes catalyze the excision
106 of nucleoside monophosphates from nucleic acids in the 3’-to-5’ direction, using a
107 mechanism that depends on two divalent metal ions and a reactive water molecule
108 (16-18). Five conserved active site residues arranged in three canonical motifs (I, II
109 and III; Fig. 1) orchestrate ExoN activity (14, 19-21). Additionally, the domain
110 incorporates two zinc finger (ZF) motifs (10), ZF1 and ZF2 (Fig. 1), that were
111 hypothesized to contribute to the structural stability and catalytic activity of ExoN,
112 respectively (20).

113 The predicted 3’-to-5’ exoribonuclease activity of the CoV ExoN domain was first
114 confirmed *in vitro*, in biochemical assays using recombinant SARS-CoV nsp14 and
115 different synthetic RNA substrates (19). Originally, residues D90/E92 (motif I), D243
116 (motif II), and D273 (motif III) were identified as putative active site residues of SARS-
117 CoV nsp14 (14, 19). However, the SARS-CoV nsp14 crystal structure revealed E191
118 rather than D243 to be the acidic active residue in Motif II, demonstrating that ExoN is
119 in fact a DEEDh enzyme (20). By using reverse genetics of the alphacoronavirus
120 human coronavirus 229E (HCoV-229E), Minskaia *et al.* demonstrated that inactivation
121 of the ExoN active site results in failure to recover infectious viral progeny (19).

122 Interestingly, a quite different phenotype was described for the corresponding ExoN-
123 knockout mutants of two betacoronaviruses, mouse hepatitis virus (MHV) and SARS-
124 CoV. While ExoN inactivation decreased replication fidelity in these viruses, conferring
125 a 'mutator phenotype', the mutants were viable, both in cell culture (22, 23) and in
126 animal models (24). These findings suggested that ExoN may indeed be part of an
127 error correction mechanism. Subsequently, the ability of ExoN to excise 3'-terminal
128 mismatched nucleotides from a double-stranded (ds) RNA substrate was
129 demonstrated *in vitro* using recombinant SARS-CoV nsp14 (25). Furthermore, this
130 activity was shown to be strongly enhanced (up to 35-fold) by the presence of nsp10,
131 a small upstream subunit of the CoV replicase (26). The two subunits were proposed
132 to operate, together with the nsp12-RdRp, in repairing mismatches that may be
133 introduced during CoV RNA synthesis (21, 27). In cell culture, MHV and SARS-CoV
134 mutants lacking ExoN activity exhibit increased sensitivity to mutagenic agents like 5-
135 fluoracil (5-FU), compounds to which the wild-type virus is relatively resistant (28, 29).
136 Recently, ExoN activity was also implicated in CoV RNA recombination, as an MHV
137 ExoN knockout mutant exhibited altered recombination patterns, possibly reflecting its
138 involvement in other activities than error correction during CoV replication and
139 subgenomic mRNA synthesis (30). Outside the order *Nidovirales*, arenaviruses are the
140 only other RNA viruses known to employ an ExoN domain, which is part of the
141 arenavirus nucleoprotein and has been implicated in fidelity control (31) and/or immune
142 evasion, the latter by degrading viral dsRNA (32, 33). Based on results obtained with
143 TGEV and MHV ExoN knockout mutants, also the CoV ExoN activity was suggested
144 to counteract innate responses (34, 35).

145 In the meantime, CoV nsp14 was proven to be a bifunctional protein by the discovery
146 of an (N7-guanine)-methyltransferase (N7-MTase) activity in its C-terminal domain

147 (36)(Fig. 1). This enzymatic activity was further corroborated *in vitro*, using biochemical
148 assays with purified recombinant SARS-CoV nsp14. The enzyme was found capable
149 of methylating cap analogues or GTP substrates, in the presence of S-adenosyl
150 methionine (SAM) as methyl donor (36, 37). The N7-MTase was postulated to be a
151 key factor for equipping CoV mRNAs with a functional 5'-terminal cap structure, as N7-
152 methylation is essential for cap recognition by the cellular translation machinery (25).
153 Although, the characterization of the nsp14 N7-MTase active site and reaction
154 mechanism was not completed, alanine scanning mutagenesis and *in vitro* assays with
155 nsp14 highlighted several key residues (Fig. 1)(36, 38, 39). Moreover, crystal
156 structures of SARS-CoV nsp14 in complex with its nsp10 co-factor (PDB entries 5C8U
157 and 5NFY) revealed several unique structural and functional features (Ma et al., 2015;
158 Ferron et al., 2018). These combined structural and biochemical studies confirmed that
159 the two enzymatic domains of nsp14 are functionally distinct (36) and physically
160 independent (20, 21). Still, the two activities are structurally intertwined, as it seems
161 that the N7-MTase activity depends on the integrity of the N-terminal ExoN domain,
162 whereas the flexibility of the protein is modulated by a hinge region connecting the two
163 domains (21).

164 Coronaviruses are abundantly present in mammalian reservoir species, including
165 bats, and pose a continuous zoonotic threat (40-43). To date, seven CoVs that can
166 infect humans have been identified, and among these the severe acute respiratory
167 syndrome coronavirus 2 (SARS-CoV-2) is currently causing an unprecedented
168 pandemic outbreak. The previous CoV to emerge, in 2012, was the Middle East
169 respiratory syndrome coronavirus (MERS-CoV) (44). Due to zoonotic transfer from
170 dromedary camels and subsequent nosocomial transmission, MERS-CoV still
171 continues to circulate and cause serious human disease, primarily in the Arabian

172 Peninsula (45). Occasional spread to other countries has also occurred, including an
173 outbreak with 186 confirmed cases in South Korea in 2015 (46-48). Like SARS-
174 CoV/SARS-CoV2 and MHV, MERS-CoV is classified as a member of the
175 *betacoronavirus* genus, although it belongs to a different lineage (subgenus) of that
176 cluster (49, 50). The current lack of approved therapeutics and vaccines to prevent or
177 treat CoV infections, as well as the general threat posed by emerging CoVs,
178 necessitates the further in-depth characterization of CoV replication and replicative
179 enzymes. In this context, the quite different phenotypes described for ExoN knockout
180 mutants of other CoVs (see above) prompted us to study the importance of this enzyme
181 for MERS-CoV replication. To this end, using both reverse genetics and biochemical
182 assays with recombinant nsp14, we engaged in an extensive site-directed
183 mutagenesis study, targeting all active site residues of the MERS-CoV ExoN domain.
184 Strikingly, in contrast to what was observed for other betacoronaviruses, our studies
185 revealed that ExoN inactivation renders MERS-CoV RNA synthesis undetectable and
186 results in failure to recover viable virus progeny. Our biochemical evaluation of nsp14
187 mutants suggests that this is not caused by inadvertent side-effects of ExoN
188 inactivation on N7-MTase activity. Our combined data suggest that MERS-CoV ExoN
189 and/or nsp14 play a more direct and fundamental role in CoV RNA synthesis than
190 merely safeguarding the long-term fidelity of replication, and can be considered a
191 prominent target for the development of antiviral drugs.

192

193 **RESULTS**

194

195 **ExoN inactivation is lethal for MERS-CoV**

196 Previous studies into CoV ExoN function involved its biochemical characterization
197 (based almost exclusively on the SARS-CoV version of the enzyme) and the
198 phenotypic analysis of (predicted) ExoN knockout virus mutants, generated using
199 reverse genetics approaches. The latter studies yielded replication-incompetent ExoN
200 knockout mutants for the alphacoronaviruses HCoV-229E (19) and transmissible
201 gastroenteritis virus (TGEV) (34). However, the equivalent mutants of the
202 betacoronaviruses SARS-CoV and MHV-A59 were somewhat crippled but clearly
203 viable, while displaying a 15- to 20-fold increased mutation rate (Eckerle, Lu et al. 2007,
204 Eckerle, Becker et al. 2010). An alignment of CoV nsp14 amino acid sequences is
205 presented in Fig. 1, including SARS-CoV-2, which emerged during the course of this
206 project. It highlights the key motifs/residues of the two enzymatic domains of nsp14,
207 as well as other structural elements, like the nsp10 binding site, the hinge region
208 connecting the ExoN and N7-MTase domains, and three previously identified zinc
209 finger domains (20, 21). The alignment also illustrates the generally high degree of
210 nsp14 sequence conservation across different CoV (sub)genera.

211 In the present study, we targeted all five predicted active site residues of the MERS-
212 CoV ExoN domain (D90, E92, E191, D273, and H268) by replacing them with alanine
213 as well as more conservative substitutions (D to E or Q; E to D or Q). This yielded a
214 total of 14 ExoN active site mutants (Fig. 2A), including the D90A/E92A motif-I double
215 mutant (DM) that was frequently used in studies of the viable ExoN knockout mutants
216 for MHV and SARS-CoV. A BAC-based MERS-CoV reverse genetics system (51, 52),
217 based on the sequence of the EMC/2012 isolate of MERS-CoV (53), served as the

218 starting point to evaluate our ExoN mutants by transfection of full-length RNA that was
219 obtained by *in vitro* transcription using T7 RNA polymerase.

220 Transcripts were electroporated into BHK-21 cells, which lack the DPP4 receptor
221 required for natural MERS-CoV infection (Chan, Chan et al. 2013, Raj, Mou et al. 2013)
222 but are commonly used to launch engineered CoV mutants because of their excellent
223 survival of the electroporation procedure (19, 22, 23, 34, 51, 54). As BHK-21 cells have
224 a severely compromised innate immune response (55), they would seem an
225 appropriate cell line to launch ExoN knockout mutants also in case the enzyme would
226 be needed to counter innate immunity (Becares, Pascual-Iglesias et al. 2016, Case, Li
227 et al. 2018). To amplify any progeny virus released, transfected BHK-21 cells were
228 mixed with either innate immune-deficient (Vero) or -competent (HuH7) cells, which
229 both are naturally susceptible to MERS-CoV infection.

230 In stark contrast to what was previously described for MHV and SARS-CoV,
231 mutagenesis of ExoN active site residues was found to fully abrogate MERS-CoV
232 replication. When transfected cell cultures were analyzed using immunofluorescence
233 microscopy at 2 days post transfection (d p.t.), abundant signal was always observed
234 for wild-type MERS-CoV, but no sign of virus replication was observed for 13 out of 14
235 mutants tested (Fig. 2), regardless whether Vero or HUH7 cells were used for
236 propagation of recombinant virus. Furthermore, infectious progeny was not detected
237 when transfected cell culture supernatants were analyzed in plaque assays (Fig. 2 and
238 data not shown). The single exception was the mutant carrying the conservative E191D
239 replacement in ExoN motif II (Fig. 1), which was alive but somewhat crippled, as will
240 be discussed in more detail below. These results were consistent across a large
241 number of independent repeats (>10 for several of the mutants), performed with RNA
242 transcribed from independently engineered (and fully sequenced) duplicate full-length

243 cDNA clones. The non-viable phenotype of MERS-CoV ExoN mutants in both cell
244 types suggests that innate immune responses did not influence the outcome of these
245 experiments.

246

247 **ExoN inactivation abrogates MERS-CoV RNA synthesis**

248 For a selection of MERS-CoV ExoN knockout mutants, intracellular RNA was isolated
249 from transfected cell cultures after 48 h and analyzed by in-gel hybridization and RT-
250 PCR to more rigorously verify the lack of viral RNA synthesis (Fig. 3). In this analysis,
251 a non-viable MERS-CoV mutant with an in-frame 100-aa deletion in the nsp12-RdRp
252 domain was used as a negative control (NC) for viral RNA synthesis, in order to assess
253 and correct for the detection of any residual full-length RNA transcript that might still
254 be present at this timepoint after transfection. Upon direct in-gel hybridization analysis
255 using a ³²P-labeled probe recognizing the 3' end of all viral mRNAs, the characteristic
256 nested set of MERS-CoV transcripts could only be detected for the E191D mutant and
257 the wt control virus (Fig. 3). Even after a 28-day exposure of the phosphor imager
258 screen (data not shown), signal could not be detected for any of the other mutants.

259 The lack of MERS-CoV-specific RNA synthesis was further analyzed using RT-PCR
260 assays specifically detecting genomic RNA or subgenomic mRNA₃. RNA
261 accumulation was evaluated at 1 and 2 d p.t. for seven selected ExoN active site
262 mutants (D90A, D90E, E191A, E191D, D273A, H268A, DM) using samples from two
263 independent experiments both comprising duplicate transfections for each mutant.
264 Again, MERS-CoV-specific genomic and subgenomic RNA synthesis was only
265 detected for the E191D mutant and the wt virus control (data not shown). For all other
266 mutants, the RT-PCR assays yielded Ct values equivalent to those obtained for
267 samples from mock-infected cells and the replication-deficient NC mutant. In

268 conclusion, with the exception of E191D (see below), all our engineered ExoN active
269 site mutations abrogated viral RNA synthesis completely, suggesting that in the case
270 of MERS-CoV the enzyme is indispensable for basic replication in cell culture.

271

272 **Characterization of rMERS-CoV-nsp14-E191D replication kinetics and 5-FU** 273 **sensitivity**

274 Among our ExoN active site mutants, only the E191D mutant yielded viable progeny
275 (Fig. 2). The mutant appeared to be genetically stable as the substitution was
276 preserved upon multiple consecutive passages in HuH7 or Vero cells (data not shown).
277 Interestingly, the E191D mutation transforms the DEEDh catalytic motif into the
278 DEDDh motif that is characteristic for members of the exonuclease family to which the
279 CoV ExoN belongs (56). In fact, when comparing ExoN sequences from different
280 nidovirus taxa (14, 19) the equivalent of E191 alternates between E and D (57), in line
281 with the observation that this mutation is tolerated in MERS-CoV ExoN.

282 To characterize the E191D mutant in more detail, its replication and fitness in cell
283 culture were analyzed. Full-length genome sequencing of passage 2 of the E191D
284 mutant virus revealed that it had acquired two other mutations when compared with
285 the recombinant wt control: a synonymous mutation in the nsp2-coding region (U→C
286 at nt position 2,315) and a non-synonymous mutation (C→A at nt position 6,541)
287 specifying an A1235D substitution in the betacoronavirus-specific marker (βSM)
288 domain of nsp3, which has been predicted to be a non-enzymatic domain (58) and is
289 absent in alpha- and delta-coronaviruses (59, 60). Thus, we assumed that any
290 changes in viral replication were likely caused by the E191D mutation in nsp14 ExoN.
291 The same virus stock was used to assess growth kinetics in HuH7 cells (Fig. 4B) and
292 Vero cells (Fig. 4A), which were found to be very similar for wt and mutant virus. Still,

293 the E191D mutant was found to be somewhat crippled, yielding smaller plaque sizes
294 and somewhat lower progeny titers in HuH7 cells (Fig. 4 B-C), but not in Vero cells
295 (Fig. 4A).

296 We next examined the sensitivity of E191D and wt virus to the mutagenic agent 5-
297 FU, which intracellularly is converted into a nucleoside analogue that is incorporated
298 into viral RNA (61, 62). Previously, MHV and SARS-CoV ExoN knockout mutants were
299 found to exhibit increased sensitivity to 5-FU treatment, in particular in multi-cycle
300 experiments, which was attributed to a higher mutation frequency in the absence of
301 ExoN-driven error correction (28). We employed this same assay to assess the
302 phenotype of the E191D mutant in more detail, by performing plaque assays in HuH7
303 cells in the presence of increasing 5-FU concentrations (Fig. 4C) and by growing
304 mutant and wt virus in the presence of increasing 5-FU concentrations (Fig. 4D). No
305 cytotoxicity was observed in HuH7 cells following treatment with up to 400 μ M 5-FU
306 (data not shown).

307 Plaque assays in HuH7 cells were performed for 3 days, using a standard inoculum
308 of 30 p.f.u. and an increasing amount of 5-FU in the overlay. Similar dose-dependent
309 reductions of plaque size were observed for E191D and wt virus, with E191D plaques
310 being barely visible upon treatment with 200 μ M 5-FU (Fig. 4C). In an alternative
311 experiment, Huh7 cells were infected with an m.o.i. of 1 and treated with an increasing
312 5-FU dose for 30 h, after which progeny virus titers were determined by regular plaque
313 assay. Again, both viruses exhibited a similar concentration-dependent decrease of
314 replication (Fig. 4D), although the E191D mutant appeared to be somewhat more
315 sensitive to the mutagenic agent, yielding ~1-log lower progeny titers than wt virus
316 upon treatment with 5-FU concentrations between 100 and 400 μ M. These
317 experiments demonstrated that E191D and wt MERS-CoV are comparably sensitive to

318 5-FU treatment, suggesting that ExoN functionality is not strongly affected by the
319 E191D substitution.

320

321 **ExoN zinc finger motifs are important for viral replication**

322 Studies addressing the structural biology and biochemistry of SARS-CoV nsp14
323 indicated that the two ZF motifs within the ExoN domain contribute to either its
324 structural stability (ZF1) or to catalytic activity (ZF2) (20, 21). Moreover, mutagenesis
325 studies of the MHV and TGEV ZF1 domain supported their importance for viral
326 replication in cell culture (34, 54). To study the impact of similar mutations on MERS-
327 CoV replication, the nsp14 ZF1 and ZF2 domains were targeted with two mutations
328 each and their impact on virus viability was evaluated as described above. Two ZF1
329 residues (C210 and H229) were mutated from H to C or vice versa, which could
330 theoretically preserve the zinc-coordinating properties (63, 64). Two residues of the
331 non-classical ZF2 motif were also substituted (C261A and H264R) to evaluate the
332 same ZF mutations previously analyzed by Ma *et al.*, leading to disruption of ExoN
333 activity *in vitro* (20).

334 The four ZF virus mutants were launched as described above, after which a low
335 level of replication could be observed only for the H229C ZF1 mutant, for which the 2
336 d p.t. harvest yielded very small plaques and low progeny titers (Fig. 2B). For this
337 mutant, RNA synthesis could not be detected by hybridization analysis (Fig. 3), but
338 synthesis of genomic and subgenomic RNA (mRNA3) was detected by RT-PCR in
339 intracellular RNA samples harvested at 2 d p.t. (data not shown). A 6 d p.t. harvest
340 was used for full genome sequencing by NGS, which confirmed the presence of the
341 engineered nsp14 mutation in addition to the appearance of some minor genetic
342 variants (point mutations representing less than 15% of the total population) in different

343 regions of the genome, including the ORF1a domain encoding for nsp3, nsp6, nsp8,
344 and nsp9. Taken together, our observations defined the severely crippled phenotype
345 of the H229C mutant. In combination with the fact that the other ZF mutations (C210H
346 in ZF1, and C261A and H264R in ZF2) abolished all detectable MERS-CoV replication,
347 our study establishes the importance of both ExoN ZF motifs for MERS-CoV viability.
348

349 **Development of a MERS-CoV ExoN activity assay using recombinant nsp14**

350 In order to assess the impact of mutations on nsp14's enzymatic activities, we set out
351 to purify recombinant MERS-CoV nsp14 and develop an *in vitro* ExoN assay. Thus far,
352 such an assay had only been described for the equivalent SARS-CoV protein (19, 20,
353 26, 65). Wild-type and mutant MERS-CoV nsp14 proteins carrying an N-terminal His-
354 tag were expressed in *E. coli* Rosetta (DE3) pLysS. Proteins were purified by
355 immobilized metal affinity chromatography (IMAC) followed by size exclusion
356 chromatography. Upon SDS-PAGE, the purified MERS-CoV nsp14 was consistently
357 detected as a doublet (with the lower band being most abundant), migrating at the
358 expected molecular mass of ~55 kDa (Fig. 5). As a positive control, we purified SARS-
359 CoV nsp14 (26) and used it during optimization of the enzymatic assays for MERS-
360 CoV nsp14. The substrate used for ExoN activity assays was a 5'-³²P-labeled 22-
361 nucleotide (nt) long synthetic RNA, as previously used in similar assays with SARS-
362 CoV nsp14 (referred to as oligonucleotide H4 in (26)). Nucleotides 5-22 of this
363 substrate are predicted to form a hairpin with a stem consisting of seven G-C base
364 pairs and a loop of 4 A's (26), while the remaining 4 nucleotides form a 5'-terminal
365 single-stranded tail.

366 Previously, the ExoN activity of SARS-CoV nsp14 was found to be dramatically
367 stimulated by the addition of nsp10 as co-factor (26). Consequently, we also expressed

368 and purified MERS-CoV nsp10 and optimized the ExoN assay by testing different
369 molar ratios between nsp14 and nsp10 (Fig. 6A, left-hand side), different nsp14
370 concentrations (Fig. 6B, left-hand side), and by different incubation times (Fig. 7, left-
371 hand side). MERS-CoV nsp14 ExoN activity was found to be stimulated by nsp10 in a
372 dose-dependent manner (Fig. 6A), while nsp10 did not exhibit any nuclease activity by
373 itself (Fig. 6B, nsp10 lane). The full-length substrate is more completely degraded
374 when a fourfold (or higher) excess of nsp10 over nsp14 was used compared to the
375 effect of merely increasing the nsp14 concentration in the assay (Fig. 6B). Similar
376 observations were previously reported for SARS-CoV nsp14 (20, 26). Introduction of
377 the D90A/E92A motif-I double substitution resulted in a major reduction of ExoN
378 activity, although a certain level of residual activity was observed, in particular when
379 using large amounts of nsp14 (Fig. 6B, right-hand side) or a relatively high
380 nsp10:nsp14 ratio (Fig. 6A, right-hand side). Similar observations were previously
381 made for SARS-CoV nsp14 (Bouvet, Imbert et al. 2012, Ma, Wu et al. 2015).

382 Using a 4:1 ratio of nsp10 versus nsp14, MERS-CoV ExoN activity was analyzed in
383 a time-course experiment, (Fig. 7). Over time, the full-length substrate was
384 progressively converted to a set of degradation products in the size range of 6-18 nt.
385 We anticipated that the structure of the H4 RNA substrate would change from a
386 duplexed to a single-stranded conformation, upon digestion of one side of the hairpin's
387 stem by ExoN's nuclease activity. As the ExoN enzymes of other CoVs were reported
388 to prefer dsRNA substrates (19, 65), the degradation of the substrate might be slowed
389 down substantially after removal of the first couple of nucleotides from its 3' end (26).
390 This would explain the abundant degradation products of 16 and 17 nt in length that
391 were observed (Fig. 6-8), suggesting that the preference for dsRNA substrates is
392 indeed shared by MERS-CoV ExoN.

393 Degradation of the substrate could be observed within 5 min and the full-length
394 substrate was essentially gone after 30 min. A similar reaction with the nsp14 DM
395 mutant resulted in only a small amount of substrate degradation after 90 min (Fig. 7,
396 leftmost lane). Taken together, our results convincingly demonstrate the *in vitro* 3'-to-
397 5' exonuclease activity of purified MERS-CoV nsp14. As in the case of the SARS-CoV
398 enzyme, nsp10 is a critical co-factor that can strikingly upregulate MERS-CoV ExoN
399 activity *in vitro*.

400

401 **MERS-CoV nsp10 modulates nsp14 ExoN activity**

402 In order to investigate differences that might explain the variable phenotype of CoV
403 ExoN knockout virus mutants, we compared ExoN activities between SARS-CoV and
404 MERS-CoV nsp14, using the optimized *in vitro* assay described above. An incubation
405 time of 90 min was used in all experiments, unless indicated otherwise. The nsp14 and
406 nsp10 preparations of both viruses were first tested individually in an assay containing
407 the H4 RNA substrate and Mg⁺² ions (26, 66, 67). As expected, this revealed only
408 traces of exonuclease activity for both nsp14 proteins (Fig. 8, lanes 4 and lane 6).
409 When the two proteins were combined in the same reaction, a strong increase of ExoN
410 activity was observed for both nsp14-nsp10 pairs, with the SARS-CoV pair appearing
411 to be somewhat more processive than the MERS-CoV pair (Fig. 8, lanes 2 and 9).

412 The exchange of the SARS-CoV and MERS-CoV nsp10 co-factors revealed that
413 they can cross-activate the ExoN activity of nsp14 from the other virus, although some
414 changes in the pattern of degradation products were observed (Fig. 8, Lanes 1-2 and
415 lanes 8-9). However, the residual ExoN activity of the motif I double mutant (DM)
416 apparently was not affected by the choice of nsp10 co-factor (Fig. 8, compare lanes
417 11 and 12). The observed subtle changes in degradation product patterns are another

418 indication that nsp10 modulates nsp14 ExoN activity, presumably using interaction
419 surfaces that are well-conserved across CoV genera (66, 67).

420

421 **ExoN activity of MERS-CoV active site and H229C mutants**

422 Having established the optimal conditions for MERS-CoV ExoN *in vitro* activity, we
423 evaluated the impact of a subset of the DEEDh active site mutations that were used
424 during our reverse genetic analysis (Fig. 2A). For each mutant tested, two protein
425 batches were purified and analyzed independently in duplicate using the same batch
426 of MERS-CoV nsp10 for all assays. As can be seen in Fig. 9, replacement with Ala of
427 each of the five active site residues resulted in a near-complete loss of ExoN activity,
428 with the D90A, E92A, and H268A substitutions appearing to be slightly less detrimental
429 than E191A and motif III D273A. A clearly different result was again obtained with the
430 E191D mutant, which displayed an activity level comparable to that of wt nsp14,
431 corresponding with the properties of the corresponding virus mutant (Fig. 4). Overall,
432 the severe impact of active site mutations on ExoN activity was fully in line with the
433 non-viable phenotypes observed for the same mutants when tested using reverse
434 genetics (Fig. 2).

435 We also evaluated the impact of the H229C ZF1 mutation, which – despite its
436 conservative nature - yielded a crippled mutant virus (Fig. 2) and of two N7-MTase
437 mutations (discussed below). The N7-MTase mutants displayed wt nsp14-like ExoN
438 activities (Fig. 9), suggesting that – as in SARS-CoV nsp14 - ExoN and N7-MTase
439 activities are functionally separated (36). Analyzing the substrate degradation pattern
440 of the H229C mutant (Fig. 7), the enzyme seems to be somewhat crippled when
441 compared to wt nsp14. This suggests that this mutation alters ExoN activity *in vitro*,
442 potentially by affecting the structure of the ExoN domain, as ZF1 is in close proximity

443 of the nsp10 interaction surface (20). However, a similar reduction of ExoN activity was
444 observed for the E191D mutant (Fig. 7), which was much more viable than the H229C
445 mutant in the context of our reverse genetics studies. This suggests that the H229C
446 replacement may affect additional functions or interactions of the ExoN domain that
447 are important for viral RNA synthesis and viability.

448

449 **ExoN mutations do not interfere with N7-MTase activity *in vitro***

450 The nsp14 N7-MTase activity is deemed essential for formation of a functional RNA
451 cap, enabling the translation of CoV mRNAs and protecting them from degradation.
452 Consequently, at least in theory, ExoN mutations could also be detrimental for virus
453 replication if they would somehow affect the crucial enzymatic activity of the other
454 nsp14 domain. In order to evaluate this possibility, the same recombinant protein
455 preparations used in the ExoN assays (Fig. 9) were evaluated in an N7-MTase
456 biochemical assay using the synthetic cap analogues GpppA and m7GpppA (control)
457 as substrates. Moreover, nsp14 mutants R310A and D331A were used as negative
458 controls in view of their predicted involvement in the binding of the triphosphate moiety
459 of the RNA chain and the methyl donor (S-adenosylmethionine; SAM), respectively
460 (20, 26, 36). In this assay, nsp14 can methylate GpppA by transferring the [³H]CH₃
461 moiety provided by [³H]SAM. The resulting radio-labelled m7GpppA product can be
462 quantified using a DEAE filter-binding assay, followed by liquid scintillation counting,
463 and data normalization against the activity of wt control protein (25).

464 Recombinant MERS-CoV nsp14 was found to methylate GpppA, but not m7GpppA
465 (Fig. 10A), which yielded a signal that was similar to the background signal in assays
466 lacking nsp14 or substrate (data not shown). Methylation increased with time until
467 reaching a plateau after 120 min (Fig. 10B). The N7-MTase activity of the various

468 nsp14 mutants was compared with that of wt nsp14 after reaction times of 30 and 120
469 min (Fig. 10C). While the R310A and D331A control mutations fully inactivated the N7-
470 MTase activity of MERS-CoV nsp14, none of the ExoN active site mutations tested
471 was found to alter the enzyme's activity. These results again support the notion that
472 ExoN and N7-MTase domains are functionally separated, as previously demonstrated
473 for SARS-CoV-nsp14 (36). We therefore conclude that the lethal impact of ExoN
474 inactivation on MERS-CoV replication (Fig. 2A) cannot be attributed to inadvertent
475 effects on the activity of the N7-MTase domain that is present in the same nsp14
476 replicase subunit.

477

478 **DISCUSSION**

479 In this study of MERS-CoV nsp14, we demonstrate that the impact of ExoN inactivation
480 on virus viability and RNA synthesis distinguishes MERS-CoV from two other
481 betacoronaviruses, MHV and SARS-CoV. Whereas ExoN inactivation in the latter two
482 viruses yields viable mutants that are only mildly crippled and exhibit a “mutator
483 phenotype” (22, 23, 29), both conservative and alanine substitutions of MERS-CoV
484 ExoN catalytic residues abolished all detectable viral RNA synthesis (Fig. 3) and the
485 release of viral progeny (Fig. 2). The only exception was the conservative E191D
486 mutant, which was found to exhibit near-wild-type levels of ExoN activity (Fig. 7 and
487 9). Based on nsp14 conservation (Fig. 1) and the viable phenotype of SARS-CoV and
488 MHV ExoN-knockout mutants, MERS-CoV was expected to tolerate ExoN inactivation,
489 in particular since the enzyme was proposed to improve the fidelity of CoV replication
490 without being essential for RNA synthesis *per se* (10, 14, 21-23, 26, 29). This notion is
491 further supported by the fact that the CoV RdRp (nsp12) exhibits *in vitro* activity in the
492 absence of nsp14 (27). We therefore anticipated that an excess of deleterious

493 mutations would first have to accumulate before becoming detrimental to MERS-CoV
494 viability. Contrary to these expectations, an immediate and complete block of RNA
495 synthesis was observed when MERS-CoV ExoN knockout mutants were launched by
496 transfection of full-length RNA transcripts. It is noteworthy that similar observations
497 were previously made for the corresponding ExoN knockout mutants of the alpha-
498 CoVs HCoV-229E (19) and TGEV (34), the gamma-CoV avian infectious bronchitis
499 virus (IBV) (E. Bickerton, S. Keep, and P. Britton, personal communication), and –
500 according to a recent report that awaits peer review - also for SARS-CoV-2 (30), a
501 close relative of SARS-CoV.

502 None of the ExoN mutations tested had a negative effect on the *in vitro* activity of
503 the N7-MTase domain of nsp14, which is deemed essential for viral mRNA capping
504 (Fig. 10). This is consistent with previous observations for SARS-CoV nsp14, in which
505 the ExoN and N7-MTase activities were shown to be functionally distinct, though
506 structurally interconnected by a hinge region that confers flexibility (21, 36). Given their
507 unchanged N7-MTase activity, the non-viable phenotype of MERS-CoV ExoN active
508 site mutants must be attributed to a negative effect on an additional and apparently
509 critical function of the ExoN domain, which is directly involved in primary RNA synthesis
510 rather than in (longer-term) fidelity control. At present we cannot explain, why SARS-
511 CoV and MHV ExoN knockouts can apparently tolerate ExoN active site substitutions
512 that are lethal to five other CoVs (now including MERS-CoV). Within the
513 betacoronavirus group, in our experience, SARS-CoV and MHV display the most
514 robust RNA synthesis and replication in cell culture when compared to MERS-CoV and
515 SARS-CoV-2 (68-70). Possibly, the recovery of viable progeny depends on reaching
516 a minimum level of RNA synthesis, which may somehow be achieved by only the most
517 efficiently replicating CoVs. Admittedly, even bearing this possibility in mind, it remains

518 difficult to reconcile the 1- to 2-log reduction of progeny titers observed for MHV and
519 SARS-CoV ExoN-knockout mutants with the complete loss of infectious progeny
520 observed for the ExoN-knockout mutants of the other CoVs.

521 In order to eliminate technicalities that might somehow prohibit the successful
522 recovery of MERS-CoV ExoN knockout mutants and explain the phenotypic
523 differences with other CoVs, we explored various details in the transfection protocol.
524 This included the use of a DNA-launched system, similar to that used for TGEV (34),
525 and the propagation of progeny virus in both innate immune-competent
526 and -incompetent cells (Huh7 and Vero cells, respectively). However, this did not
527 change the negative outcome of our transfection experiments, which were repeated
528 more than 10 times for several mutants, always using wt and E191D MERS-CoV as
529 positive controls that proved to be consistently viable. Our extensive set of MERS-CoV
530 ExoN mutants tested, and the results obtained with various other CoVs (see above),
531 strengthen our conclusion that – in addition to its proposed role as a proofreading
532 enzyme – ExoN must have another function in CoV RNA synthesis (57).

533 As reported for MHV and SARS-CoV ExoN mutants (23, 71), possible (late)
534 reversion was observed for a few of our MERS-CoV ExoN active site mutants,
535 specifically mutants E191A, D273E, and in particular D90E, which had reverted at 6 d
536 p.t. in three out of six experiments. This suggests that these mutants exhibit a low
537 residual level of RNA synthesis that is the basis for these low-frequency events. In
538 follow-up studies with the crippled H229C ZF1 mutant, a possible pseudo-revertant
539 carrying a second-site mutation (Q19R) in nsp8 was identified in three independently
540 obtained progeny samples, providing genetic support for an interaction between nsp8
541 and nsp14, which may be relevant in the context of the association of nsp14 with the
542 tripartite RdRp complex consisting of nsp7, nsp8, and nsp12 (21, 27, 72-74). Future

543 studies will address the properties of these nsp14 ExoN knockout mutants and their
544 (pseudo) revertants in more detail.

545 In the only viable MERS-CoV ExoN active site mutant obtained, E191D, the catalytic
546 motif was changed from DEEDh to the DEDDh that is characteristic of all members of
547 the exonuclease family that ExoN belongs to (14, 15, 75). The phenotype of the E191D
548 virus mutant was comparable to that of wt virus (Fig. 4). Biochemical assays revealed
549 that E191D-ExoN enzyme is able to hydrolyze a dsRNA substrate with an activity level
550 approaching that of the wt protein (Fig. 9). Additionally, the E191D mutant behaved
551 similar as wt MERS-CoV upon treatment of infected cells with the mutagenic agent 5-
552 FU (Fig. 4C-D) (28, 71), again suggesting its ExoN activity is not dramatically altered
553 by this conservative substitution in the active site.

554 In this study, we developed an *in vitro* assay to evaluate MERS-CoV ExoN activity
555 using a largely double-stranded RNA substrate (Fig. 6 and 7). As previously observed
556 for SARS-CoV nsp14 (26), MERS-CoV ExoN activity was strongly enhanced by the
557 presence of nsp10 (Fig. 8), in line with the formation of an nsp10:nsp14 heterodimer
558 as observed in biochemical and structural studies (20, 26, 76). Slightly different
559 patterns of degradation of the H4 substrate were observed when comparing the SARS-
560 CoV and MERS-CoV ExoN enzymes *in vitro*. Likewise, the exchange of the nsp10 co-
561 factor for the nsp10 subunit of the other virus, using the same substrate and the same
562 nsp10:nsp14 ratio (1:4; Fig. 8), resulted in a slightly different pattern of substrate
563 degradation, suggesting minor differences in the interaction of the nsp10:nsp14
564 complex with this particular RNA substrate. Previously, it was demonstrated that nsp10
565 is interchangeable between CoV subgenera in its role as co-factor for the nsp16 2'-O-
566 methyltransferase, which was attributed to the high level of conservation of the nsp10-
567 nsp16 interaction surface (77). As nsp14 and nsp16 share a common interaction

568 surface on nsp10 (21, 26, 66), we explored whether a similar co-factor exchange was
569 possible in the context of nsp14's ExoN activity, which was indeed found to be the case
570 (Fig. 8). Structurally, nsp14 interacts with nsp10 figuratively similar to a “hand (nsp14)
571 over fist (nsp10)” conformation (21). In the formation of this complex, nsp10 induces
572 conformational changes in the N-terminal region of ExoN that adjusts the distance
573 between the catalytic residues in the back of the nsp14 palm and, consequently, impact
574 ExoN activity (21). The exchange of the nsp10 co-factor between the two beta-CoVs
575 might affect this conformation and, consequently, modulate the ExoN activity of the
576 nsp14:nsp10 complex.

577 Alanine substitutions of active site residues severely reduced but did not completely
578 abrogated the *in vitro* activity of MERS-CoV ExoN (Fig. 7-9), as previously shown for
579 certain SARS-CoV nsp14 mutants (20, 36). Based on the two-ion-metal catalytic
580 mechanism underlying the exonuclease activity of DEDDh family members (17, 56)
581 and the SARS-CoV nsp14 structure, it was predicted that the various ExoN motifs
582 contribute differently to the excision of nucleosides monophosphates (20, 21). Mutation
583 of ExoN catalytic residues can alter ion binding (31) or disturb the fragile chemical
584 equilibrium, as shown for conservative mutations (corresponding to E191D and
585 D273E) in the Klenow fragment, a member of the DEDDh exonuclease family, which
586 reduced ExoN activity by >96% (78). In general, all DEEDh mutations that yielded non-
587 viable virus mutants exhibited similarly low levels of residual ExoN activity *in vitro* (Fig.
588 9), indicating that each of these residues is important for catalysis.

589 Our study suggests that, in addition to the active site residues, also other motifs in
590 MERS-CoV ExoN are important for virus viability, specifically the two ZF motifs that
591 were probed using two point mutations each (Fig. 2A). In previous ZF1 studies, a
592 mutation equivalent to H229A created solubility issues during expression of

593 recombinant SARS-CoV nsp14 (20) and resulted in a partially active ExoN in the case
594 of white bream virus, a torovirus that also belongs to the nidovirus order (79). It was
595 suggested that ZF1 contributes to the structural stability of ExoN, as it is close to the
596 surface that interacts with nsp10 (20). Here, we demonstrate that the more
597 conservative H229C replacement, which converts ZF1 from a non-classical CCCH
598 type ZF motif into a classical CCCC type (63), was tolerated during recombinant protein
599 expression and yielded an ExoN that is quite active *in vitro* (Fig. 7). This likely
600 contributed to the fact that the H229C virus mutant retained a low level of viability (Fig.
601 2), although its overall crippled phenotype and the non-viable phenotype of mutant
602 C201H clearly highlight the general importance of ZF1 for virus replication. In contrast,
603 the corresponding TGEV mutant (ZF-C) was not strongly affected and could be stably
604 maintained over several passages (34) The reverse genetics data suggest that ZF2,
605 which is in close proximity to ExoN catalytic residues (20), is equally important,
606 although technical complications with expression of the C261A and H264R nsp14
607 mutants prevented us to perform *in vitro* activity assays.

608 Like the ExoN domain of the arenavirus nucleoprotein (32, 33), the CoV ExoN was
609 proposed to be involved in innate immune evasion (34, 35, 80), possibly by degrading
610 viral ds RNA that in the case of CoVs is confined to characteristic double-membrane
611 vesicles (81, 82). For TGEV, this suggestion was based on the reduced accumulation
612 of dsRNA by the ZF-C mutant, which however remains to be characterized in more
613 detail. In the absence of a TGEV ExoN activity assay, and in view of our data for the
614 equivalent MERS-CoV ZF1 mutant, it seems premature to assume that the reduced
615 levels of dsRNA in infected cells are caused by increased exonuclease activity of the
616 ZF-C ExoN mutant (34).

617 In general, the properties of viable CoV ExoN mutants warrant further analysis. In
618 future studies, the repertoire of residues probed by site-directed mutagenesis should
619 be extended beyond active site and ZF motifs, which may help in particular to establish
620 how directly reduced ExoN activity, primary viral RNA synthesis and enhanced innate
621 responses are connected. Regardless of its possible interactions with host cell
622 pathways, nsp14 clearly is a key subunit of the multi-enzyme complex that drives CoV
623 genome replication, subgenomic RNA synthesis, and RNA recombination.
624 Understanding the structure-function interplay between ExoN and other (viral and/or
625 host) components will be key to elucidating its role in CoV RNA synthesis and evolution
626 (83, 84). Taking into account the current SARS-CoV-2 pandemic, understanding the
627 phenotypic differences between ExoN knockout mutants of different CoVs may
628 contribute to the design of improved antiviral approaches, including those relying on
629 'lethal mutagenesis' or direct interference with viral RNA synthesis.

630

631

632 **MATERIALS AND METHODS**

633

634 **Cell culture**

635 Baby hamster kidney cells (BHK-21; ATCC CCL10), Vero and HuH7 cells were
636 cultured as described previously (69, 85). Vero cells were kindly provided by the
637 Erasmus Medical Center, Rotterdam, the Netherlands and HuH7 cells by Dr. Ralf
638 Bartenschlager from Heidelberg University. For transfections, cells were maintained in
639 Eagle's minimal essential medium (EMEM; Lonza) with 8% fetal calf serum (FCS;
640 Bodinco) supplemented with 100 IU/ml of penicillin and 100 µg/ml of streptomycin

641 (Sigma), and 2 mM L-Glutamine (PAA), and incubated at 37 °C with 5% CO₂. Infection
642 of Vero, Vero E6 and HuH7 cells was carried out in EMEM containing 2% FCS.

643

644 **Reverse genetics**

645 Mutations in the MERS-CoV nsp14-coding region were engineered in a bacterial
646 artificial chromosome (BAC) vector (51, 52) containing the full-length cDNA copy of
647 MERS-CoV strain EMC/2012 (44, 53), by two-step *en passant* recombineering in *E.*
648 *coli* (86). When designing the primers, a translationally silent marker mutation was
649 introduced near the site of mutagenesis in order to differentiate between the
650 occurrence of reversion and (possible) contamination with parental virus. For each
651 mutation, two mutant BACs were isolated independently, the nsp14-coding region was
652 verified by sequencing, and both BACs were used for *in vitro* run-off transcription and
653 virus launching.

654 Approximately 5 µg of BAC DNA was linearized with *NotI* and full-length RNA was
655 obtained by *in vitro* transcription with T7 RNA polymerase followed by lithium chloride
656 precipitation according to the manufacturer's protocol (mMessage-mMachine T7 Kit;
657 Ambion). 5 µg of RNA was electroporated into 5x10⁶ BHK-21 cells using the Amaxa
658 nucleofector 2b (program A-031) and Nucleofection T solution kit (Lonza). Transfected
659 BHK-21 cells were mixed with HuH7 or Vero cells in a 1:1 ratio and plated for
660 harvesting supernatants, intracellular RNA isolation and analysis by
661 immunofluorescence microscopy. Immunolabelling was performed as described
662 before (69), using antibodies recognizing double-stranded RNA (dsRNA; (87)), M
663 protein (52) or a cross-reacting antibody raised against SARS-CoV-nsp3 (88). Cells
664 were incubated at 37°C up to a maximum of 6 days post transfection (d.p.t.).
665 Supernatants were collected when full cytopathic effect was observed or at the end of

666 the experiment. Virus titers were determined by plaque assay in HuH7 and Vero cells
667 (89). In order to confirm the presence of engineered mutations in viral progeny, HuH7
668 and Vero cells were infected with supernatants harvested from transfected cells and
669 intracellular RNA was isolated at 18 h post infection as described above. cDNA was
670 synthesized by reverse transcription using RevertedAid H minus reverse transcriptase
671 (ThermoFischer Scientific) and random hexamer primers (Promega), in combination
672 with a primer targeting the 3' end of the MERS-CoV genome. The full-length genome
673 or the nsp14-coding region were amplified by PCR using MyTaq DNA polymerase
674 (Bioline) and after purification the PCR product was sequenced by Sanger sequencing.
675 Genome sequencing by NGS was performed as described before (70). All work with
676 live recombinant MERS-CoV was done in a biosafety level 3 laboratory at Leiden
677 University Medical Center.

678

679 **Analysis of viral RNA synthesis**

680 Isolation of intracellular RNA was performed by lysing infected cell monolayers with
681 TriPure isolation reagent (Roche Applied Science) according to the manufacturer's
682 instructions. After purification, intracellular RNA samples were loaded onto a 1.5%
683 agarose gel containing 2.2 M formaldehyde, which was run overnight at low voltage
684 overnight in MOPS buffer (10 mM MOPS (sodium salt) (pH 7), 5 mM sodium acetate,
685 1 mM EDTA). Dried agarose gels were used for direct detection of viral mRNAs by
686 hybridization with a ³²P-labeled oligonucleotide probe (5'-
687 GCAAATCATCTAATTAGCCTAATC-3') that is complementary to the 3'-terminal
688 sequence of MERS-CoV genome and all subgenomic mRNAs. After hybridization,
689 RNA bands were visualised (using exposure times of up to 28 days) and quantified by

690 phosphorimaging using a Typhoon-9410 variable mode scanner (GE Healthcare) and
691 ImageQuant TL software (GE Healthcare).

692 PCR primers and Taqman probes targeting ORF1a (junction of nsp2-nsp3 coding
693 region), the nucleocapsid (N) protein gene, or the leader-body TRS junction of
694 subgenomic mRNA3 were designed and analyzed for multiplex quality using Beacon
695 Designer™ Software (Premier Biosoft). Reverse transcription (RT) was performed
696 using RevertedAid H minus reverse transcriptase (ThermoFischer Scientific) and a mix
697 of specific reverse primers targeting ORF1a, ORF8, or subgenomic RNA 3 (primer
698 sequences used available upon request). The mRNA derived from the cellular β -actin
699 gene was used as a reference housekeeping gene. Tagged primers were used to
700 differentiate between positive- and negative-stranded viral RNA. Samples were
701 assayed by Taqman multiplex real-time PCR using TaqMan Universal Master Mix II
702 and a CFX384 Touch™ Real Time PCR detection system (BioRad). A standard curve
703 was obtained using an *in vitro* transcript derived from a synthetic plasmid that
704 contained all PCR targets. cDNA was obtained as described above. Each RNA sample
705 was analyzed in triplicate.

706

707 **Plaque reduction assay**

708 HuH7 cells seeded in 6-well clusters were infected with recombinant MERS-CoV at
709 low MOI (30 PFU/well) for 1 h at 37°C. Subsequently, the inoculum was replaced with
710 2 ml of a 1.2% suspension of Avicel (RC-581; FMC Biopolymer (90) in DMEM
711 (containing 2% FCS and antibiotics) and serial dilutions of 5-FU (F6627, Sigma-
712 Aldrich) or Ribavirin (R9644, Sigma-Aldrich) ranging from 0 to 400 μ M. Cells were
713 incubated at 37°C for 72 h, fixed with 7.4% formaldehyde, and plaques were visualized
714 using crystal violet staining.

715 To compare the effect of 5-FU treatment on the progeny titers of wt and nsp14-
716 E191D rMERS-CoV, confluent monolayers of HuH7 were incubated for 30 min at 37°C
717 with solvent or a range of 5-FU concentrations. The drug was then removed and cells
718 were infected at an MOI of 0.1 during 1 h at 37°C. After removal of the inoculum, EMEM
719 containing 2% FCS and solvent or a matching concentration of 5-FU was added to the
720 wells. Supernatants were collected after 30h and rMERS-CoV progeny titers were
721 determined by plaque assay. All drug-treated samples were normalized to the
722 untreated vehicle control, and values were expressed as fold change compared to
723 untreated virus titers.

724

725 **Expression and purification of recombinant CoV nsps**

726 SARS-CoV nsp10 and nsp14 were produced as described before (25) and used as a
727 positive control in all biochemical assays. A MERS-CoV nsp10 expression construct
728 was kindly provided by Dr. Deyin Guo (77) (91) and purified according to their protocol.
729 All MERS-CoV nsp14 constructs were cloned into expression vector pDEST14 with an
730 N-terminal His₆-tag using the Gateway system (25). MERS-CoV nsp14 mutant
731 expression plasmids were generated by Quikchange site-directed mutagenesis using
732 Accuzyme DNA polymerase (Bioline) following the manufacturer's instructions.
733 pDEST14 plasmids expressing MERS-CoV nsp14 were transformed into competent *E.*
734 *coli* strain Rosetta (DE3) pLyS (Novagen) and cultured in Luria-Bertani (LB) broth
735 supplemented with 100 µg/ ml of ampicillin and 30 µg/ ml of chloramphenicol. Protein
736 expression was induced at an optical density (OD_{600nm}) of 0.8 by adding 50 µM of
737 isopropyl-β-D-a-thiogalactopyranoside (IPTG; Bioline). After 24 h at 13°C, induced
738 cells were harvested and lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM
739 NaCl, 5 mM β-mercaptoethanol, 5% glycerol, 1 mM PMSF, and 20 mM imidazole (92).

740 Next, the lysate was centrifuged at 12,000xg for 30 min, and the soluble fraction was
741 column-purified by immobilized metal ion affinity chromatography using Nickel
742 sepharose high performance beads (17526802, GE Healthcare). The eluate was
743 fractionated by gel filtration on a Superdex-200 Increase 10/300GL column (GE
744 Healthcare) in buffer containing 30 mM HEPES, pH 7.5, 300 mM NaCl, and 5%
745 glycerol. In the end, proteins were concentrated using ultrafiltration devices with a
746 molecular mass cut-off of 30 kDa (Millipore), and protein concentrations were
747 measured using spectrophotometry. All purified proteins were analyzed by SDS-PAGE
748 followed by Coomassie blue staining as well as by Western blot using a mouse
749 monoclonal antibody against the 6xHis-Tag (Novagen). Protein aliquots were stored at
750 -80°C in 50% glycerol (v/v) and used for enzymatic assays.

751

752 **Exonuclease activity assay**

753 Synthetic RNA H4 (26) was radiolabeled at its 5' end using T4 polynucleotide kinase
754 (Epicentre) and [γ -³²P]ATP (Perkin Elmer). Unless stated otherwise in figures or
755 legends, reactions contained 200 nM of recombinant nsp14, 800 nM of nsp10, and 750
756 nM of radiolabeled substrate in 40 mM Tris-HCl pH 7.5 containing 5 mM of MgCl₂ and
757 1 mM of DTT. After incubation at 37°C for up to 90min, reactions were stopped by
758 addition of an equal volume of loading buffer containing 96% formamide and 10mM
759 EDTA. Samples were then loaded on 7M urea-containing 20% (wt/vol) polyacrylamide
760 gels (acrylamide/bisacrylamide ratio 19:1) buffered with 0.5x Tris-borate-EDTA and run
761 at high voltage (1600 V). Results were visualized by phosphorimaging as described
762 above.

763

764 **N7-methyltransferase activity assay**

765 Methyltransferase assays were performed in 40 mM Tris-HCl, pH 8.0, 5 mM DTT, 2
766 μM of ^7Me GpppA or GpppA RNA cap analogue (New England Biolabs), 10 μM
767 adenosyl-methionine (AdoMet, ThermoFisher), 0.03 $\mu\text{Ci}/\mu\text{l}$ [^3H]AdoMet (PerkinElmer)
768 (25). In each reaction, MERS-CoV or SARS-CoV nsp14 was added to a final
769 concentration of 500 or 250 nM, respectively. Reactions were incubated at 30°C for up
770 to 120min, and were stopped by the addition of 10-fold volume of 100 μM ice-cold
771 Adenosyl-Homocysteine (AdoHcy; ThermoFischer). Then, samples were spotted on a
772 DEAE filtermat (PerkinElmer) pre-wet with Tris-HCl pH 8.0 buffer. Filtermats were
773 washed twice with 10 mM ammonium formate (Sigma-Aldrich), pH 8.0, twice with MiliQ
774 water, and once with absolute ethanol (Sigma-Aldrich). After air drying for 10 min,
775 filtermats were cut and relevant pieces transferred to individual tubes. Betaplate
776 scintillation fluid (PerkinElmer) was added and the amount of ^3H -label bound was
777 measured in counts per minute (cpm) using a Wallac scintillation counter. For relative
778 quantification, incorporation measurements for mutant proteins were normalized to
779 values obtained with the wt control nsp14. Samples were measured in duplicate in
780 each experiment.

781

782 **ACKNOWLEDGMENTS**

783 N.S.O. was supported by the Marie Skłodowska-Curie ETN European Training
784 Network 'ANTIVIRALS' (EU Grant Agreement No. 642434). C.C.P and E.J.S. were
785 supported in part by TOP-GO grant 700.10.352 from the Netherlands Organization for
786 Scientific Research. We thank Yvonne van der Meer for technical support and Linda
787 Boomaars for providing SARS-and MERS-CoV nsp10 and nsp14 expression
788 constructs. We are grateful to Etienne Decroly, Francois Ferron and Bruno Canard
789 (University of Aix-Marseille, France) for helpful discussions. We kindly acknowledge

790 the sharing of unpublished information on IBV ExoN knockout mutants by Dr. Erica
791 Bickerton and colleagues (Pirbright Institute, U.K.).

792 **FIGURE LEGENDS**

793

794 **Figure 1** - Alignment of nsp14 amino acid sequences from selected coronaviruses.
795 Sequences of the ExoN and N7Mtase domains in MERS-CoV (NC-019843); SARS-
796 CoV (NC_004718); SARS-CoV-2 (NC_045512.2); MHV (NP_045298); HCoV-229E
797 (NC_002645); TGEV (AJ271965); and IBV (NP_040829) were used for the analysis.
798 The different domains indicated on the top are based on the SARS-CoV-nsp14
799 secondary structure (PDB 5NFY (21)). Fully conserved residues are boxed in dark grey
800 with white letters (above 70% conservation), whereas partially conserved residues are
801 displayed in lighter shades of grey. Catalytic residues and residues involved in
802 formation of zinc fingers are marked with asterisks and circles, respectively. Full circles
803 indicate zinc fingers targeted by mutagenesis (Fig. 2A) while two black arrows identify
804 the two N7Mtase domain residues mutated to generate the methyltransferase negative
805 control used in biochemical assays. The alignment was generated using Clustal
806 Omega (93) and edited using Jalview version 2.11 (94).

807

808 **Figure 2** - (A) Phenotype of MERS-CoV nsp14 ExoN mutants used in this study,
809 scored at 2 d p.t.. (B) Comparison of plaque phenotype of selected ExoN mutants in
810 HuH7 cells. Plaque assays were performed using supernatants harvested from
811 transfected cells at 2 d p.t., which were diluted 10^{-4} for wt and mutant E191D mutant
812 and used in undiluted form for the H229C and DM mutants.

813

814 **Figure 3** - Impact of ExoN inactivation on intracellular RNA synthesis. In-gel
815 hybridization analysis of intracellular RNA isolated after 2 or 3 days post transfection
816 of transfected BHK-21 cells, which were subsequently mixed with HuH7 or Vero cells

817 as indicated. Purified RNA was separated in an agarose gel and probed with a
818 radiolabeled oligonucleotide probe recognizing the MERS-CoV genome and
819 subgenomic mRNAs.

820

821 **Figure 4** - Characterization of growth kinetics of rMERS-nsp14-E191D and its
822 sensitivity to 5-FU treatment. (A) Vero cells or (B) HuH7 cells were infected at an MOI
823 of 3, supernatant was harvested at the indicated time points, and viral progeny titers
824 were measured by plaque assay from two independent experiments using duplicates
825 (n=4; mean \pm sd is presented). (C) Plaque phenotype in HuH7 cells of rMERS-CoV
826 nsp14-E191D and wt control in the absence or presence of the mutagenic agent 5-FU.
827 (D) Dose response curve of wt and E191D mutant MERS-CoV in the presence of 5-
828 FU concentrations up to 400 μ M (m.o.i. of 1; n=4; mean \pm sd is presented). Statistical
829 significance compared to wt at each time point (A and B) or concentration (D) was
830 determined by paired t-test. All statistical analysis is denoted by asterisks: *, $P < 0.05$;
831 ** $p < 0.005$.

832

833 **Figure 5** - Expression and purification of recombinant MERS-CoV nsp14. N-terminally
834 His-tagged wt and mutant MERS-CoV nsp14 (~55 kDa) was expressed in *E. coli*,
835 affinity purified, and analyzed in a 10% SDS-PAGE gel that was stained with
836 Coomassie Blue. The molecular masses of the protein marker (Invitrogen) are given
837 in kDa.

838

839 **Figure 6** - Optimization of MERS-CoV nsp14 *in vitro* ExoN assay conditions. The
840 substrate for the assay was a 22-nt long synthetic RNA (H4) that was ³²P-labeled at its
841 5' terminus (*p-RNA). Cleavage products were separated by polyacrylamide gel

842 electrophoresis and visualized by autoradiography. (A) Analysis of ExoN activity in the
843 presence of an increasing amount of nsp10, using wt MERS-CoV-nsp14 (left) and the
844 ExoN double knockout mutant (DM, D90A/E92A; right). The RNA substrate was
845 hydrolyzed for 90 min at 37°C using a fixed concentration of nsp14 (200 nM) and an
846 increasing nsp10 concentration, ranging from 0 to 1600 nM. (B) Evaluation of the ExoN
847 activity of an increasing concentration (200 to 2000 nM) of wt or DM nsp14 in the
848 presence of a fixed amount of nsp10 (200 nM).

849

850 **Figure 7** - Time course analysis of the *in vitro* ExoN activity of MERS-CoV nsp14. The
851 ExoN activity of different recombinant nsp14 proteins (wt, D90A/E92A, E191D and
852 H229C) was evaluated by incubating 200 nM of nsp14 and 800 nM of nsp10 for 0, 5,
853 30, 60 and 90 min at 37°C. As controls, individual proteins (800 nM) were incubated
854 for 90 min. For technical details, see the legend to Fig. 6.

855

856 **Figure 8** - Cross-activation of the *in vitro* activity of SARS-CoV and MERS-CoV nsp14
857 by heterologous nsp10. The nsp10 co-factor was exchanged in ExoN assays
858 performed with MERS-CoV and SARS-CoV nsp14, using a 1:4 ratio between nsp14
859 and nsp10 and a 90-min incubation at 37°C. For technical details, see the legend to
860 Fig. 5.

861

862 **Figure 9** - *In vitro* ExoN activity of MERS-CoV nsp14 mutants. Residues from the
863 DEDDh catalytic motif and ZF1 motif of the nsp14 ExoN domain and the nsp14
864 N7MTase domain were mutated as indicated. Assays were performed using a 1:4 ratio
865 between nsp14 and nsp10 and a 90-min incubation at 37°C. For technical details, see
866 the legend to Fig. 6.

867

868 **Figure 10** - *In vitro* N7-MTase activity of MERS-CoV nsp14 mutants. The N7-MTase
869 activity of recombinant nsp14 was analyzed *in vitro* by filter binding assay using
870 synthetic cap analogues as substrate. (A) Increasing concentrations of MERS-CoV
871 nsp14 were incubated with GpppA and m⁷GpppA in the presence of [³H]SAM for 30
872 min. (B) The ability of nsp14 to methylate GpppA or m⁷GpppA was determined after
873 reaction times between 0 and 120 min at 30°C . (C) The ability of nsp14 mutants to
874 methylate GpppA was measured in four times in duplicate. Values were normalized to
875 the wt control (n=8; mean ± sd are shown).

876

877 **REFERENCES**

878

- 879 1. Vignuzzi M, Stone JK, Arnold JJ, Cameron CE, Andino R. 2006. Quasispecies diversity
880 determines pathogenesis through cooperative interactions in a viral population. *Nature*
881 439:344-8.
- 882 2. Eigen M, Schuster P. 1977. The hypercycle. A principle of natural self-organization. Part A:
883 Emergence of the hypercycle. *Naturwissenschaften* 64:541-65.
- 884 3. Domingo E, Sheldon J, Perales C. 2012. Viral quasispecies evolution. *Microbiol Mol Biol Rev*
885 76:159-216.
- 886 4. Sanjuan R. 2010. Mutational fitness effects in RNA and single-stranded DNA viruses: common
887 patterns revealed by site-directed mutagenesis studies. *Philos Trans R Soc Lond B Biol Sci*
888 365:1975-82.
- 889 5. Sanjuan R, Nebot MR, Chirico N, Mansky LM, Belshaw R. 2010. Viral mutation rates. *J Virol*
890 84:9733-48.
- 891 6. Steinhauer DA, Domingo E, Holland JJ. 1992. Lack of evidence for proofreading mechanisms
892 associated with an RNA virus polymerase. *Gene* 122:281-8.
- 893 7. Drake JW, Holland JJ. 1999. Mutation rates among RNA viruses. *Proc Natl Acad Sci U S A*
894 96:13910-3.
- 895 8. Eigen M. 2002. Error catastrophe and antiviral strategy. *Proc Natl Acad Sci U S A* 99:13374-6.
- 896 9. Eigen M. 1971. Selforganization of matter and the evolution of biological macromolecules.
897 *Naturwissenschaften* 58:465-523.
- 898 10. Nga PT, Parquet Mdel C, Lauber C, Parida M, Nabeshima T, Yu F, Thuy NT, Inoue S, Ito T,
899 Okamoto K, Ichinose A, Snijder EJ, Morita K, Gorbalenya AE. 2011. Discovery of the first
900 insect nidovirus, a missing evolutionary link in the emergence of the largest RNA virus
901 genomes. *PLoS Pathog* 7:e1002215.
- 902 11. Lauber C, Goeman JJ, Parquet Mdel C, Nga PT, Snijder EJ, Morita K, Gorbalenya AE. 2013. The
903 footprint of genome architecture in the largest genome expansion in RNA viruses. *PLoS*
904 *Pathog* 9:e1003500.
- 905 12. Saberi A, Gulyaeva AA, Brubacher JL, Newmark PA, Gorbalenya AE. 2018. A planarian
906 nidovirus expands the limits of RNA genome size. *PLoS Pathog* 14:e1007314.
- 907 13. Bukhari K, Mulley G, Gulyaeva AA, Zhao L, Shu G, Jiang J, Neuman BW. 2018. Description and
908 initial characterization of metatranscriptomic nidovirus-like genomes from the proposed new
909 family *Abysoviridae*, and from a sister group to the *Coronavirinae*, the proposed genus
910 *Alphaletovirus*. *Virology* 524:160-171.
- 911 14. Snijder EJ, Bredenbeek PJ, Dobbe JC, Thiel V, Ziebuhr J, Poon LL, Guan Y, Rozanov M, Spaan
912 WJ, Gorbalenya AE. 2003. Unique and conserved features of genome and proteome of SARS-
913 coronavirus, an early split-off from the coronavirus group 2 lineage. *J Mol Biol* 331:991-1004.
- 914 15. Zuo Y, Deutscher MP. 2001. Exoribonuclease superfamilies: structural analysis and
915 phylogenetic distribution. *Nucleic Acids Res* 29:1017-26.
- 916 16. Deutscher MP, Marlor CW. 1985. Purification and characterization of *Escherichia coli* RNase
917 T. *J Biol Chem* 260:7067-71.
- 918 17. Beese LS, Steitz TA. 1991. Structural basis for the 3'-5' exonuclease activity of *Escherichia coli*
919 DNA polymerase I: a two metal ion mechanism. *EMBO J* 10:25-33.
- 920 18. Steitz TA, Steitz JA. 1993. A general two-metal-ion mechanism for catalytic RNA. *Proc Natl*
921 *Acad Sci U S A* 90:6498-502.
- 922 19. Minskaia E, Hertzog T, Gorbalenya AE, Campanacci V, Cambillau C, Canard B, Ziebuhr J. 2006.
923 Discovery of an RNA virus 3'->5' exoribonuclease that is critically involved in coronavirus RNA
924 synthesis. *Proc Natl Acad Sci U S A* 103:5108-13.

- 925 20. Ma Y, Wu L, Shaw N, Gao Y, Wang J, Sun Y, Lou Z, Yan L, Zhang R, Rao Z. 2015. Structural basis
926 and functional analysis of the SARS coronavirus nsp14-nsp10 complex. *Proc Natl Acad Sci U S*
927 *A* 112:9436-41.
- 928 21. Ferron F, Subissi L, Silveira De Morais AT, Le NTT, Sevajol M, Gluais L, Decroly E, Vonnrhein C,
929 Bricogne G, Canard B, Imbert I. 2018. Structural and molecular basis of mismatch correction
930 and ribavirin excision from coronavirus RNA. *Proc Natl Acad Sci U S A* 115:E162-E171.
- 931 22. Eckerle LD, Lu X, Sperry SM, Choi L, Denison MR. 2007. High fidelity of murine hepatitis virus
932 replication is decreased in nsp14 exoribonuclease mutants. *J Virol* 81:12135-44.
- 933 23. Eckerle LD, Becker MM, Halpin RA, Li K, Venter E, Lu X, Scherbakova S, Graham RL, Baric RS,
934 Stockwell TB, Spiro DJ, Denison MR. 2010. Infidelity of SARS-CoV Nsp14-exonuclease mutant
935 virus replication is revealed by complete genome sequencing. *PLoS Pathog* 6:e1000896.
- 936 24. Graham RL, Becker MM, Eckerle LD, Bolles M, Denison MR, Baric RS. 2012. A live, impaired-
937 fidelity coronavirus vaccine protects in an aged, immunocompromised mouse model of lethal
938 disease. *Nat Med* 18:1820-6.
- 939 25. Bouvet M, Debarnot C, Imbert I, Selisko B, Snijder EJ, Canard B, Decroly E. 2010. In vitro
940 reconstitution of SARS-coronavirus mRNA cap methylation. *PLoS Pathog* 6:e1000863.
- 941 26. Bouvet M, Imbert I, Subissi L, Gluais L, Canard B, Decroly E. 2012. RNA 3'-end mismatch
942 excision by the severe acute respiratory syndrome coronavirus nonstructural protein
943 nsp10/nsp14 exoribonuclease complex. *Proc Natl Acad Sci U S A* 109:9372-7.
- 944 27. Subissi L, Posthuma CC, Collet A, Zevenhoven-Dobbe JC, Gorbalenya AE, Decroly E, Snijder EJ,
945 Canard B, Imbert I. 2014. One severe acute respiratory syndrome coronavirus protein
946 complex integrates processive RNA polymerase and exonuclease activities. *Proc Natl Acad Sci*
947 *U S A* 111:E3900-9.
- 948 28. Smith EC, Blanc H, Surdel MC, Vignuzzi M, Denison MR. 2013. Coronaviruses lacking
949 exoribonuclease activity are susceptible to lethal mutagenesis: evidence for proofreading
950 and potential therapeutics. *PLoS Pathog* 9:e1003565.
- 951 29. Graepel KW, Lu X, Case JB, Sexton NR, Smith EC, Denison MR. 2017. Proofreading-Deficient
952 Coronaviruses Adapt for Increased Fitness over Long-Term Passage without Reversion of
953 Exoribonuclease-Inactivating Mutations. *MBio* 8.
- 954 30. Gribble J, Pruijssers AJ, Agostini ML, Anderson-Daniels J, Chappell JD, Lu X, Stevens LJ, Routh
955 AL, Denison MR. 2020. The coronavirus proofreading exoribonuclease mediates extensive
956 viral recombination. *bioRxiv* doi:10.1101/2020.04.23.057786:2020.04.23.057786.
- 957 31. Yekwa E, Aphibanthammakit C, Carnec X, Picard C, Canard B, Baize S, Ferron F. 2019.
958 Arenaviridae exoribonuclease presents genomic RNA edition capacity. *bioRxiv*
959 doi:10.1101/541698:541698.
- 960 32. Hastie KM, Kimberlin CR, Zandonatti MA, MacRae IJ, Sapphire EO. 2011. Structure of the Lassa
961 virus nucleoprotein reveals a dsRNA-specific 3' to 5' exonuclease activity essential for
962 immune suppression. *Proc Natl Acad Sci U S A* 108:2396-401.
- 963 33. Russier M, Reynard S, Carnec X, Baize S. 2014. The exonuclease domain of Lassa virus
964 nucleoprotein is involved in antigen-presenting-cell-mediated NK cell responses. *J Virol*
965 88:13811-20.
- 966 34. Becares M, Pascual-Iglesias A, Nogales A, Sola I, Enjuanes L, Zuniga S. 2016. Mutagenesis of
967 Coronavirus nsp14 Reveals Its Potential Role in Modulation of the Innate Immune Response.
968 *J Virol* 90:5399-5414.
- 969 35. Case JB, Li Y, Elliott R, Lu X, Graepel KW, Sexton NR, Smith EC, Weiss SR, Denison MR. 2018.
970 Murine Hepatitis Virus nsp14 Exoribonuclease Activity Is Required for Resistance to Innate
971 Immunity. *J Virol* 92.
- 972 36. Chen Y, Cai H, Pan J, Xiang N, Tien P, Ahola T, Guo D. 2009. Functional screen reveals SARS
973 coronavirus nonstructural protein nsp14 as a novel cap N7 methyltransferase. *Proc Natl Acad*
974 *Sci U S A* 106:3484-9.

- 975 37. Jin X, Chen Y, Sun Y, Zeng C, Wang Y, Tao J, Wu A, Yu X, Zhang Z, Tian J, Guo D. 2013.
976 Characterization of the guanine-N7 methyltransferase activity of coronavirus nsp14 on
977 nucleotide GTP. *Virus Res* 176:45-52.
- 978 38. Chen Y, Tao J, Sun Y, Wu A, Su C, Gao G, Cai H, Qiu S, Wu Y, Ahola T, Guo D. 2013. Structure-
979 function analysis of severe acute respiratory syndrome coronavirus RNA cap guanine-N7-
980 methyltransferase. *J Virol* 87:6296-305.
- 981 39. Case JB, Ashbrook AW, Dermody TS, Denison MR. 2016. Mutagenesis of S-Adenosyl-l-
982 Methionine-Binding Residues in Coronavirus nsp14 N7-Methyltransferase Demonstrates
983 Differing Requirements for Genome Translation and Resistance to Innate Immunity. *J Virol*
984 90:7248-7256.
- 985 40. Li X, Song Y, Wong G, Cui J. 2020. Bat origin of a new human coronavirus: there and back
986 again. *Sci China Life Sci* 63:461-462.
- 987 41. Pyrc K, Berkhout B, van der Hoek L. 2007. Identification of new human coronaviruses. *Expert*
988 *Rev Anti Infect Ther* 5:245-53.
- 989 42. Munster VJ, Koopmans M, van Doremalen N, van Riel D, de Wit E. 2020. A Novel Coronavirus
990 Emerging in China - Key Questions for Impact Assessment. *N Engl J Med* 382:692-694.
- 991 43. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CL, Chen HD,
992 Chen J, Luo Y, Guo H, Jiang RD, Liu MQ, Chen Y, Shen XR, Wang X, Zheng XS, Zhao K, Chen QJ,
993 Deng F, Liu LL, Yan B, Zhan FX, Wang YY, Xiao GF, Shi ZL. 2020. A pneumonia outbreak
994 associated with a new coronavirus of probable bat origin. *Nature* 579:270-273.
- 995 44. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. 2012. Isolation of a
996 novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med* 367:1814-20.
- 997 45. Cotten M, Watson SJ, Zumla AI, Makhdoom HQ, Palser AL, Ong SH, Al Rabeeah AA, Alhakeem
998 RF, Assiri A, Al-Tawfiq JA, Albarrak A, Barry M, Shibl A, Alrabiah FA, Hajjar S, Balkhy HH,
999 Flemban H, Rambaut A, Kellam P, Memish ZA. 2014. Spread, circulation, and evolution of the
1000 Middle East respiratory syndrome coronavirus. *mBio* 5.
- 1001 46. Rabaan AA, Al-Ahmed SH, Bazzi AM, Al-Tawfiq JA. 2017. Dynamics of scientific publications
1002 on the MERS-CoV outbreaks in Saudi Arabia. *J Infect Public Health* 10:702-710.
- 1003 47. Farag E, Sikkema RS, Vinks T, Islam MM, Nour M, Al-Romaihi H, Al Thani M, Atta M, Alhajri
1004 FH, Al-Marri S, AlHajri M, Reusken C, Koopmans M. 2018. Drivers of MERS-CoV Emergence in
1005 Qatar. *Viruses* 11.
- 1006 48. Al-Tawfiq JA, Memish ZA. 2016. Drivers of MERS-CoV transmission: what do we know? *Expert*
1007 *Rev Respir Med* 10:331-8.
- 1008 49. Siddell SG, Walker PJ, Lefkowitz EJ, Mushegian AR, Adams MJ, Dutilh BE, Gorbalenya AE,
1009 Harrach B, Harrison RL, Junglen S, Knowles NJ, Kropinski AM, Krupovic M, Kuhn JH, Nibert M,
1010 Rubino L, Sabanadzovic S, Sanfacon H, Simmonds P, Varsani A, Zerbini FM, Davison AJ. 2019.
1011 Additional changes to taxonomy ratified in a special vote by the International Committee on
1012 Taxonomy of Viruses (October 2018). *Arch Virol* 164:943-946.
- 1013 50. Coronaviridae Study Group of the International Committee on Taxonomy of V. 2020. The
1014 species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and
1015 naming it SARS-CoV-2. *Nat Microbiol* 5:536-544.
- 1016 51. Almazan F, DeDiego ML, Sola I, Zuniga S, Nieto-Torres JL, Marquez-Jurado S, Andres G,
1017 Enjuanes L. 2013. Engineering a replication-competent, propagation-defective Middle East
1018 respiratory syndrome coronavirus as a vaccine candidate. *mBio* 4:e00650-13.
- 1019 52. Rabouw HH, Langereis MA, Knaap RC, Dalebout TJ, Canton J, Sola I, Enjuanes L, Bredenbeek
1020 PJ, Kikkert M, de Groot RJ, van Kuppeveld FJ. 2016. Middle East Respiratory Coronavirus
1021 Accessory Protein 4a Inhibits PKR-Mediated Antiviral Stress Responses. *PLoS Pathog*
1022 12:e1005982.
- 1023 53. van Boheemen S, de Graaf M, Lauber C, Bestebroer TM, Raj VS, Zaki AM, Osterhaus AD,
1024 Haagmans BL, Gorbalenya AE, Snijder EJ, Fouchier RA. 2012. Genomic characterization of a
1025 newly discovered coronavirus associated with acute respiratory distress syndrome in
1026 humans. *MBio* 3.

- 1027 54. Eckerle LD, Brockway SM, Sperry SM, Lu X, Denison MR. 2006. Effects of mutagenesis of
1028 murine hepatitis virus nsp1 and nsp14 on replication in culture. *Adv Exp Med Biol* 581:55-60.
- 1029 55. Habjan M, Penski N, Spiegel M, Weber F. 2008. T7 RNA polymerase-dependent and -
1030 independent systems for cDNA-based rescue of Rift Valley fever virus. *J Gen Virol* 89:2157-
1031 66.
- 1032 56. Bernad A, Blanco L, Lazaro JM, Martin G, Salas M. 1989. A conserved 3'----5' exonuclease
1033 active site in prokaryotic and eukaryotic DNA polymerases. *Cell* 59:219-28.
- 1034 57. Ogando NS, Ferron F, Decroly E, Canard B, Posthuma CC, Snijder EJ. 2019. The Curious Case
1035 of the Nidovirus Exoribonuclease: Its Role in RNA Synthesis and Replication Fidelity. *Front*
1036 *Microbiol* 10:1813.
- 1037 58. Neuman BW, Joseph JS, Saikatendu KS, Serrano P, Chatterjee A, Johnson MA, Liao L, Klaus JP,
1038 Yates JR, 3rd, Wuthrich K, Stevens RC, Buchmeier MJ, Kuhn P. 2008. Proteomics analysis
1039 unravels the functional repertoire of coronavirus nonstructural protein 3. *J Virol* 82:5279-94.
- 1040 59. Neuman BW. 2016. Bioinformatics and functional analyses of coronavirus nonstructural
1041 proteins involved in the formation of replicative organelles. *Antiviral Res* 135:97-107.
- 1042 60. Lei J, Kusov Y, Hilgenfeld R. 2018. Nsp3 of coronaviruses: Structures and functions of a large
1043 multi-domain protein. *Antiviral Res* 149:58-74.
- 1044 61. Noordhuis P, Holwerda U, Van der Wilt CL, Van Groenigen CJ, Smid K, Meijer S, Pinedo HM,
1045 Peters GJ. 2004. 5-Fluorouracil incorporation into RNA and DNA in relation to thymidylate
1046 synthase inhibition of human colorectal cancers. *Ann Oncol* 15:1025-32.
- 1047 62. Greenhalgh DA, Parish JH. 1990. Effect of 5-fluorouracil combination therapy on RNA
1048 processing in human colonic carcinoma cells. *Br J Cancer* 61:415-9.
- 1049 63. Abbehausen C. 2019. Zinc finger domains as therapeutic targets for metal-based compounds
1050 - an update. *Metallomics* 11:15-28.
- 1051 64. Imanishi M, Matsumura K, Tsuji S, Nakaya T, Negi S, Futaki S, Sugiura Y. 2012. Zn(II) binding
1052 and DNA binding properties of ligand-substituted CXHH-type zinc finger proteins.
1053 *Biochemistry* 51:3342-8.
- 1054 65. Chen P, Jiang M, Hu T, Liu Q, Chen XS, Guo D. 2007. Biochemical characterization of
1055 exoribonuclease encoded by SARS coronavirus. *J Biochem Mol Biol* 40:649-55.
- 1056 66. Bouvet M, Lugari A, Posthuma CC, Zevenhoven JC, Bernard S, Betzi S, Imbert I, Canard B,
1057 Guillemot JC, Lecine P, Pfefferle S, Drosten C, Snijder EJ, Decroly E, Morelli X. 2014.
1058 Coronavirus Nsp10, a critical co-factor for activation of multiple replicative enzymes. *J Biol*
1059 *Chem* 289:25783-96.
- 1060 67. Rosas-Lemus M, Minasov G, Shuvalova L, Inniss NL, Kiryukhina O, Wiersum G, Kim Y,
1061 Jedrzejczak R, Enders M, Jaroszewski L, Godzik A, Joachimiak A, Satchell KJF. 2020. The crystal
1062 structure of nsp10-nsp16 heterodimer from SARS-CoV-2 in complex with S-
1063 adenosylmethionine. *bioRxiv* doi:10.1101/2020.04.17.047498:2020.04.17.047498.
- 1064 68. de Wilde AH, Falzarano D, Zevenhoven-Dobbe JC, Beugeling C, Fett C, Martellaro C,
1065 Posthuma CC, Feldmann H, Perlman S, Snijder EJ. 2017. Alisporivir inhibits MERS- and SARS-
1066 coronavirus replication in cell culture, but not SARS-coronavirus infection in a mouse model.
1067 *Virus Res* 228:7-13.
- 1068 69. de Wilde AH, Raj VS, Oudshoorn D, Bestebroer TM, van Nieuwkoop S, Limpens RW,
1069 Posthuma CC, van der Meer Y, Barcena M, Haagmans BL, Snijder EJ, van den Hoogen BG.
1070 2013. MERS-coronavirus replication induces severe in vitro cytopathology and is strongly
1071 inhibited by cyclosporin A or interferon-alpha treatment. *J Gen Virol* 94:1749-60.
- 1072 70. Ogando NS, Dalebout TJ, Zevenhoven-Dobbe JC, Limpens RW, van der Meer Y, Caly L, Druce
1073 J, de Vries JJC, Kikkert M, Bárcena M, Sidorov I, Snijder EJ. 2020. SARS-coronavirus-2
1074 replication in Vero E6 cells: replication kinetics, rapid adaptation and cytopathology. *bioRxiv*
1075 doi:10.1101/2020.04.20.049924:2020.04.20.049924.
- 1076 71. Graepel KW, Agostini ML, Lu X, Sexton NR, Denison MR. 2019. Fitness Barriers Limit
1077 Reversion of a Proofreading-Deficient Coronavirus. *J Virol* 93.

- 1078 72. Kirchdoerfer RN, Ward AB. 2019. Structure of the SARS-CoV nsp12 polymerase bound to
1079 nsp7 and nsp8 co-factors. *Nat Commun* 10:2342.
- 1080 73. Yin W, Mao C, Luan X, Shen DD, Shen Q, Su H, Wang X, Zhou F, Zhao W, Gao M, Chang S, Xie
1081 YC, Tian G, Jiang HW, Tao SC, Shen J, Jiang Y, Jiang H, Xu Y, Zhang S, Zhang Y, Xu HE. 2020.
1082 Structural basis for inhibition of the RNA-dependent RNA polymerase from SARS-CoV-2 by
1083 remdesivir. *Science* doi:10.1126/science.abc1560.
- 1084 74. Hillen HS, Kokic G, Farnung L, Dienemann C, Tegunov D, Cramer P. 2020. Structure of
1085 replicating SARS-CoV-2 polymerase. *Nature* doi:10.1038/s41586-020-2368-8.
- 1086 75. Barnes MH, Spacciapoli P, Li DH, Brown NC. 1995. The 3'-5' exonuclease site of DNA
1087 polymerase III from gram-positive bacteria: definition of a novel motif structure. *Gene*
1088 165:45-50.
- 1089 76. Pan J, Peng X, Gao Y, Li Z, Lu X, Chen Y, Ishaq M, Liu D, Dediego ML, Enjuanes L, Guo D. 2008.
1090 Genome-wide analysis of protein-protein interactions and involvement of viral proteins in
1091 SARS-CoV replication. *PLoS One* 3:e3299.
- 1092 77. Wang Y, Sun Y, Wu A, Xu S, Pan R, Zeng C, Jin X, Ge X, Shi Z, Ahola T, Chen Y, Guo D. 2015.
1093 Coronavirus nsp10/nsp16 Methyltransferase Can Be Targeted by nsp10-Derived Peptide In
1094 Vitro and In Vivo To Reduce Replication and Pathogenesis. *J Virol* 89:8416-27.
- 1095 78. Derbyshire V, Grindley ND, Joyce CM. 1991. The 3'-5' exonuclease of DNA polymerase I of
1096 *Escherichia coli*: contribution of each amino acid at the active site to the reaction. *EMBO J*
1097 10:17-24.
- 1098 79. Durzynska I, Sauerwald M, Karl N, Madhugiri R, Ziebuhr J. 2018. Characterization of a
1099 bafinivirus exoribonuclease activity. *J Gen Virol* 99:1253-1260.
- 1100 80. Kindler E, Thiel V. 2014. To sense or not to sense viral RNA--essentials of coronavirus innate
1101 immune evasion. *Curr Opin Microbiol* 20:69-75.
- 1102 81. Knoops K, Kikkert M, Worm SH, Zevenhoven-Dobbe JC, van der Meer Y, Koster AJ, Mommaas
1103 AM, Snijder EJ. 2008. SARS-coronavirus replication is supported by a reticulovesicular
1104 network of modified endoplasmic reticulum. *PLoS Biol* 6:e226.
- 1105 82. Snijder EJ, Limpens R, de Wilde AH, de Jong AWM, Zevenhoven-Dobbe JC, Maier HJ, Faas F,
1106 Koster AJ, Barcena M. 2020. A unifying structural and functional model of the coronavirus
1107 replication organelle: Tracking down RNA synthesis. *PLoS Biol* 18:e3000715.
- 1108 83. Ulferts R, Mettenleiter TC, Ziebuhr J. 2011. Characterization of Bafinivirus main protease
1109 autoprocessing activities. *J Virol* 85:1348-59.
- 1110 84. Snijder EJ, Decroly E, Ziebuhr J. 2016. The Nonstructural Proteins Directing Coronavirus RNA
1111 Synthesis and Processing. *Adv Virus Res* 96:59-126.
- 1112 85. Nedialkova DD, Gorbalenya AE, Snijder EJ. 2010. Arterivirus Nsp1 modulates the
1113 accumulation of minus-strand templates to control the relative abundance of viral mRNAs.
1114 *PLoS Pathog* 6:e1000772.
- 1115 86. Tischer BK, Smith GA, Osterrieder N. 2010. En passant mutagenesis: a two step markerless
1116 red recombination system. *Methods Mol Biol* 634:421-30.
- 1117 87. Weber F, Wagner V, Rasmussen SB, Hartmann R, Paludan SR. 2006. Double-stranded RNA is
1118 produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by
1119 negative-strand RNA viruses. *J Virol* 80:5059-64.
- 1120 88. Snijder EJ, van der Meer Y, Zevenhoven-Dobbe J, Onderwater JJ, van der Meulen J, Koerten
1121 HK, Mommaas AM. 2006. Ultrastructure and origin of membrane vesicles associated with the
1122 severe acute respiratory syndrome coronavirus replication complex. *J Virol* 80:5927-40.
- 1123 89. van den Worm SH, Eriksson KK, Zevenhoven JC, Weber F, Zust R, Kuri T, Dijkman R, Chang G,
1124 Siddell SG, Snijder EJ, Thiel V, Davidson AD. 2012. Reverse genetics of SARS-related
1125 coronavirus using vaccinia virus-based recombination. *PLoS One* 7:e32857.
- 1126 90. Matrosovich M, Matrosovich T, Garten W, Klenk HD. 2006. New low-viscosity overlay
1127 medium for viral plaque assays. *Virol J* 3:63.

- 1128 91. Chen CC, Kruger J, Sramala I, Hsu HJ, Henklein P, Chen YM, Fischer WB. 2011. ORF8a of SARS-
1129 CoV forms an ion channel: experiments and molecular dynamics simulations. *Biochim*
1130 *Biophys Acta* 1808:572-9.
- 1131 92. Aouadi W, Eydoux C, Coutard B, Martin B, Debart F, Vasseur JJ, Contreras JM, Morice C,
1132 Querat G, Jung ML, Canard B, Guillemot JC, Decroly E. 2017. Toward the identification of viral
1133 cap-methyltransferase inhibitors by fluorescence screening assay. *Antiviral Res* 144:330-339.
- 1134 93. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M,
1135 Soding J, Thompson JD, Higgins DG. 2011. Fast, scalable generation of high-quality protein
1136 multiple sequence alignments using Clustal Omega. *Mol Syst Biol* 7:539.
- 1137 94. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. 2009. Jalview Version 2--a
1138 multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25:1189-91.

1139

1140

Fig. 1

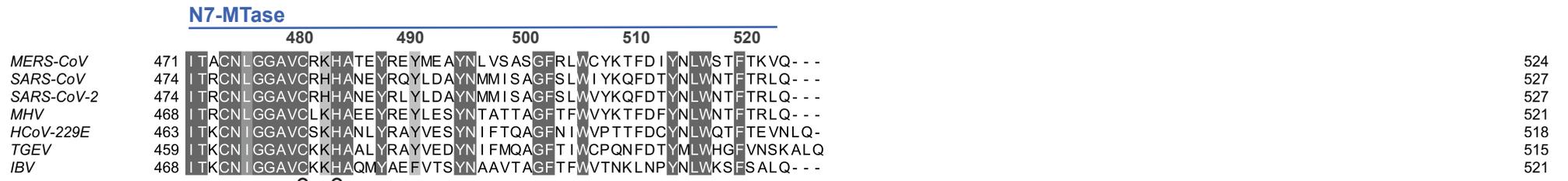
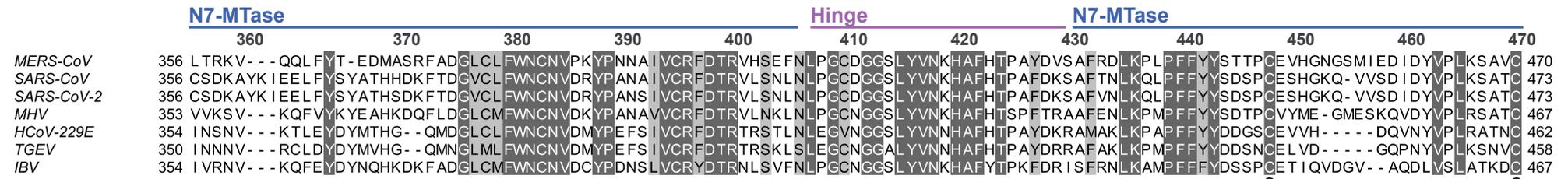
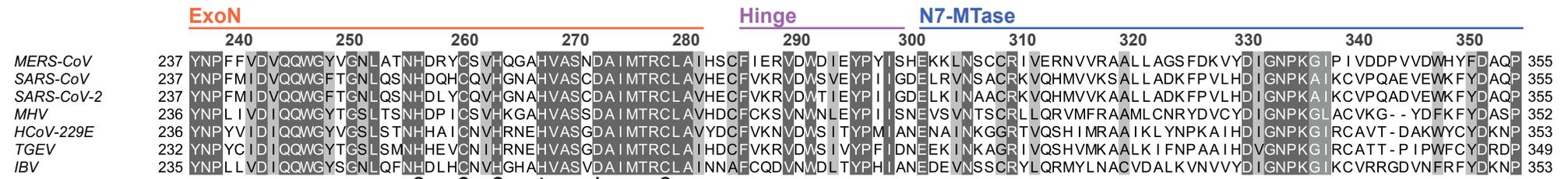
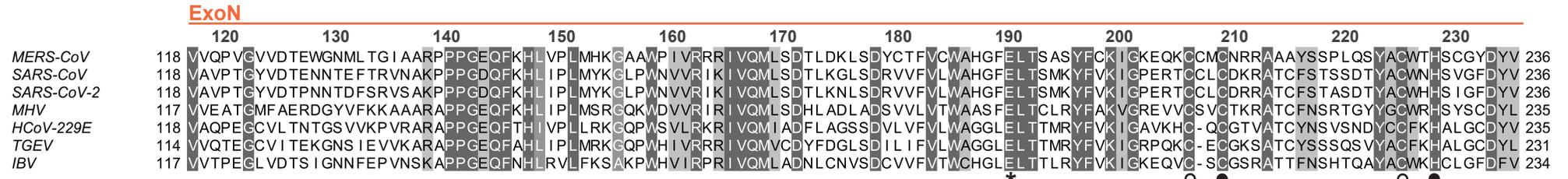
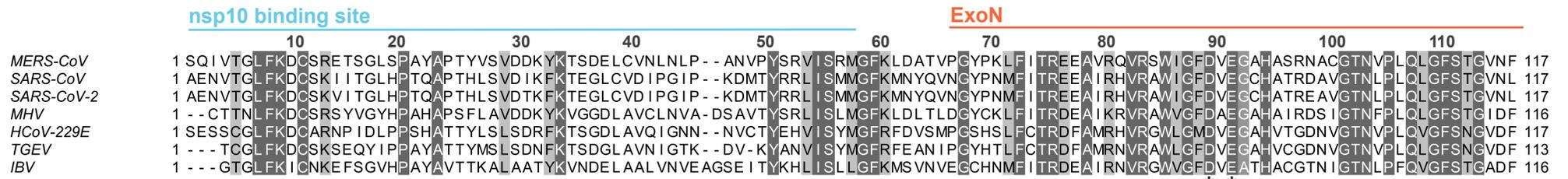


Fig. 2

A

	nsp14 ExoN mutation	Number of transfections performed	Phenotype
DEEDh Motif	D90A	9	non-viable
	D90Q	5	non-viable
	D90E	6	non-viable
	E92A	7	non-viable
	E92D	4	non-viable
	E92Q	4	non-viable
	E191A	8	non-viable
	E191D	18	viable; mildly crippled
	E191Q	4	non-viable
	D273A	8	non-viable
	D273E	5	non-viable
	D273Q	3	non-viable
	H268A	7	non-viable
	D90A/ E92A (DM)	15	non-viable
ZF1	C210H	5	non-viable
	H229C	5	viable; severely crippled
ZF2	C261A	4	non-viable
	H264R	4	non-viable

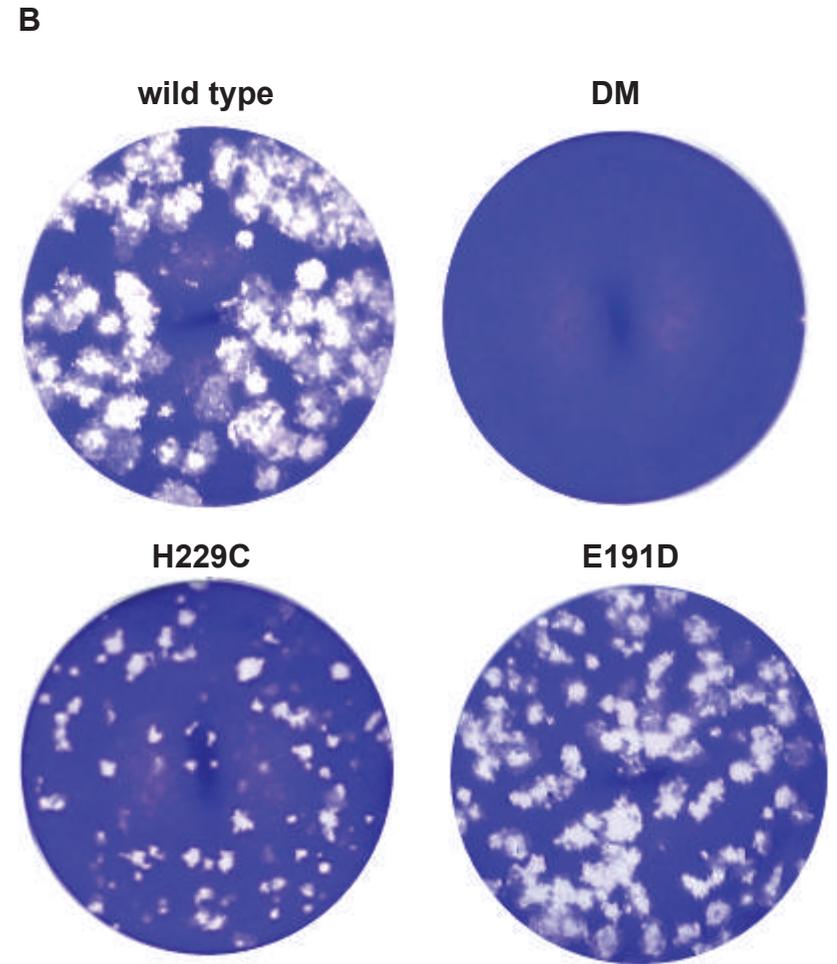
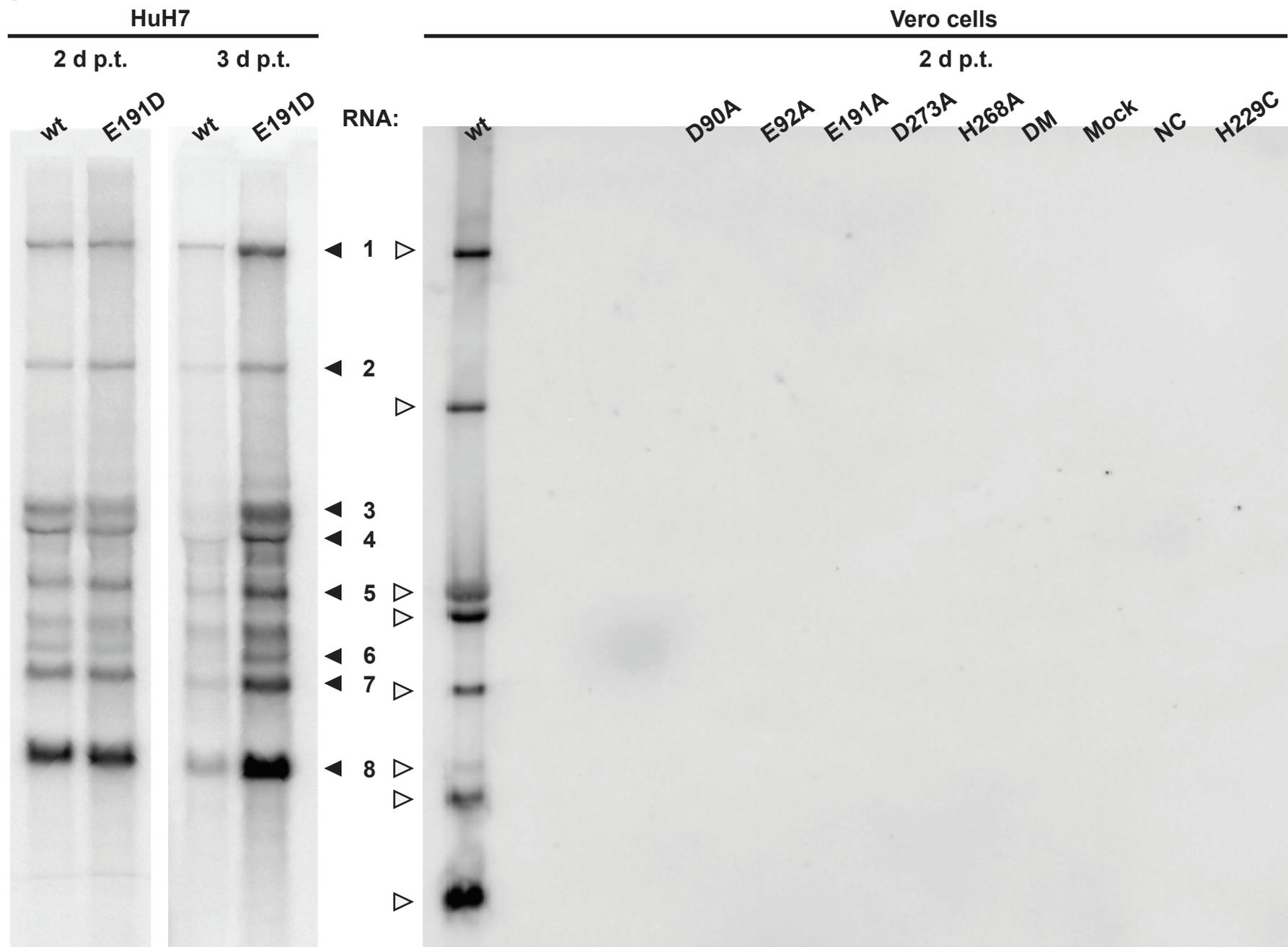


Fig. 3



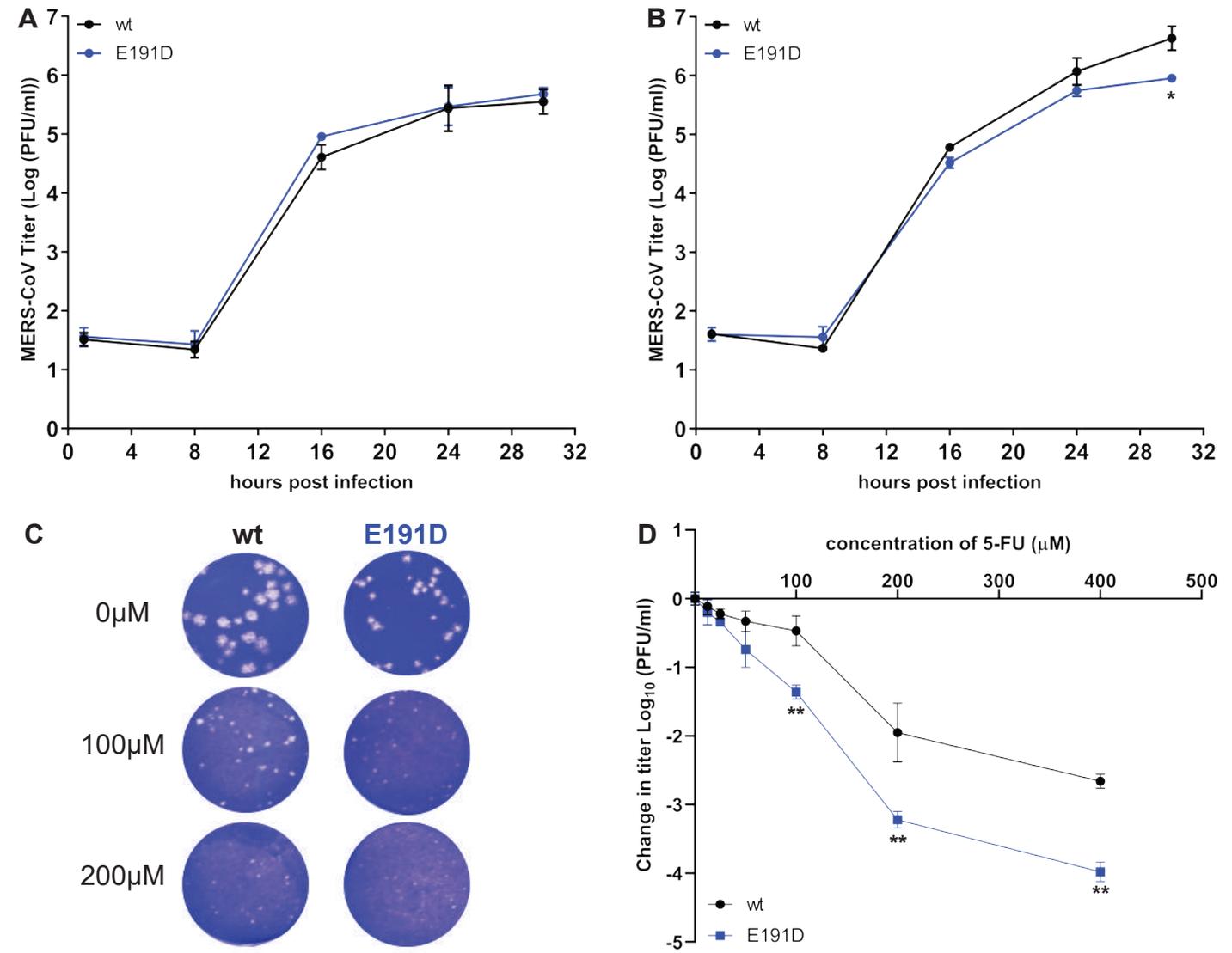


Fig. 5

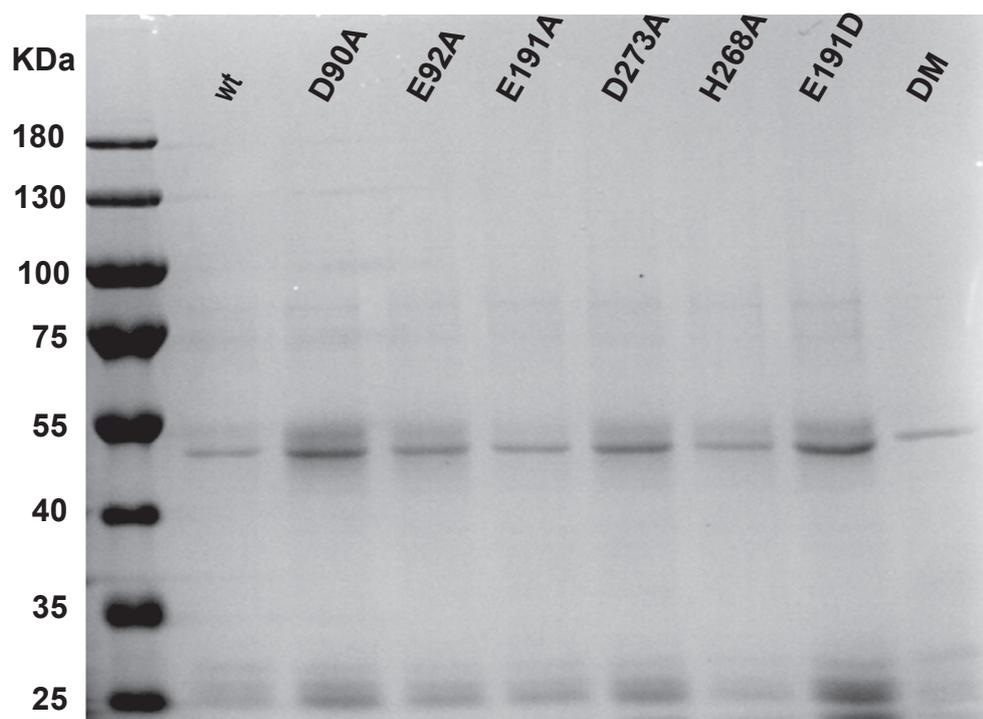


Fig. 6

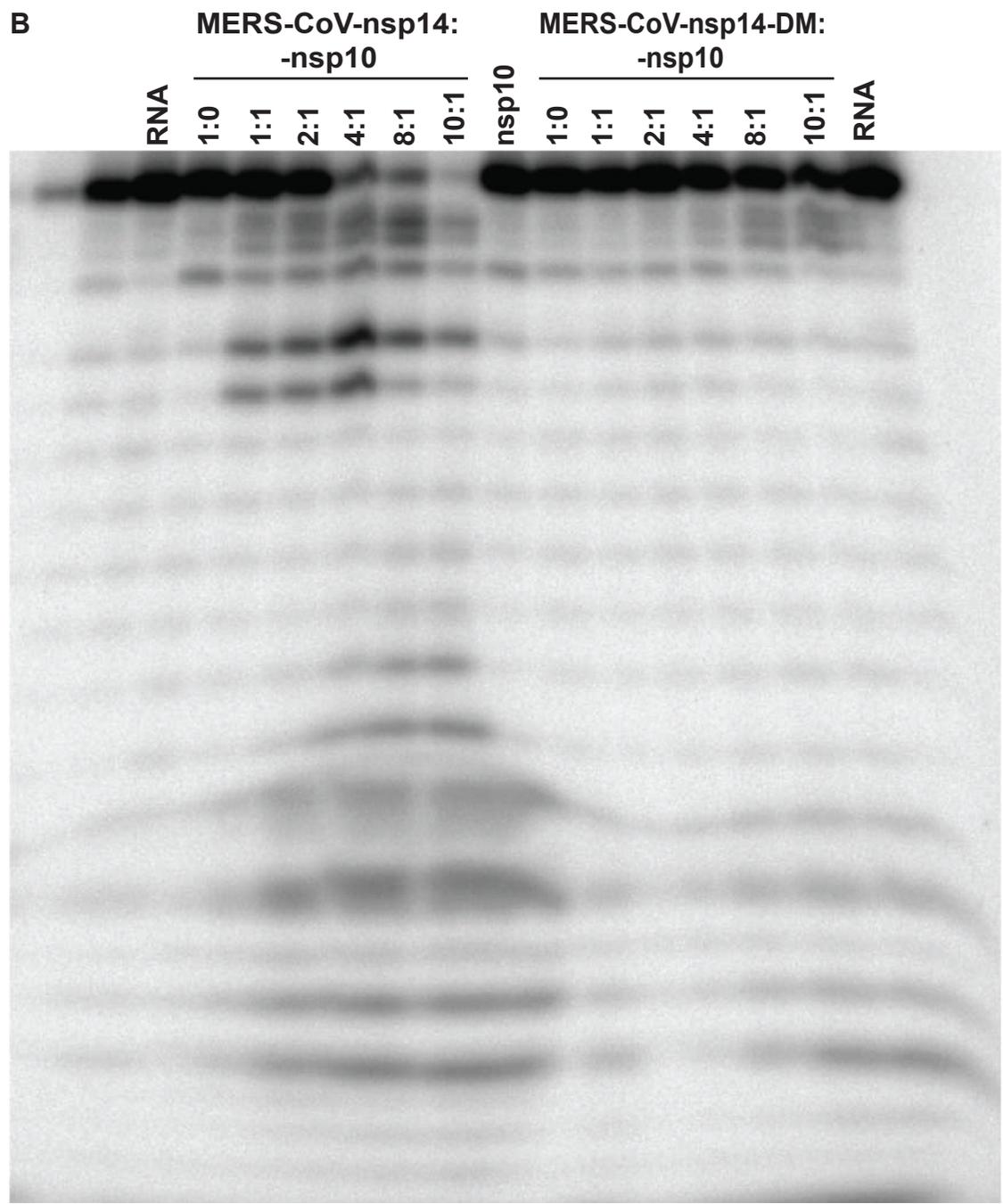
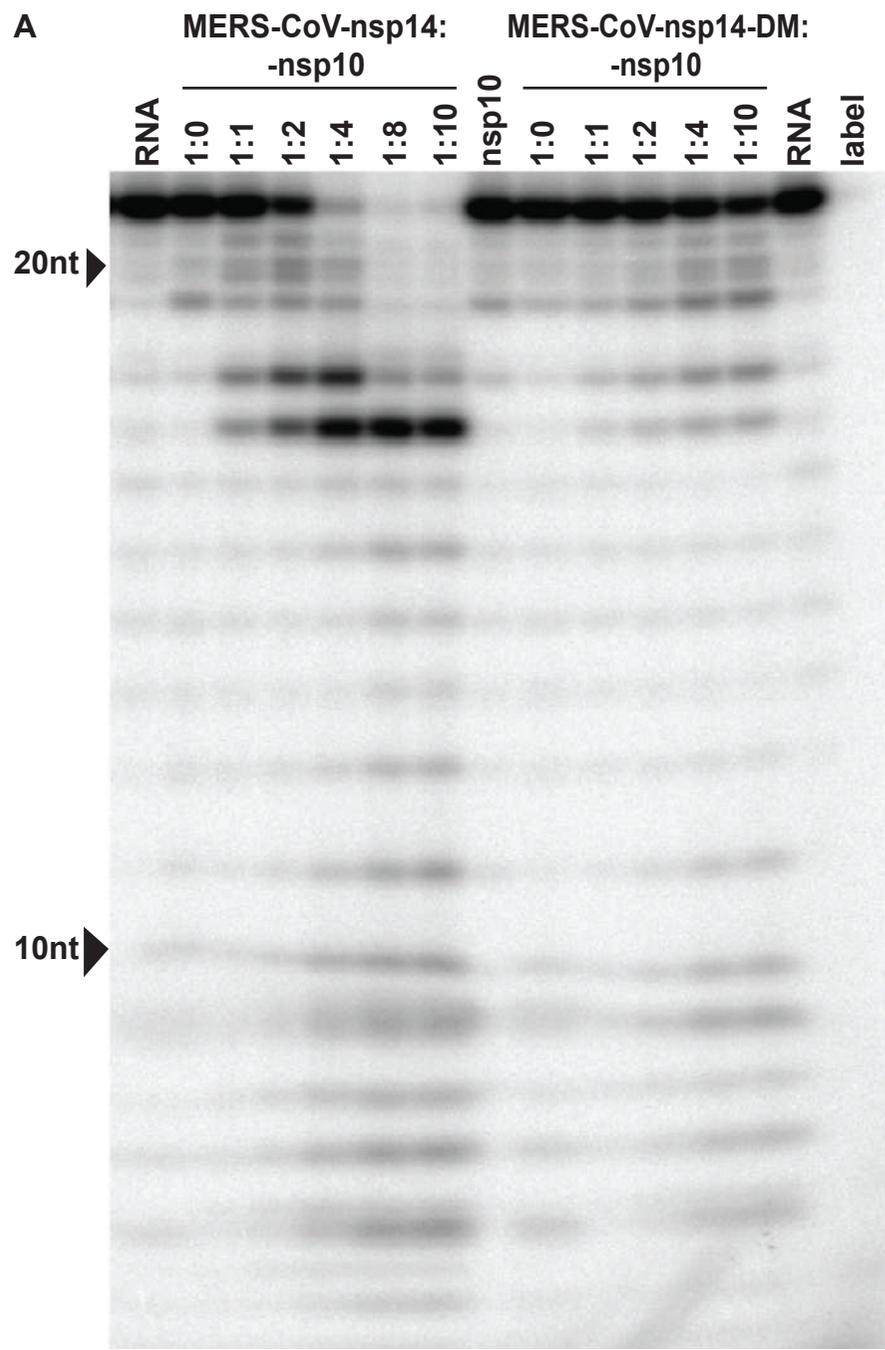


Fig. 7

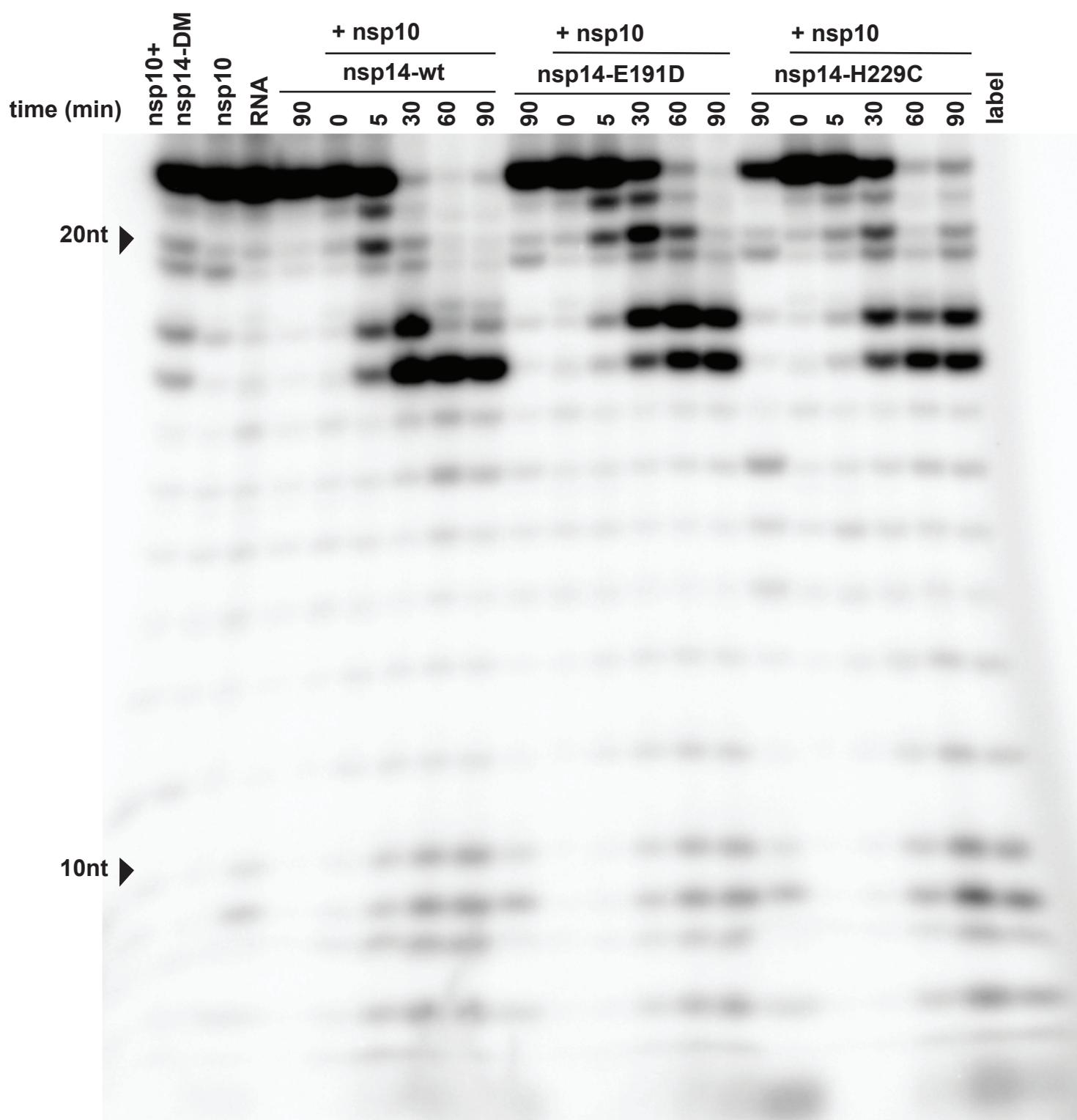


Fig. 8

