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1 ***Macro1* domain residue F156: a hallmark of SARS-CoV-2**
2 **de-MARylation specificity**

3
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45

46 **Abstract**

47 SARS-CoV-2 is a large, enveloped and positive sense single stranded RNA virus. Its genome codes
48 for 16 non-structural proteins. The largest protein of this complex is nsp3, that contains a well
49 conserved *Macro1* domain. Viral *Macro* domains were shown to bind to mono-ADP-ribose (MAR)
50 and poly-ADP-ribose (PAR) in their free form or conjugated to protein substrates. They carry ADP-
51 ribose hydrolase activities implicated in the regulation of innate immunity. SARS-CoV-2 and
52 SARS-CoV show widely different induction and handling of the host interferon response. Herein,
53 we have conducted a mutational study on the key amino-acid residue F156 in SARS-CoV-2,
54 pinpointed by bioinformatic and structural studies, and its cognate residue N157 in SARS-CoV.
55 Our data suggest that the exchange of these residues slightly modifies ADP-ribose binding, but
56 drastically impacts de-MARylation activity. Alanine substitutions at this position hampers PAR
57 binding, abolishes MAR hydrolysis of SARS-CoV-2, and reduces by 70% this activity in the case of
58 SARS-CoV.

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60 **Keywords:** SARS-CoV-2, *Macro* domain, ADP-ribose, de-MARylation, ADP-ribose binding.

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72 1. Introduction

73 Severe Acute Respiratory Syndrome (SARS) Coronavirus 2 (SARS-CoV-2) is the virus associated
74 with the pandemic of coronavirus disease appeared in 2019 (COVID-19). SARS-CoV-2 is a
75 member of the family *Coronaviridae*, the subfamily *Coronavirinae* and the subgenus sarbecoviruses
76 (Pal et al., 2020). SARS-CoV-2 belongs to the β -coronavirus genus together with the previously
77 identified SARS-CoV and MERS-CoV. All of these β -coronaviruses have been associated with
78 human fatal diseases (L. Lu et al., 2020). SARS-CoV is the agent of SARS that caused 774 deaths
79 and 8096 confirmed cases from 2002 to 2003 (Cherry, 2004). MERS-CoV emerged in 2012
80 spreading in the Middle East, with 2494 positive cases and 858 deaths (R. Lu et al., 2020).
81 Although the mortality rate of SARS-CoV-2 (<2%) is low compared to that of SARS-CoV (9.6%)
82 and MERS (35%), it spreads faster among humans. The mortality rate associated with SARS-CoV-2
83 infection can fluctuate considerably according to countries, patient age and health condition. Future
84 epidemiological studies will shed light on the possible under or over estimation of the actual death
85 rates (Abdelghany et al., 2021; Fani et al., 2020; Ioannidis, 2021; Pustake et al., 2022). Rapidly
86 evolving vaccine strategies prove effective, but are constantly challenged by the emergence of new
87 variants (Kim et al., 2021), advising the identification of viral druggable targets.

88
89 SARS-CoV-2 carries a positive-sense single-stranded RNA genome with a length of 29.9 kb (Wu et
90 al., 2020), which codes for two large open reading frames (ORF1a and ORF1ab), the latter
91 generated by a ribosomal frame-shift close to the ORF1a end. They lead to the production of two
92 large polyproteins subsequently processed to 16 non-structural proteins nsp1 to 16. The last third of
93 the genome encodes viral structural proteins: spike (S), envelope (E), membrane (M), nucleocapsid
94 protein (N) and accessory proteins. The translation of this last part of the genome occurs *via* the
95 production of individual subgenomic RNAs. Structural proteins ensure genomic packaging,
96 encapsidation, virion formation and release (Kirtipal et al., 2020). Among the nsps, nsp3 is the
97 largest multi-domain protein component of the replication/transcription complex (RTC), with an
98 average molecular mass of about 200 kDa. Its architecture is however not absolutely conserved
99 within CoV genera, due to duplication or deletion of some domains. In addition to two
100 transmembrane regions (TM1 and TM2), eight regions remain conserved: the ubiquitin-like domain
101 1 (Ubl1), the hypervariable region (Glu-rich or acidic domain), a *Macro* domain (also named “X
102 domain”), the ubiquitin-like domain 2 (Ubl2), the papain-like protease 2 (PL2^{pro}), a zinc-finger
103 domain (ectodomain), the Y1 and CoV-Y domains of unknown functions (Lei et al., 2018). SARS-
104 CoV and SARS-CoV-2 contains three tandem *Macro*- like domains (*Macro1* to 3). *Macro2* and
105 *Macro3* interact with nucleic acids, whereas *Macro1* is involved in ADP-ribose binding and
106 hydrolysis (Tan et al., 2009).

107

108 *Macro* domains are widely distributed among life kingdoms and are also coded by some (+) RNA
109 viruses including *Togaviridae*, *Hepeviridae* and *Coronaviridae*, as mentioned above (Lei et al.,
110 2018; Rack et al., 2016). These domains are able to bind various mono-ADP-ribose derivatives,
111 including ADP-ribose 1” phosphate (Appr1p), O-acetyl-ADP-ribose, and PAR, in a free form or
112 conjugated to protein or RNA substrates (Munnur et al., 2019; Munnur and Ahel, 2017). ADP-
113 ribosylation is a ubiquitous post-translational modification affecting protein activity, interactions,
114 ubiquitination and targeting of proteins for degradation. The reaction is driven by ADP-ribosyl
115 transferases (ARTs), which promote the addition of one (MAR) or multiple/poly (PAR) ADP-ribose
116 moieties onto charged amino acid residues, including Ser, Thr, Tyr, Arg, Lys, His, and Cys of target
117 proteins. To date the ART superfamily comprises 23 families. One of the best characterized is the
118 ARTD family, encompassing Poly-ADP-Ribosyl Polymerases (PARPs), which use nicotinamide
119 adenine dinucleotide (NAD⁺) as a substrate (Lüscher et al., 2022). The PARP subfamily comprises
120 ~17 members in humans. Their ADP-ribosylation activities are implicated in DNA repair, chromatin
121 remodeling, transcriptional regulation, cell signalling, inflammation, and immune response (Brady
122 et al., 2019). PARPs 1, 5a,7, 9, 10, 11, 12, 13 and 14 are involved in the regulation of innate
123 immunity and provide an anti-viral effect *via*: (i) promoting viral proteins proteasome degradation;
124 (ii) inhibiting translation machinery; (iii) inhibiting viral replication; (iv) stimulating the formation
125 of stress granules; and (v) inducing interferon (IFN) signaling and interferon stimulated genes
126 (ISGs) (Fehr et al., 2020; Hoch, 2021; Zhu et al., 2021; Zhu and Zheng, 2021).

127

128 ADP-ribosylation can be reversed by two enzyme families, namely: ADP-ribosyl hydrolases
129 (ARHs) and *Macro* domains, including viral *Macro* domains (Egloff et al., 2006; Fehr et al., 2016;
130 Li et al., 2016; Malet et al., 2006; McPherson et al., 2017). Hydrolysis activities are believed to
131 counter the above-mentioned PARPs anti-viral effects. This assumption is supported by several
132 *Macro* domain mutagenesis studies in which key residues implicated in ADP-ribose binding showed
133 a reduction in virus replication and virulence of Hepatitis E virus (HEV), alphaviruses and several
134 coronaviruses (Fehr et al., 2018). Hence, catalytic mutants of murine hepatitis virus (MHV) *Macro*
135 domain failed to induce acute hepatitis in mice, and MHV growth was restricted in cells culture
136 unless IFN receptor knockout cells were used, or PARPs inhibitors were added (Eriksson et al.,
137 2008; Fehr et al., 2015; Grunewald et al., 2019). In HCoV-229, a *Macro* domain mutant virus
138 becomes susceptible to IFN type I and II, and is unable to suppress the activation of ISGs (Kuri et
139 al., 2011). In mouse adapted SARS-CoV, mutation of the *Macro* domain ADP-ribose binding pocket
140 renders the virus more susceptible to cytokines, including IFN, TNF and IL-6, and protects mice
141 from lethal infection (Fehr et al., 2016). Consistent with these data, SARS-CoV-2 infection alters
142 PARP family gene expression and disrupts NAD⁺ biosynthesis (Heer et al., 2020). The accumulated
143 data highlight

144 the importance of ADP-ribosylation in establishing infection, thus making *Macro* domain an
145 interesting enzyme to understand virus-host interactions and their mitigations.

146

147 Amino-acid sequence comparison of nsp domains between SARS-CoV-2 and SARS-CoV reveals
148 that the SARS-CoV-2 *Macro1* domain shares 71% identity with SARS-CoV, hence being the most
149 divergent amongst nsps (Frick et al., 2020; Lin et al., 2020). The crystal structure of SARS-CoV-2
150 *Macro1* domain was solved in its free form (apo) and complexed to various ligands including 2-(N-
151 morpholino) ethanesulfonic acid (MES), AMP and ADP-ribose. The reported structures follow the
152 classical architecture described for *Macro* domains, with 7 β -sheets sandwiched between two layers
153 of α -helixes (Alhammad et al., 2021; Frick et al., 2020; Lin et al., 2020; Michalska et al., 2020).
154 Structural comparison studies between SARS-CoV-2 and SARS-CoV *Macro1* domains point to an
155 unexpected difference at the heart of the *Macro1* domain active site: the F156 residue in SARS-
156 CoV-2 corresponds to N157 residue in SARS-CoV. A divergence at this position suggests a
157 significant change in the orientation of the ADP-ribose adenine group in the binding pocket between
158 the *Macro1* domains of these two viruses, which might impact considerably ADP-ribose binding
159 and/or hydrolysis kinetics, and might translate into difference on innate immune response observed
160 against sarbecoviruses.

161

162 In the present study, we focus on SARS-CoV-2 F156 and SARS-CoV N157 *Macro1* domain
163 residues. Through mutagenesis and functional studies, we evaluate the role of this particular residue
164 on *Macro1* domain stability, ligand binding and ADP-ribose derivatives hydrolysis activity.

165

166 **2. Materials and methods**

167 **2.1. Expression and Purification of the SARS-CoVs *Macro1*** 168 **domain**

169 The cDNA encoding nsp3 sequence of SARS-CoV (residues 182 to 355, GenBank #AY291315)
170 and SARS-CoV-2 (residues 207 to 375, NCBI accession YP_009725299.1) were codon optimized
171 and cloned into the pET28 vector (Novagen)-TWIST Bioscience. Mutagenesis was performed by
172 PCR using Phusion High-Fidelity DNA Polymerase (NEB), as recommended by the manufacturer.
173 Primers used for mutant generation are compiled in Table S1. The recombinant proteins were
174 expressed in competent *E. coli* cells (C41 (DE3) plys). A detailed protocol on expression and
175 purification of SARS-CoV was previously reported (Malet et al., 2006). The expression of SARS-
176 CoV-2 recombinant protein was done in Turbo Broth medium (Cat#0104 AthernaES). The induction
177 was carried with 50 μ M of IPTG (O.D_{600 nm} of 0.6) at 25°C for 12-14 hours. At the end of the
178 incubation time, the cultures were centrifuged at 9000 rpm at 4°C for 30 min, and the pellets were

179 kept at -80°C until purification. Thawed bacterial pellet from *Macro1* domain of SARS-CoV-2 was
180 resuspended in lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 20 mM MgSO₄, 5% glycerol,
181 20 µg/mL DNase, 0.25mg/mL lysozyme and 1 mM PMSF) at 4°C for 1 hour. The cell lysate was
182 sonicated and the supernatant was collected by centrifugation at 20,000 rpm for 25 min at 4°C.
183 Immobilized metal affinity chromatography (IMAC) purification was performed using Ni-NTA
184 column (GE Healthcare). The filtered supernatant was loaded into the column equilibrated with
185 binding buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5% Glycerol and 10mM Imidazole). The
186 column was then washed with 10 column volume of wash buffer (50 mM Tris-HCl, pH 8, 150mM
187 NaCl, 5% Glycerol and 30 mM Imidazole) followed by a second wash with buffer supplemented
188 with 1 M NaCl. Recombinant protein was eluted with elution buffer (50 mM Tris-HCl, pH 8, 150
189 mM NaCl, 5% Glycerol and 200 mM Imidazole). The fractions containing the purified protein were
190 then pooled and dialysed against gel filtration (GF) buffer (50 mM Tris-HCl pH 8, 150 mM NaCl,
191 5% glycerol). In addition, a second purification step of size-exclusion chromatography was
192 performed by injecting the protein into a Superdex 75 column (GE Healthcare) equilibrated with GF
193 buffer. The eluted protein was concentrated in Vivaspin centrifugal concentrator (Sartotius) and
194 stored at -80°C. The presence of the proteins in the fractions of interest was confirmed by western
195 blot, using an anti-histidine antibody (Penta-His HRP, Quiagen). Mutants *Macro1* domains were
196 expressed and purified following the same conditions as the corresponding wild-type recombinant
197 proteins.

198

199 **2.2. Poly (ADP-ribose) synthesis**

200 PARylated protein was obtained through auto-ADP-ribosylation of human PARP1 (hPARP1)
201 (Sigma SRP0192) in 300µL reaction volume, in the presence of 100 mM Tris pH 8, 10 mM MgCl₂,
202 2 mM DTT, 0.8 units hPARP1 and 250 µM NAD⁺. The reaction was carried at room temperature
203 (RT) for 2 h under moderate agitation. PARylation was stopped by diluting the reaction in 20mL of
204 dot blot buffer (10mM Tris pH 8,150 mM NaCl and 0.05 % Tween). The diluted reaction was
205 directly used for the *Macro1* domain binding assay.

206

207 **2.3. Binding assay**

208 Binding affinity of ADP-ribose to SARS-CoV and SARS-CoV-2 was performed in Immobilon-PSQ
209 PVDF membrane (MERCK Cat #ISEQ85R). The binding reactions were carried out by spotting
210 serial dilutions (from 250 to 1.9 pmol) of recombinant *Macro1* domain proteins on nitrocellulose
211 membrane using Minifold II dot blot apparatus (Schleicher & Schuell). Bovine serum albumin
212 (BSA) was used as a negative control for the assay. The membrane was blocked with dot blot buffer
213 supplemented with 4% skimmed milk, for 1 h, at RT. The blocked membrane was then incubated

214 with auto-ADP-ribosylated hPARP1 for 1h at RT under constant agitation. The unbound material
215 was removed by three extensive washes with dot blot buffer. The primary antibody anti-PAR
216 binding reagent (Sigma Cat #MABE1031) was used diluted 1:1500 in dot blot buffer with 1 % non-
217 fat milk for 2 h, at RT. The secondary antibody, anti-rabbit IgG (Dako), was diluted 1/2000 in dot
218 blot buffer and incubated with the membrane for 1 h, at RT. The membrane was washed three times
219 in dot blot buffer after each anti-body. Immunoreactive signals were revealed using ECL reagent
220 (Cat. # 170–5061, Bio-Rad) and visualized using Amersham™ ImageQuant™ 800 Immager
221 system. Images were analyzed and quantified using ImageJ software (Schneider et al., 2012). Each
222 experiment was repeated three times. Band's intensity was normalized to the total protein load.

223

224 **2.4. Isothermal titration calorimetry (ITC)**

225 ITC experiments were performed at 20°C using an Microcal iTC200 (Malvern Panalytical). Purified
226 recombinant SARS-CoV and SARS-CoV-2 WT and mutant proteins were used at 150µM. The
227 interaction was assessed in GF buffer, using 75 to 2000µM of ADP-ribose as injected ligand. Heat
228 of dilution were measured by injecting the ligand into the protein solution. Titration curves were
229 fitted with the MicroCal Origin software, assuming a one-site binding model, and enthalpy (ΔH),
230 entropy changes (ΔS), dissociation equilibrium constants (K_D) and stoichiometry were extracted.

231

232 **2.5. De-MARylation and de-PARYlation assays**

233 MARylated human PARP3 (hPARP3) was obtained by incubating 10µM of full length hPARP3
234 (Sigma SRP0194) in 25mM Tris pH 8, 100mM NaCl, 2 mM DTT, and 200µM NAD⁺ for 30
235 minutes at 30°C. De-MARylation activity was assessed by incubating 1µM of MARylated hPARP3
236 with 150 nM of recombinant *MacroI* domains at 37°C for a period of 0 to 30 min in the same
237 buffer. The reaction was stopped by adding mPAGE™ 4X LDS Millipore sample buffer and heating
238 at 95°C for 5 min. Autoribosylated hPARP3 protein without any *MacroI* domain served as negative
239 control. Reactions were loaded on a 4 to 20% mPAGE bis-tris precasted gel (Millipore
240 Cat#MP42G12). After migration, protein bands were transferred to nitrocellulose membrane
241 (Amersham Cat#10600008) overnight at 4°C. The membranes were blocked with 4% non-fat milk
242 in TBS-Tween buffer for 1 h. The primary antibody anti-MAR binding reagent (Sigma Cat #
243 MABE1076) was used at a final dilution of 1/2500 in TBS-T with 1% non-fat milk for 1h. The
244 membrane was then processed as described above in the dot blot binding assay. Quantification was
245 done using ImageJ program. The results were normalized to the value of the negative control.
246 The de-PARYlation activity was performed by incubating of 1 or 5µM of SARS-CoV and SARS-
247 CoV-2 *MacroI* domain with 0.4 U of auto-PARYlated hPARP1. The reactions were incubated for 1h
248 at 37°C in the reaction buffer (100 mM Tris pH 8, 10 mM MgCl₂ and 2 mM DTT). After the

249 incubation time, the samples were subjected to the same process described above in de-MARylation
250 activity. The primary antibody used to detect the PAR non-removed was anti-PAR binding reagent
251 (Sigma Cat # MABE1076) diluted 1:1500 in TBS-T with 1% non-fat milk.

252

253 **2.6. Protein stabilization assessment**

254 *2.6.1 Thermal shift assay (TSA)*

255 Protein thermal shift assays were performed to determine the stability of SARS-CoV and SARS-
256 CoV-2 *Macro1* domains proteins in presence of increases concentration of ADP-ribose (10 μ M to
257 1000 μ M). For that end, *Macro1* domain proteins were diluted in TSA buffer (20mM HEPES pH 8
258 and 150mM NaCl) to a final concentration of 2 μ M. The test was performed in MicroAmp[®] Fast
259 Optical 96-well reaction plates from Applied Biosystems. Protein thermal shift dye kit (Applied
260 biosystems, Thermo Fisher Scientific) was used diluted in water, according to manufacturer's
261 instructions. After mixing all components in the wells, the plate was sealed and put in the 7500
262 FastReal-Time PCR System (Applied Biosystems). The reaction was started by a first incubation
263 step at 25°C for 2 min. The temperature was then increased with a ramp rate of 0.5°C per min to
264 95°C. Normalized melting curves were obtained with GraphPad Prism. The melting temperatures
265 (T_m) were calculated from the inflection point of the melting curves.

266

267 *2.6.2 HoTMuSiC tools*

268 HoTMuSiC tools was used to predict the change in melting temperature (T_m) upon point mutations.
269 For that purpose, the protein structure (SARS-CoV (PDB: 2FAV) and SARS-CoV-2 (PDB: 6WOJ)
270 and the melting temperatures of the wild-type proteins (SARS-CoV (T_m =319K) and SARS-CoV-
271 2 (T_m =321K)), determined by TSA, were supplied.

272

273 **3. Results and discussion**

274 **3.1. ADP-ribose binding coordination**

275 The overall structure of the CoV *Macro1* domain consists of six α -helices and one seven-stranded
276 β -sheet. The β -sheet (β_1 - β_2 - β_7 - β_6 - β_3 - β_5 - β_4) is topped and bottomed by 3 α -helices (α_1 , α_2 , and
277 α_3 and α_4 , α_5 , and α_6 respectively) thus delimiting a groove where the ligand binds (Fig 1).
278 Comparative protein sequence analysis of SARS-CoV and SARS-CoV-2 *Macro1* domains (Fig 1A)
279 reveals that most of the differences are located at the extremities of the domain namely the α_1 helix
280 and α_1 - β_3 loop at the N-terminus, and the α_6 helix at the C-terminus. Divergent residues between
281 the two closely related viruses represent ~ 30 % of the sequence in accordance to previous reports
282 (Frick et al., 2020; Lin et al., 2020). The structural basis of *Macro1* domain and ADP-ribose
283 interactions have been characterized (Alhammad et al., 2021; Egloff et al., 2006; Frick et al., 2020;

284 Lin et al., 2020; Malet et al., 2009; Michalska et al., 2020). These studies pointed out that the ADP-
285 ribose is partially buried in a chief hydrophobic cleft encompassing, in the case of SARS-CoV-2,
286 the C-terminal end of β strands β_3 , β_5 , β_6 , β_7 and two loops, β_3 - α_2 and β_6 - α_5 (Fig 1B and C). The
287 cleft accommodating ADP-ribose creates four contact zones. The first contact zone forms a
288 hydrophobic patch between the residues I23, V49, P125, V155 and F156 (all conserved except the
289 latter), which interacts with adenine and points it toward the polar D at position 22. Residues
290 interacting directly with ADP ribose are well conserved among various *Macro* domains. The residue
291 D22 correspond to D23 in SARS-CoV (Fig 1E) and is present in other *Coronavirinae* members,
292 alpha-like viruses and non-viral *Macro* domains (Allen et al., 2003; Cho et al., 2016; Fehr et al.,
293 2018; Rack et al., 2020). Mutagenesis studies showed that these residues are crucial for ADP-
294 ribosyl hydrolase and ADP-ribose-1'-phosphatase activities, and drastically impact viral replication
295 and virulence (reviewed in (Fehr et al., 2018)). The second contact zone, a conserved glycine rich
296 stretch (residues 46-48), accommodates the diphosphate moiety of ADP-ribose. Phosphate groups
297 connecting adenosine moiety to the distal ribose interact with amide backbones of the main chain of
298 residues within the loop β_3 - α_2 and β_6 - α_5 region. The α -phosphate forms hydrogen bonds with
299 V49 and I131 while β -phosphate interacts with S128, G130 and F132 (Fig 1 D and E). Distal ribose
300 fits tightly into the pocket formed by the nitrogen bonds between the amide group of G48, G46 and
301 side chain of N40 and 1', 2' and 3'-hydroxyl groups of the ribose. The third contact zone implicates
302 F132 and I131, which stabilize the proximal ribose. The fourth contact zone, involving L126,
303 together with A154 and D157, supports the distal ribose *via* water-hydrogen bonding (Alhammad et
304 al., 2021; Frick et al., 2020; Lin et al., 2020; Michalska et al., 2020).

305

306 In SARS-CoV-2 the adenosine moiety is partially stacked by F156, which interacts edge-to-face
307 with the aromatic ring system (Fig 1C and D). This residue corresponds to N157 in SARS-CoV (Fig
308 1E). The proximity of phenylalanine to the adenosine ring is only observed in SARS-CoV-2 among
309 betacoronaviruses, even if other hydrophobic residues are present at that position. These data, in
310 concert with previous 3D studies, might suggest a different alignment of ADP-ribose adenine group
311 in the binding pocket of SARS-CoV-2.

312

313 **3.2. Expression and Purification of recombinant *Macro1*** 314 **domain proteins**

315 In order to understand the influence of F156 on ADP-ribose binding and hydrolysis kinetics, we
316 generated several substitutions leading to: (i) the exchange of F156 and N157 between *Macro1*
317 domains of SARS-CoV-2 and SARS-CoV, respectively; (ii) the substitution of the target residues to
318 alanine. To set a negative reference for comparison, mutations of D22 and D23, for SARS-CoV-2
319 and SARS-CoV, respectively, were also conducted. Recombinant *Macro1* domain proteins, with a

320 N-terminal 6 x His tag, of 172 amino acids (aa) and 168 aa, corresponding to SARS-CoV and
321 SARS-CoV-2 respectively, were successfully expressed in *E. coli* (Fig 2A) and purified. Bands of
322 about 20 kDa were observed in soluble purification fractions, and enriched throughout the
323 purification process (Fig 2B). Despite being a 20.8 kDa protein, the *Macro1* domain of SARS-CoV-
324 2 migrates slightly higher than that of SARS-CoV (20.9 kDa). The difference in the gel migration
325 could be related to the difference in SDS adsorption of these two proteins. Besides, SARS-CoV-2
326 shows slight differences in purification buffer preferences as compared to SARS-CoV. First,
327 significant amounts of SARS-CoV-2 were lost during the washing steps at the imidazole
328 concentration used for SARS-CoV (60 mM). Thus, imidazole concentration was decreased to 30
329 mM. In contrast, salt concentration had to be increased to eliminate more tightly bound
330 contaminants. After two purification steps (IMAC and gel filtration), proteins of interest were pure,
331 as evidenced by SDS-PAGE after Coomassie blue staining (Fig 2C). Identities of WT SARS-
332 CoV-2 and SARS-CoV recombinant *Macro1* domains were confirmed by Matrix Assisted Laser
333 Desorption Ionization - Time of Flight (MALDI-TOF) mass spectrometry.

334

335 **3.3. Assessment of CoV *Macro1* domains ADP-ribose binding**

336 In order to accurately evaluate the characteristics of generated mutants, we started by setting the
337 reference values for our WT *Macro1* domain proteins, in comparison to earlier reports. Hence, dot
338 blot assay, previously reported (Egloff et al., 2006; Li et al., 2016; Malet et al., 2009), was first
339 conducted using auto-PARylated hPARP1 and increasing amounts of WT *Macro1* domains (Fig 3A,
340 middle panel), to assess PAR binding to WT *Macro1* domain proteins. Our results show that auto-
341 PARylated hPARP1 binds to SARS-CoV and SARS-CoV-2 WT, and is detected using an anti-PAR
342 antibody, in a concentration dependent manner (Fig.3A upper and middle panel). The quantified
343 binding signal displays drastic differences, up to 75%, in PAR binding of SARS-CoV regarding to
344 SARS-CoV-2 at various *Macro1* domain concentrations (Fig 3A, upper panel). N to F and F to N
345 substitution in SARS-CoV and SARS-CoV-2, respectively do not impact PAR binding (Fig. 3A,
346 lower panel). However, alanine substitutions at that position decrease PAR binding for both *Macro1*
347 domains. Alanine replacements of the conserved aspartate residue (D23A and D22A for SARS-CoV
348 and SARS-CoV-2, respectively) abrogate PAR binding (Fig. 3A, lower panel). PAR binding was
349 previously reported for SARS-CoV (Egloff et al., 2006; Li et al., 2016) and SARS-COV-2 (Lin et
350 al., 2020). Therefore, our data confirm preceding studies. Moreover, the differential in PAR binding
351 capacity between SARS-CoV and SARS-CoV-2 illustrated in Fig 3A might suggest a lower PAR
352 binding capacity of SARS-CoV-2 *Macro1* domain, compared to SARS-CoV, exemplified by the
353 need of high concentrations of protein for the binding to occur. Differences in PAR binding were
354 also noticed between SARS-CoV and alphaviruses (Egloff et al., 2006; Malet et al., 2009), the
355 former being less prone to PAR binding compared to the latter. Knowing that PAR polymers can

356 vary in size and complexity (Han et al., 2011; Jankevicius et al., 2013), this might suggest that
357 viruses have different PARylated targets.

358

359 Next, we determined the dissociation constants and thermodynamic parameters of ADP-ribose using
360 ITC (Fig 3B, C, D and E). Previous studies investigated *Macro1* domain binding parameters
361 towards ADP-ribose using ITC (Isothermal titration calorimetry) for various human pathogenic
362 coronaviruses, including SARS-CoV and SARS-CoV-2. The reported K_D values vary between 10
363 and 24 μM for SARS-CoV, and 10 and 17 μM for SARS-CoV-2 (Cho et al., 2016; Egloff et al.,
364 2006; Frick et al., 2020; Lin et al., 2020). The differences being due to slight variations in
365 experimental protocols and constructs design. In our hands, computed K_D values for SARS-CoV
366 and SARS-CoV-2 correspond to $5.9 \pm 2.9 \mu\text{M}$ and $11.5 \pm 5.7 \mu\text{M}$, respectively (Table 1). As expected,
367 the substitution of SARS-CoV-2 D22A and SARS-CoV D23A, tested as negative controls, had a
368 detrimental effect on the ADP-ribose binding. The computed K_D for SARS-CoV D23A *Macro1*
369 domain is $28.9 \pm 1.99 \mu\text{M}$ (Table 1). Unfortunately, in the case of SARS-CoV-2, binding kinetics
370 could not be determined accurately because of precipitation issues, paralleling observations made
371 for Chikungunya virus (CHIKV) (McPherson et al., 2017). To determine if the presence of F156
372 SARS-CoV-2 influences ADP-ribose binding into the pocket, K_D values were measured for the
373 generated mutants. Substitution of F156N in SARS-CoV-2 and N157F in SARS-CoV slightly
374 affects ADP-ribose binding affinities (Table 1), with measured values of $6.17 \pm 1.66 \mu\text{M}$ for SARS-
375 CoV N157F and $9.17 \pm 1.14 \mu\text{M}$ for SARS-CoV-2 F156N. Alanine replacement in SARS-CoV
376 N157A led to a 2-fold increase in K_D ($13.5 \pm 1.67 \mu\text{M}$) compared to WT. Conversely, SARS-CoV-2
377 F156A substitution had minor consequences on ADP-ribose affinity, with a K_D of $9.8 \pm 3.43 \mu\text{M}$.
378 One can notice that enthalpy and entropy values are similar between SARS-CoV and SARS-CoV-2
379 WT proteins. The mutations of interest do not change drastically ΔH values, nevertheless leaning
380 towards the disruption of energetically favourable noncovalent interactions. Besides, a slight trend
381 of higher sensitivity for SARS-CoV-2 is observed, with up to 7-fold increase in ΔS value for SARS-
382 CoV-2 F156A mutant; while overall stable for SARS-CoV mutant proteins (Table 1).

383

384 **Table 1. Thermodynamic parameters for CoV *Macro1* domains determined by ITC.**

CoV <i>Macro1</i> domain	$K_D(\mu\text{M})$	$\Delta H(\text{kcal/mol})$	$\Delta S(\text{cal/mol/deg})$
SARS-CoV	5.9 ± 2.4	-10.35 ± 0.21	-11.4
SARS-CoV-2	11.5 ± 5.7	-10.14 ± 0.13	-12.0
SARS-CoV-N157F	6.17 ± 1.66	-9.528 ± 0.17	-8.66
SARS-CoV-2 F156N	9.17 ± 1.14	-8.037 ± 0.2	-4.36
SARS-CoV N157A	13.5 ± 1.67	-9.352 ± 0.043	-9.63
SARS-CoV-2 F156A	9.8 ± 3.43	-7.225 ± 0.37	-1.72
SARS-CoV D23A	28.9 ± 1.99	-7.962 ± 0.13	-6.39
SARS-CoV-2 D22A	ND		

385 K_D (dissociation constant), ΔH (enthalpy), ΔS (entropy), ND (Not determined)

387 Hence, even if SARS-CoV-2 F156N substitution drives the mutant toward SARS-CoV K_D value,
388 single amino acid substitution is not sufficient, in this context, for a complete mimicking of SARS-
389 CoV ADP-ribose binding characteristics. Interestingly, the simultaneous mutation of SARS-CoV-2
390 V24I/E25Q/F156N, mimicking MERS-CoV sequence, generated a *Macro1* domain with K_D value
391 similar to that obtain for MERS-CoV ($\sim 3 \mu\text{M}$) (Cho et al., 2016; Lin et al., 2021, 2020). More
392 recently, while this paper was under revision, Tsika *et al* 2022, reported a study investigating the
393 inhibitory effect of remdesivir metabolite GS-441524 on SARS-CoV, MERS-CoV and SARS-CoV-
394 2 *Macro1* domains. They showed that F156 plays a key role with adjacent residues in the selective
395 binding of GS-441524 to SARS-CoV-2 *Macro1* domain. In that context, they exchanged F and N
396 residues between SARS-CoV-2 and MERS-CoV, showing that the substitution N154F in the later
397 has no effect on ADP-ribose binding, similar to what we are reporting for SARS-CoV, possessing an
398 N residue at that position. In their hands, the computed K_D value for SARS-CoV-2 F156N was 8.2
399 μM , in accordance with our study (Tsika et al., 2022). Previous mutational studies on CHIKV
400 *Macro* domain and human *MacroD2*, targeting residues in the vicinity of the ADP-ribose binding
401 pocket, including the residues corresponding to G48 and G130 in SARS-CoV-2 *Macro1* domain,
402 disrupt the binding of ADP-ribose (Li et al., 2016; Malet et al., 2009; McPherson et al., 2017). In
403 SARS-CoV-2 the proximity of F156 to D22 may impact the strength of hydrogen bonds formation,
404 which could influence the side chain orientation of the ADP-ribose adenine base, as pointed out by
405 the crystallographic studies. Structural determination of the *Macro1* domain in the apo form as well
406 as in complex to various ligands highlighted that ADP-ribose binding pocket is dynamic and
407 flexible, adapting to different ADP-ribose derivatives. The loop in between $\beta 7$ and $\alpha 6$, harboring the
408 F156 residue, participates to a hydrophobic patch accommodating the adenine portion of the ADP
409 ribose. This patch, including A21, I23, V49, L126 and A154 (Fig1. D and E), facilitates hydrogen
410 bonding between adenine's N6 atom and β -carboxyl group of D22 and insures the correct
411 positioning of water molecules, crucial for catalysis (Alhammad et al., 2021; Correy et al., 2022;
412 Lin et al., 2020; Michalska et al., 2020). Our results demonstrate that the swap of N and F between
413 the two *Macro1* domains or even a mutation to A at this position do not hamper ADP-ribose
414 binding. However, alanine substitutions of the aspartate at positions 22 and 23 (in SARS-CoV-2 and
415 SARS-CoV, respectively) hinder it. Hence, our data support the need of a synergistic effect between
416 several molecular determinants for ADP-ribose positioning in the binding pocket.

417 **3.4. Altered de-MARylation in CoV *Macro1* domains mutants**

418 Viral *Macro* domains were shown to possess MAR and PAR hydrolytic activities leading to the
419 removal of ADP-ribose derivatives from MARylated and PARylated proteins (Aguilar et al., 2022;
420 Alhammad et al., 2021; Eckeï et al., 2017; Jankevicius et al., 2013; Li et al., 2016; Rosenthal et al.,

421 2013). De-PARylation activity can be assessed by different ways including: (i) the use of
422 radiolabeled [P^{32}] NAD⁺ for PARPs auto-PARylation followed by the addition of the *Macro* domain
423 containing protein and the quantification of PAR signal removal after SDS-PAGE migration; (ii) the
424 use of cold auto-PARylated PARPs coupled to anti-PAR antibody detection following gel migration.
425 In our hands, using the second method, de-PARylation activity was neither detected for SARS-CoV
426 nor for SARS-CoV-2 *Macro1* domains (Fig 4A), at concentrations ranging from 0.1 to 10 μ M. Our
427 results are in accordance with previously published results on MacroD2 family, including viral
428 *Macro* domains (Alhammad et al., 2021; Ecker et al., 2017; Jankevicius et al., 2013; Rosenthal et
429 al., 2013). All these data underline the low PAR hydrolysis potential of viral *Macro* domains when
430 compared to the Poly ADP-ribose glycohydrolase (PARG), the prototype of PAR hydrolytic
431 enzymes, reversing the action of PARP enzymes in cells. Nevertheless, removal of radiolabelled
432 PAR from auto-PARylated hPARP1, even if weak, was observed for Hepatitis E virus (HEV),
433 Sindbis virus (SINV), Venezuelan equine encephalitis virus (VEEV) and SARS-CoV. In the case of
434 HEV the activity was prompted by the presence of the helicase domain (Aguilar et al., 2022; Li et
435 al., 2016). Furthermore, *Macro* domain's constructs used in different studies, cited above, varies
436 slightly. The generated proteins present a shift of 3 to 8 amino acids at the NT and/or the CT. This
437 might suggest that if this activity is indeed biologically relevant in the case of viral *Macro* domains,
438 they may need additional molecular determinants to fulfill it.

439

440 To assess the effect of CoV *Macro1* domain mutation on protein de-MARylation, hPARP3 protein
441 was used as a substrate in time course hydrolysis reactions (Fig 4 B and Fig. S1). hPARP3 is known
442 to MARylate different substrates such as Tankyrase 1, the mitotic components NuMa, PARP1 and
443 its self (Rodriguez-Vargas et al., 2019). Hence, MARylated hPARP3 was incubated in the presence
444 of various CoV *Macro1* domain mutants at a 10:1 substrate-enzyme ration, otherwise the hydrolysis
445 was too rapid. After the indicated time points, the reactions were subjected to mPAGE. Western blot
446 using the anti-MAR reagent allows de-MARylation assessment by the removal of the MAR signal
447 (Fig 4B, upper panel). CoV *Macro1* domain load was controlled by mPAGE Coomassie blue
448 staining (Fig 4B). Load controls for hPARP3, corresponding to Ponceau membrane staining are
449 shown in (Fig 4B). Auto-MARylated hPARP3 without CoV *Macro1* domain served as a control for
450 zero hydrolysis. Bands intensities were quantified and fitted in nonlinear regression curves (Fig 4B,
451 lower panel). Both SARS-CoV-2 and SARS-CoV *Macro1* domains are able to remove almost
452 completely MAR from hPARP3 within 30 minutes. Substrate decay is more important in SARS-
453 CoV-2 (70-90% loss after 5-10 minutes) compared to SARS-CoV (30-70%) (Fig 4B, lower panel),
454 in accordance with recently reported data (Alhammad et al., 2021). The effect of *Macro1* domain
455 mutations on hPARP3 de-MARylation is intriguing. First, substitutions of the conserved D22 and
456 D23 by alanine in SARS-CoV-2 and SARS-CoV, added to the study as negative controls for

457 hydrolysis activity, abolish totally the de-MARylation activity, corroborating the crucial role of this
458 aspartic acid in the enzymatic activity. SARS-CoV-2 F156A mutant shows a complete loss in
459 hydrolysis activity for the first 10 min. MAR hydrolysis hardly reaches 40% of WT value after 30
460 min. Alanine substitution in SARS-CoV has also a negative effect on de-MARylation, as observed
461 for SARS-CoV N157A mutant. At 10 min the de-MARylation activity is reduced by 70% but not
462 completely abolished. Intriguingly, SARS-CoV N157F and SARS-CoV-2 F156N mutants behaved
463 as the mimicked parental *Macro1* domain. Hence, SARS-CoV N157F mutant shows an increased
464 hydrolysis activity similar to that of SARS-CoV-2; whereas SARS-CoV-2 F156N MAR hydrolysis
465 was impeded, reaching SARS-CoV values. It is interesting to note that in the study reported by
466 Tsika et al, the MAR hydrolysis activity, tested on MARylated PARP10 at a 1:1 substrate-enzyme
467 ratio, is slightly higher in SARS-CoV compared to SARS-CoV-2. However, the F156N mutation of
468 the later led to the shift of the activity toward SARS-CoV and MERS-CoV values (Tsika et al.,
469 2022), supporting the assumption that a F at that position has an impact on MAR-hydrolysis. The
470 exact catalysis mechanism of *Macro* domains is still under debate, confronting two possible modes
471 of action. One of which involves a water molecule, in the vicinity of the α -phosphate, acting as the
472 nucleophile. The second relaying on the involvement of a nucleophilic residue, H45 in the case of
473 SARA-CoV-2, mediating water molecule activation (Rack et al. 2016 and Correy et al. 2022).
474 Considering these results as well as available structural data, one can hypothesize that the aromatic
475 structure of phenylalanine in the hydrophobic cavity creates a stacking interaction with the adenine
476 moiety, thus stabilizing the distal ribose, undergoing the nucleophilic attack, in the active site
477 groove. This explanation might account for: (i) the improved activity observed for SARS-CoV-2
478 compared to SARS-CoV; and (ii) the detrimental effect on de-MARylation activity observed for
479 SARS-CoV-2 F156A mutant. Still, the SARS-CoV N157A mutant does not lose completely the
480 hydrolytic activity, suggesting that water molecule network might display significant differences
481 between the two viruses.

482 **3.5. Phenylalanine in the hydrophobic cavity stabilizes the** 483 ***Macro1* domain groove.**

484 To relate the observed activities to a possible modification of mutant proteins thermostability, CoV
485 *Macro1* domain sequences were subjected to the HoTMuSiC tool, to evaluate changes in melting
486 temperature under point mutations, on the basis of its experimental 3D structure (Pucci et al., 2020).
487 The obtained predictions, listed in Table 2, corroborate de-MARylation activity results. Thus,
488 SARS-CoV-2 F156N has a negative ΔT_m (-3.77) value, causing protein destabilization; while
489 SARS-CoV N157F displays a ΔT_m of +1.01, favoring thermostability. Alanine substitution at these
490 positions impacts negatively protein stability with computed ΔT_m values of - 3.39 and -0.49 for
491 SARS-CoV-2 F156A and SARS-CoV N157A, respectively. These data endorse that F156, in SARS-
492 CoV-2, and its corresponding N157 residue in SARS-CoV are key players in *Macro1* domain de-

493 MARYlation activity. Mutation of the key aspartate residue to alanine, leading to a complete loss in
 494 hydrolytic activity, has also a deleterious impact on protein stability with a ΔT_m of -2.35 and -1.93
 495 for SARS-CoV-2 D22A and SARS-CoV D23A *Macro1* domain, respectively (Table 2).

496
 497 Subsequently, to verify experimentally the impact of the studied mutations on *Macro1* domain
 498 stability, WT and mutant proteins were subjected to thermal shift assay in presence of increases
 499 concentrations of ADP-ribose. The data is compiled in the Fig 5. The experimental ΔT_m reported in
 500 the table 2 correspond to the values measured at the highest ADP-ribose concentrations in Fig 5.
 501 Thus, the F156N substitution in SARS-CoV-2 decreases the ΔT_m (-1.1K) compared to the WT. In
 502 the case of N157F in SARS-CoV, no significant change is observed. However, the ΔT_m are highly
 503 impaired in all alanine mutants. F156A exhibits a significant decay of ΔT_m ~(-4K) in SARS-CoV-2
 504 and ΔT_m (-3,4K) in the case of N157A in SARS-CoV. As expected, substitutions of aspartic acid by
 505 alanine drastically disrupted *Macro1* domain stability, supporting the importance of this residue in
 506 ADP-ribose stabilization through hydrogen bonding (Correy et al., 2022). In spite of the little
 507 differences between ΔT_m absolute values, the experimental data corroborate the HoTMuSiC
 508 predicted values.

509

510 **Table 2. Changes in melting temperature for CoV *Macro1* domain mutants determined by**
 511 **HoTMuSiC and TSA.**

512

CoV <i>Macro1</i> domain mutants	HoTMuSiC ΔT_m (K)	Experimental ΔT_m (K)
SARS-CoV-N157F	1.01	-0.3
SARS-CoV-2 F156N	-3.77	-1.1
SARS-CoV N157A	-0.39	-3.4
SARS-CoV-2 F156A	-3.39	-3.9
SARS-CoV D23A	-2.35	-4.5
SARS-CoV-2 D22A	-1.93	-5.1

513 ΔT_m (melting temperature), K (Kelvin)

514

515 Despite a high similarity in terms of sequence and function among viral *Macro* domains, notable
 516 differences in their affinity for ADP-ribose and catalytic activity indeed exist, affecting the state of
 517 ADP-ribosylation substrates in the cell. Multiple studies link viral *Macro* domain ADP-ribose
 518 hydrolase activity to viral pathogenesis. Mutations targeting residues D23, N41, H46 and G131 in
 519 SARS-CoV (Fig 1E) and equivalent positions in other RNA viruses, were shown to be deleterious
 520 for ADP-ribose binding/hydrolysis activity, PARP activation, and viral virulence (Egloff et al.,
 521 2006; Grunewald et al., 2019; Lin et al., 2020; McPherson et al., 2017; Michalska et al., 2020). As
 522 an example, in the case of SARS-CoV N41 catalytic residue, the *Macro1* domain mutant N41A is
 523 devoid of MAR-hydrolase activity and elicits more significant IFN, ISGs and pro-inflammatory
 524 cytokines production than the wild type virus in infected mice (Fehr et al., 2016). In MHV,

525 mutation of the D residue (D16, numbered D1329 in ORF1a polyprotein) to A has a severe impact
526 on virus replication and the mutant virus is defective in blocking IFN production (Grunewald et al.,
527 2019; Jankevicius et al., 2013). The phenomenon is emphasized when this mutation is combined to
528 the substitution N30A (numbered N1347A in ORF1a polyprotein). The latter corresponds to N40 in
529 SARS-CoV-2, involved in distal ribose interaction, through hydrogen bond formation with the C3'
530 hydroxyl of the ADP-ribose (Correy et al., 2022; Voth et al., 2021). A recent study reports the
531 impact of SARS-CoV-2 N40 mutation on PARP9 activation and IFN signaling (Russo et al., 2021),
532 highlighting the importance of this residue in immune escape *via* STAT signaling. The substitution
533 of D10A, G32E and G112E in CHIKV *Macro* domain, corresponding to D22, G48 and G130 in
534 SARS-CoV-2 *Macro1* domain, decreased the MAR-hydrolase activity. The mutants were unable to
535 produce viable CHIKV (McPherson et al., 2017). Also, the D22V mutation in SARS-CoV-2 and
536 D23A in SARS-CoV impedes its activity, stressing the crucial role of this residues in the hydrolysis
537 activity (Fehr et al., 2016; Rack et al., 2020). Former studies reported *Macro* domains with reduced
538 affinity for ADP-ribose retaining high MAR hydrolase activity or *vice versa*. Yet, studies on CHIKV
539 *Macro* domain showed that Y114 substitution, corresponding to F132 in SARS-CoV-2 and situated
540 in the stretch accommodating the diphosphate moiety of ADP-ribose, impaired MAR hydrolysis
541 activity but not ADP-ribose binding, hampering viral replication (McPherson et al., 2017). In
542 accordance with these results, mutagenesis of V133 and Y114 residues in other *Macro* domains,
543 corresponding to I131 and F132 and in SARS-CoV-2, also disrupted the hydrolysis activities
544 without affecting ADP-ribose binding (Jankevicius et al., 2013; Zhang et al., 2015). Moreover,
545 substitution of conserved Y126 by F in *E. coli Macro* domain containing YmdB protein,
546 corresponding to F132 in SARS-CoV-2, showed a higher hydrolysis activity, highlighting the
547 importance of the phenyl group for the catalytic activity (Zhang et al., 2015). Interestingly, F132L
548 replacement in SARS-CoV-2 impedes MAR hydrolysis activity, as in CHIKV, but F156L
549 substitution did not show a drastic effect on ADP-ribose hydrolysis from auto-ribosylated PARP14
550 WWE-CAT after 1 hour of reaction (Rack et al., 2020). In our hands, when the incubation is done
551 for one hour, no differences are observed, since all the ADP-ribose is quickly removed.

552

553 The change of Asparagine by phenylalanine in SARS-CoV-2 seems to enhance π - π stacking
554 interactions at the ADP-ribose site due to the flexibility of the aromatic side chain of this residue.
555 Recently, a number of identified compounds, targeting SARS-CoV-2 *Macro1* domain, were shown
556 to interact closely with F156 through π -stacking (Correy et al., 2022; Roy et al., 2022; Schuller et
557 al., 2021). Structural data highlighted the importance of F156N substitution in SARS-CoV-2 and
558 our experimental data brought some explanations on how this substitution is impacting the
559 hydrolysis activity of SARS-CoV and SARS-CoV-2 *Macro1* domains. Hence, the phenylalanine is a
560 key residue for stabilizing the protein, as well as an important residue to consider **for** the antiviral

561 design (Ni et al., 2021; Tsika et al., 2022). It is pointing out the importance of the hydrophobic
562 patch at that position in SARS-CoV-2, and highlights the fact that many questions remain regarding
563 the role of non-conserved and/or non-catalytic residues.

564

565 Thus, understanding how this residue and corresponding residues in other CoVs impact activity and
566 ultimately infection of the virus could contribute significantly to drug design of pan-coronavirus
567 *Macro1* domain inhibitors.

568

569 **4. Conclusions**

570 Finally, the importance of F156 residue in SARS-CoV-2 for MAR hydrolysis and ADP-ribose
571 binding was investigated experimentally. Mutational analysis conducted in the present paper shed
572 some light on functional differences in terms of ADP-ribose binding affinities and MAR hydrolysis
573 activities of SARS-CoV and of SARS-CoV-2 *Macro1* domains, boarding our knowledge on *Macro*
574 domains function, a pre-requisite for anti-viral drug design.

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599

600 **6. Declaration of competing interest**

601 The authors declare that they have no known competing financial interests or
602 personal relationships that could have appeared to influence the work reported in this
603 paper.

604 **7. Credit authorship contribution statement**

605 **Oney Ortega Granda:** Conceptualization, Investigation, Methodology, Software, Writing –
606 Original Draft, Review & Editing. **Karine Alvarez:** Resources, Writing – Review & Editing.
607 **Maria J. Mate-Perez:** Methodology, Writing – Review & Editing. **Bruno Canard:** Resources,
608 Writing – Review & Editing. **François Ferron:** Conceptualization, Investigation, Resources,
609 Software, Writing – Review & Editing. **Nadia Rabah:** Conceptualization, Investigation,
610 Methodology, Project Administration, Resources, Software, Writing – Original Draft, Writing –
611 Review & Editing.

612

613

614 **8. Figure captions**

615 **Fig 1. Sequence alignment and structure superposition of SARS-CoV with SARS-CoV-2**
616 ***Macro1* domain proteins.** (A) SeaView comparison of SARS-CoV and SARS-CoV-2 *Macro1*
617 domains. Secondary-structure elements are represented above the alignment, indicating the number
618 and position of β -sheets and α -helices. Residues with strict identity are marked in red box, whereas
619 residues considered as highly similar are dyed in red and framed in blue. Sequences and 3D
620 structure of SARS-CoV (PDB: 2FAV) and SARS-CoV-2 (PDB: 6WOJ) *Macro1* domains were
621 extracted from PDB. Superposition and the alignment were made with Chimera (Pettersen et al.,
622 2004) and visualized using the ESPript (Robert and Gouet, 2014). (B) SARS-CoV (blue) and
623 SARS-CoV-2 (green) structure superposition with ADP-ribose. The secondary structures are labeled
624 and ADP-ribose molecule is shown in yellow sticks with oxygens (red), nitrogens (blue) and water
625 (red spheres). (C) An expanded close-up view of the ADP-ribose binding groove superposition
626 highlights the amino acids with major divergence N157 in SARS-CoV and F156 in SARS-CoV-2.
627 ADP-ribose coordinates with their interacting amino acids in the binding cleft of SARS-CoV-2 (D)
628 and SARS-CoV (E). Chemical structure of ADP-ribose and corresponding amino acids are exposed
629 according to stick and balls model. Interaction of covalent bonds of ADP-ribose are shown in purple
630 and of amino acid residues in brown. Hydrogen bonds formed between residues and ligand are
631 represented as green dashed lines with the bond length as numeric numbers. Surrounding residues,
632 in the hydrophobic pocket, in contact with ADP-ribose, are displayed as red eyelash symbols.
633 Diagrams were generated by using LigPlot+ (Laskowski and Swindells, 2011).

634
635 **Fig 2. Expression and purification of recombinant SARS-CoV and SARS-CoV-2 *Macro1***
636 **domains.** (A) Western blot probing the expressed recombinant proteins insoluble (IF) and soluble
637 (SF) expression fraction. (B) SDS-PAGE of bacterially expressed SARS-CoV-2 *Macro1* domain
638 following IMAC purification (E1-9: elution fractions; W: wash; UB: unbound material). (C) SDS-
639 PAGE of SARS-CoV-2 *Macro1* domain elution fraction (GF1 to 5) after Gel filtration
640 chromatography.

641
642 **Fig 3. ADP-ribose binding to SARS-CoV and SARS-CoV-2 recombinant *Macro1* domains.** (A)
643 Upper Panel: Dot blot binding signal quantification of WT proteins using ImageJ software. Average
644 and standard deviation were computed using the GraphPad Prism program. Middle and lower
645 panels: Dot blot auto-PARylated hPARP1 binding to SARS-CoV and SARS-CoV-2 WT and mutant
646 recombinant proteins. BSA is used as a negative control for PAR binding. (B, C, D and E) ITC
647 analysis of SARS-CoV and SARS-CoV-2 WT and mutant proteins titrated with ADP-ribose. Top
648 panels show the heat variation upon injection as a function of time. Bottom panels represent the
649 curve of integrated raw data as a function of the molar concentration of injectant.

650 **Fig 4. de-PARylation and de-MARylation by CoV *Macro1* domains.** (A) Western blot of auto-
651 PARylated hPARP1 with SARS-CoV and SARS-CoV-2 *Macro1* domains. (B, upper panel) Western
652 blot with anti-MAR binding reagent, assessing MAR hydrolysis from hPARP3. Ponceau red
653 staining of WB membrane for hPARP3 protein load control. mPAGE Coomassie blue staining for
654 *Macro1* domain protein load control. The results are representative of three independent
655 experiments. (B, lower panel) Bands intensity were quantified using ImageJ software and fitted to
656 a nonlinear regression curve. Error bars indicate standard deviation.

657

658 **Fig 5. Effect of mutation on the thermal stability of SARS-CoV and SARS-CoV-2 *Macro1***
659 **domains.** Melting temperature profiles of SARS-CoV, SARS-CoV-2 WT and mutant *Macro1*
660 domains in presence of increasing concentrations of ADP-ribose. See Material and Methods for
661 experimental details. Error bars indicate standard deviation.

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Fig 2.

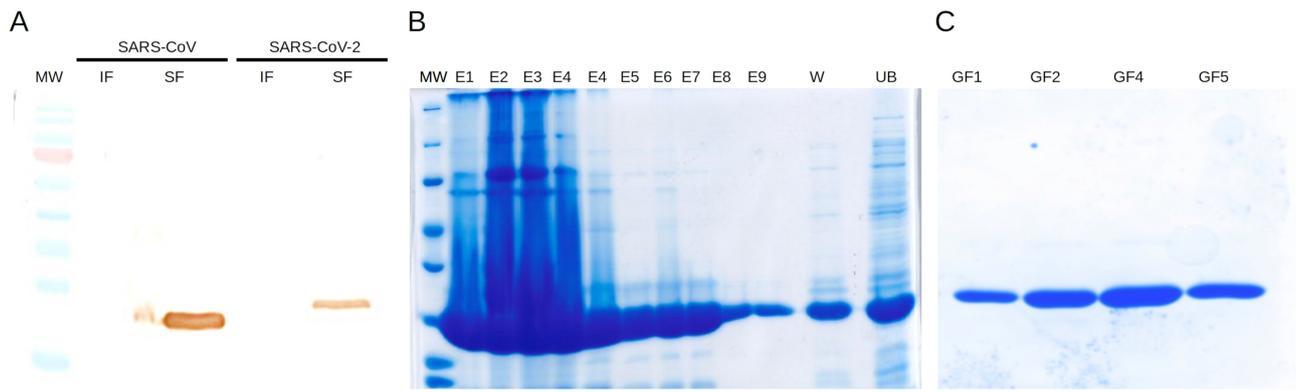


Fig 3.

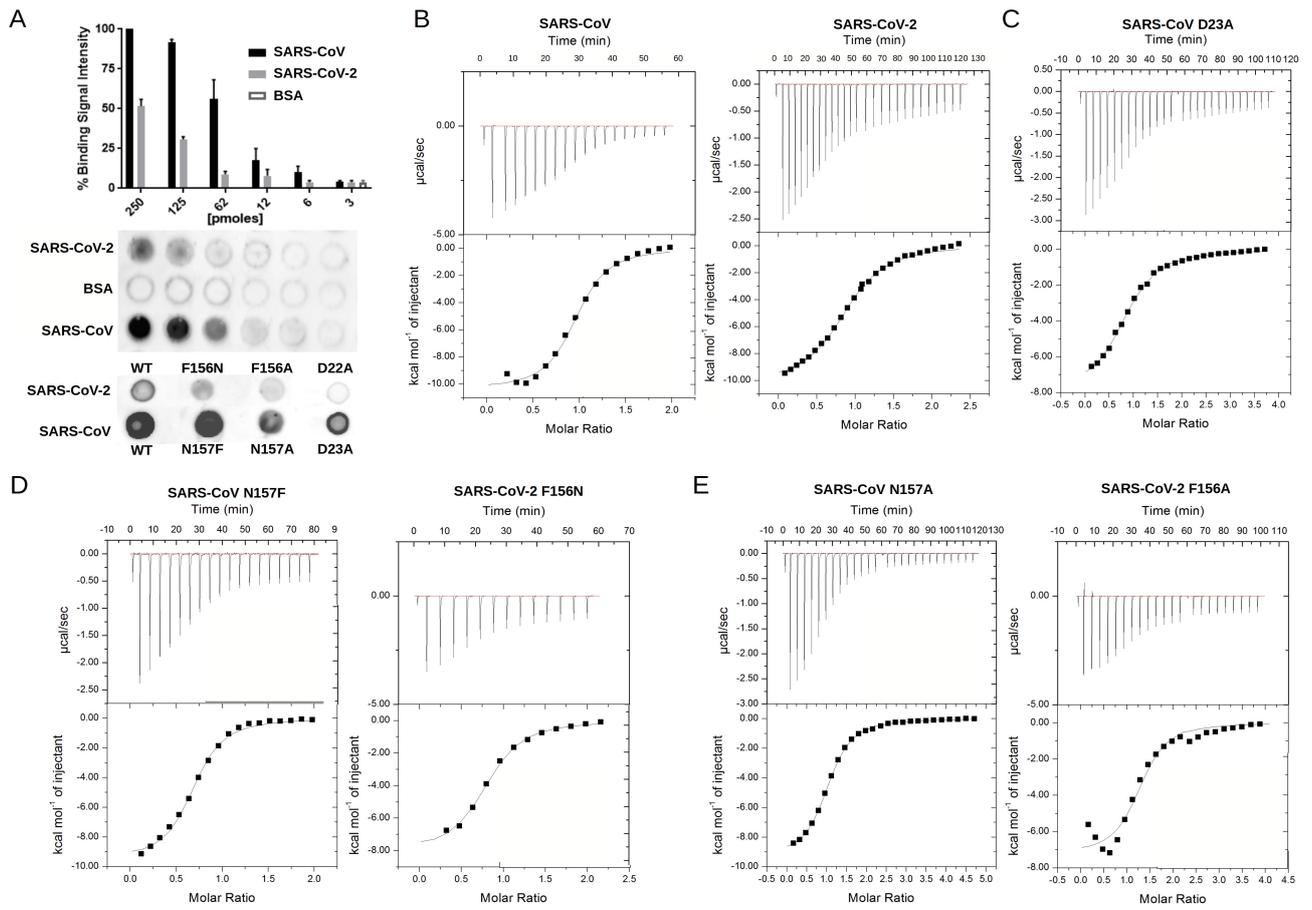


Fig 4.

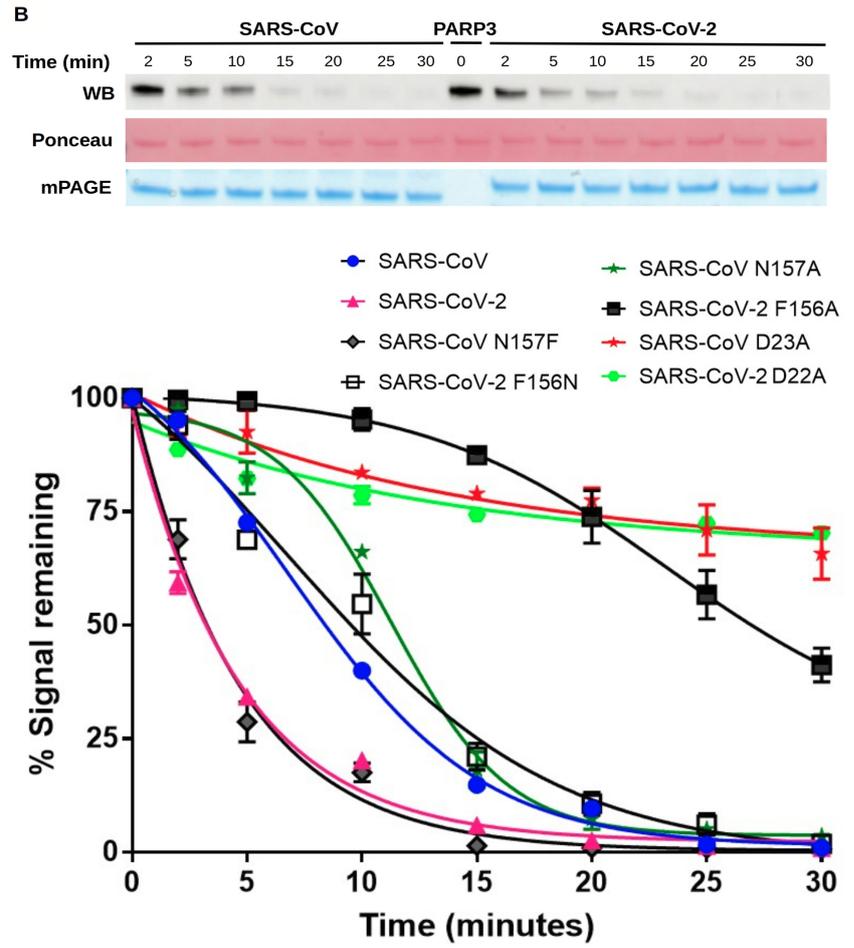
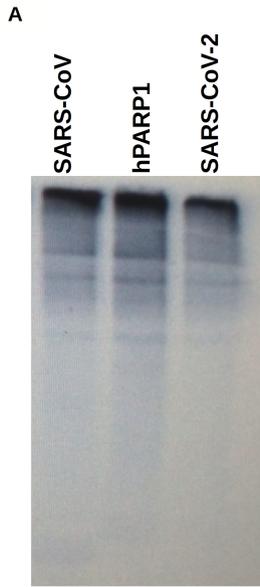
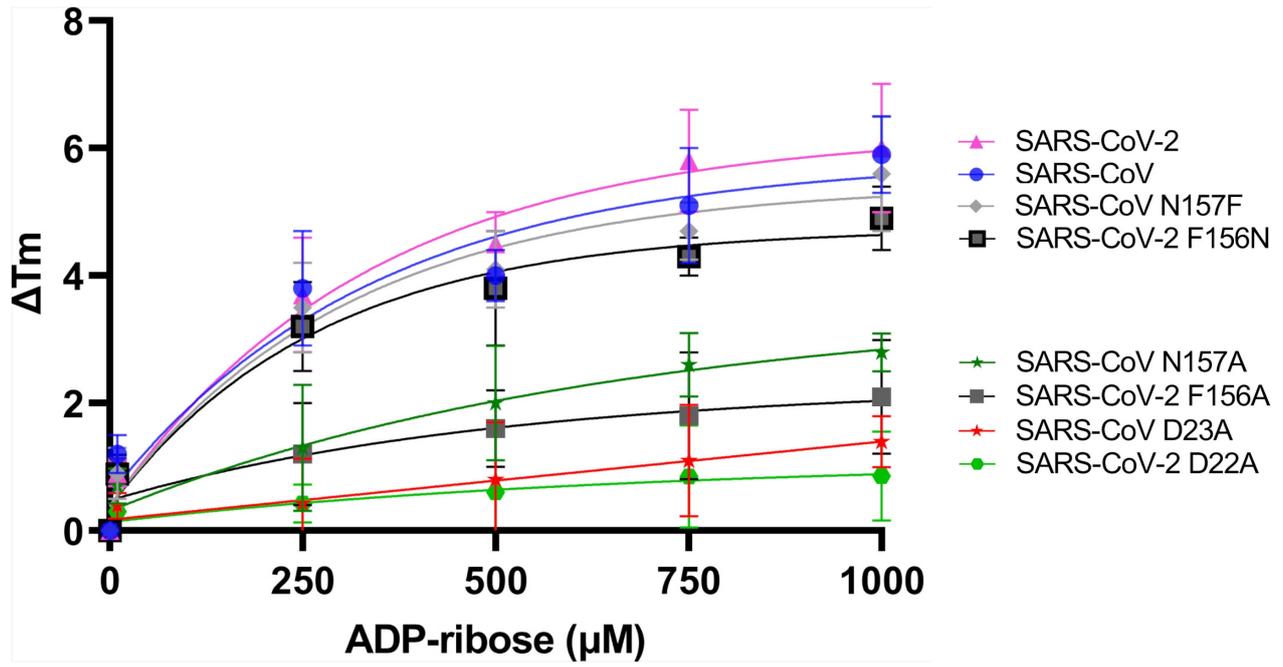


Fig 5.



688 Table S1. Primers template of CoV *Macro1* domains mutants.

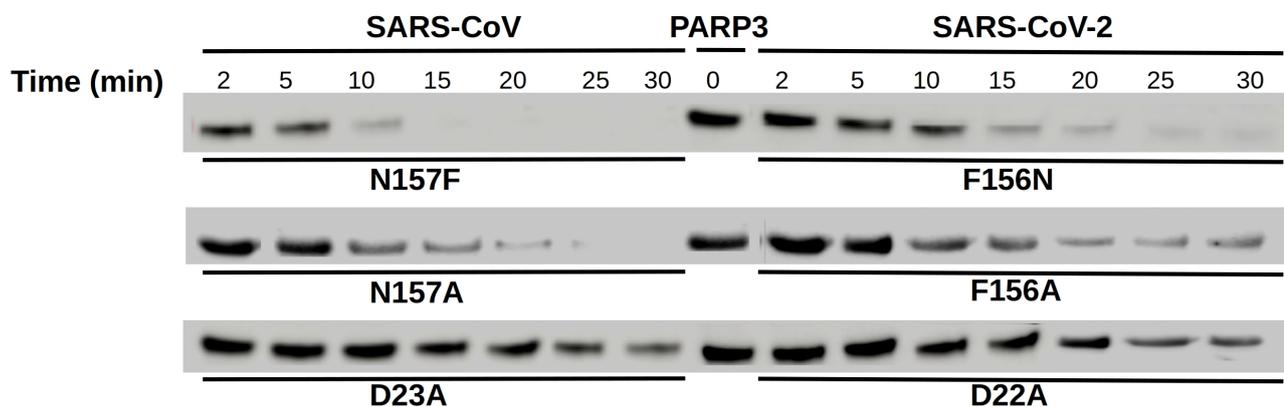
CoV <i>Macro1</i> domain mutants	Forward Primer Sequence (5' to 3')	Reverse Primer Sequence (5' to 3')
SARS-CoV-N157F	5'-ACCCAGGTTTACATCGCGGTGTTGATAA-3'	5'-AACCTGTTCTGACAGGGCTTTATCGAACACCG-3'
SARS-CoV-2 F156N	5'-GTACCAACGTGTACCTGGCGGTGAATGATAA-3'	5'-CTGGAGACTAATTTGTCATACAAAATTTTATCATTACCCG-3'
SARS-CoV N157A	5'-ACCCAGGTTTACATCGCGGTGGCCGATAA-3'	5'-AACCTGTTCTGACAGGGCTTTATCGGCCACCG-3'
SARS-CoV-2 F156A	5'-GTACCAACGTGTACCTGGCGGTGGCTGATAA-3'	5'-CTGGAGACTAATTTGTCATACAAAATTTTATCAGCCACCG-3'
SARS-CoV D23A	5'-GATAATGTCGCCATTAAGTGCGTAGCTATTGTG-3'	5'-CACT-CTGTGCTTCTTTACAATAGCTACGC-3'
SARS-CoV-2 D22A	5'-CAATGTGTACATAAAAATGCGGCCATTGTTG-3'	5'-CCTTTTTTGCTTCTTCAACAATGGCCGCATT-3'

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693 Figure S1. de-MARylation activity by CoV *Macro1* domains mutants. Western blot of auto-
 694 MARylated hPARP3 with SARS-CoV and SARS-CoV-2 *Macro1* domains mutants.