

1 **Host specific sensing of coronaviruses and picornaviruses by the CARD8 inflammasome**

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3 **Authors**

4 Brian V. Tsu¹, Rimjhim Agarwal¹, Nandan S. Gokhale³, Jessie Kulsuptrakul⁴, Andrew P. Ryan¹,
5 Lennice K. Castro¹, Christopher M. Beierschmitt¹, Elizabeth A. Turcotte⁵, Elizabeth J. Fay¹,
6 Russell E. Vance^{5,6}, Jennifer L. Hyde², Ram Savan³, Patrick S. Mitchell^{2*}, Matthew D.
7 Daugherty^{1*}

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9 **Affiliations:**

10 ¹Department of Molecular Biology, University of California, San Diego; La Jolla, CA, USA.

11 ²Department of Microbiology, University of Washington; Seattle, WA, USA.

12 ³Department of Immunology, University of Washington; Seattle, WA, USA.

13 ⁴Molecular and Cellular Biology Graduate Program, University of Washington; Seattle, WA,
14 USA.

15 ⁵Division of Immunology and Pathogenesis, University of California, Berkeley; Berkeley, CA,
16 USA.

17 ⁶Howard Hughes Medical Institute, University of California, Berkeley; Berkeley, CA, USA.

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19 *Corresponding authors. Matthew D. Daugherty (mddaugherty@ucsd.edu) and Patrick S.
20 Mitchell (psmitche@uw.edu)

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22 **Short title: Coronavirus and picornavirus proteases activate the CARD8 inflammasome**

23

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25 **viral proteases, coronaviruses, picornaviruses, SARS-CoV-2, COVID-19**

26

27 **Abstract**

28 Hosts have evolved diverse strategies to respond to microbial infections, including the detection
29 of pathogen-encoded proteases by inflammasome-forming sensors such as NLRP1 and CARD8.
30 Here, we find that the 3CL protease (3CL^{pro}) encoded by diverse coronaviruses, including
31 SARS-CoV-2, cleaves a rapidly evolving region of human CARD8 and activates a robust
32 inflammasome response. CARD8 is required for cell death and the release of pro-inflammatory
33 cytokines during SARS-CoV-2 infection. We further find that natural variation alters CARD8
34 sensing of 3CL^{pro}, including 3CL^{pro}-mediated antagonism rather than activation of megabat
35 CARD8. Likewise, we find that a single nucleotide polymorphism (SNP) in humans reduces
36 CARD8's ability to sense coronavirus 3CL^{pro}s, and instead enables sensing of 3C proteases
37 (3C^{pro}) from select picornaviruses. Our findings demonstrate that CARD8 is a broad sensor of
38 viral protease activities and suggests that CARD8 diversity contributes to inter- and intra-species
39 variation in inflammasome-mediated viral sensing and immunopathology.

40 **Introduction**

41 Effector-triggered immunity (ETI) is a host defense strategy by which innate immune sensors
42 recognize pathogens via the detection of pathogen-specific activities (1-5). A subset of
43 eukaryotic ETI sensors form inflammasomes—large, intracellular immune complexes that
44 activates a pro-inflammatory caspase, predominantly caspase-1 (CASP1), to initiate
45 inflammatory signaling via interleukin (IL)-1 β and IL-18 and pyroptotic cell death through
46 cleavage of the pore-forming protein Gasdermin D (GSDMD) (6-8). In humans, the activity of
47 viral proteases can be sensed by the inflammasome-forming sensors CARD8 and NLRP1 (9-12).
48 CARD8 is comprised of a disordered N-terminal region and a C-terminal function-to-fund
49 domain (FIIND) and caspase activation and recruitment domain (CARD). The FIIND undergoes
50 self-cleavage resulting in a bipartite sensor, with the disordered N-terminus acting as a ‘tripwire’
51 for viral proteases. For instance, proteolytic cleavage of CARD8 by the HIV-1 protease (HIV-
52 1^{pro}) leads to proteasome-dependent ‘functional degradation’ (13, 14) of the cleaved N-terminus
53 and release of the bioactive CARD-containing C-terminus, which is sufficient for inflammasome
54 assembly and activation (12). However, the extent to which CARD8 has evolved to sense other
55 viral proteases and functions as an innate immune sensor of viral infection has been unclear.

56

57 **The SARS-CoV-2 3CL^{pro} activates the human CARD8 inflammasome via proteolysis** 58 **within the disordered N-terminus**

59 To determine if the CARD8 inflammasome can sense coronavirus infection by mimicking sites
60 of viral polyprotein cleavage, we expanded our previous bioinformatic approach (11) to generate
61 a predictive model for the *Coronaviridae* main protease (3CL^{pro}, also known as nsp5 or M
62 protease), which has been shown to cleave host proteins in addition to the coronavirus

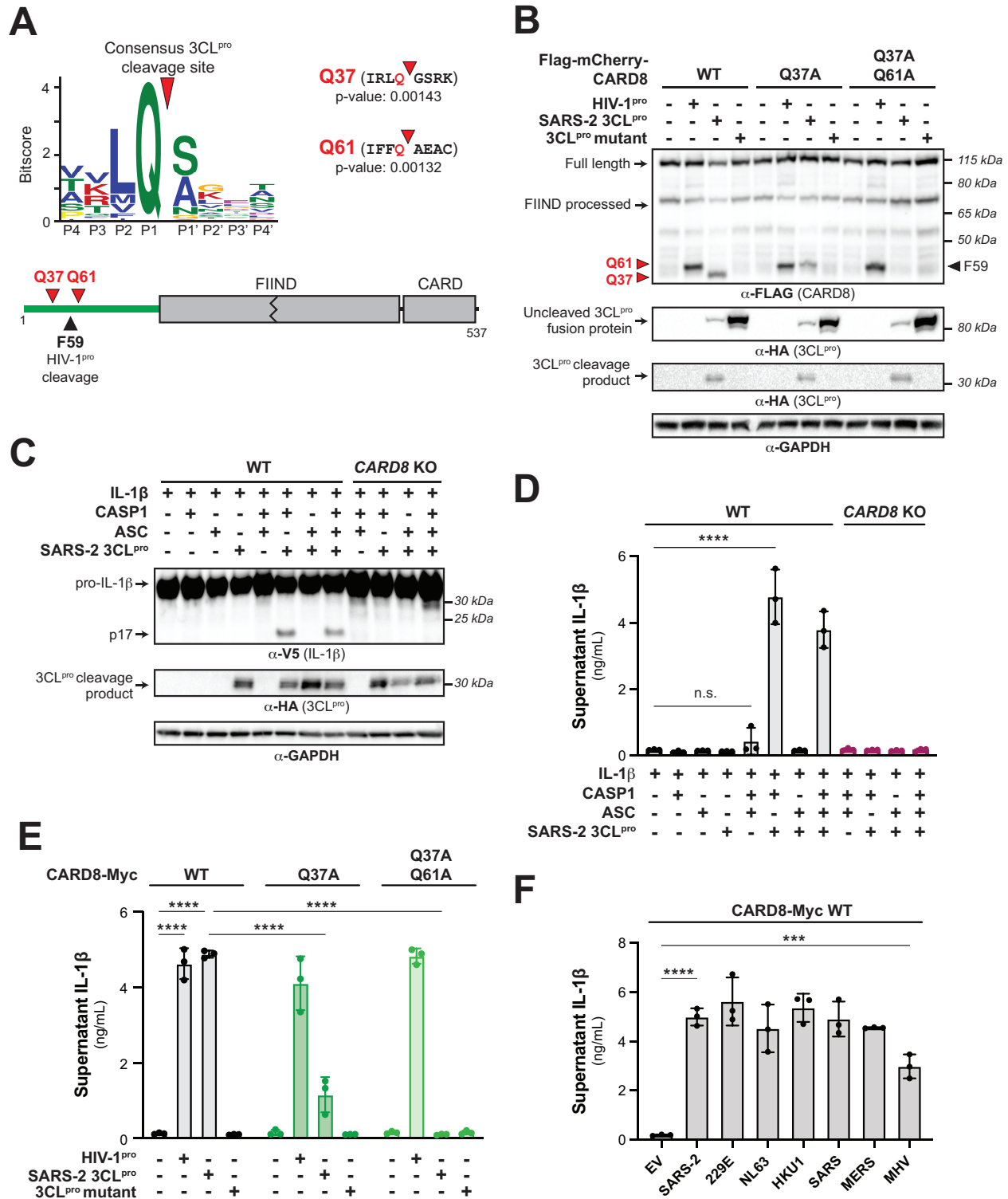
63 polyprotein (15-19). The resulting 3CL^{pro} cleavage motif, XXΦQ[G/A/S]XXX (where Φ denotes
64 a hydrophobic residue and X denotes any amino acid) (**Fig 1A, S1 Fig, S1 and S2 Tables**) is
65 broadly consistent with previous studies (20-23), and allowed us to predict two putative 3CL^{pro}
66 cleavage sites within the N-terminus of human CARD8 (**Fig 1A**).

67

68 To determine if human CARD8 is cleaved by coronavirus 3CL^{pro}, we co-expressed an N-
69 terminal 3xFlag/mCherry-tagged isoform of human CARD8 (wild-type (WT)) with 3CL^{pro} from
70 SARS-CoV-2 in HEK293T cells (**Fig 1B and S2 Fig**). We used HIV-1^{pro} as a positive control
71 since it had previously been shown to cleave CARD8 between residues F59-F60 (site F59 in **Fig**
72 **1, A and B**). We observed a ~34kDa CARD8 product in the presence of SARS-CoV-2 3CL^{pro}
73 but not the catalytically inactive C145A 3CL^{pro} mutant. The ~34kDa product is predicted to
74 result from cleavage at site Q37 (**Fig 1A**), which migrated slightly below the cleavage product of
75 HIV-1^{pro}. Mutating the putative P1 residue in the Q37 site (CARD8 Q37A) eliminated the 34kDa
76 product, confirming SARS-CoV-2 3CL^{pro} cleavage at this site. The CARD8 Q37A mutant also
77 revealed a cryptic ~37kDa 3CL^{pro}-dependent product, which matches cleavage at the predicted
78 site Q61 (**Fig 1A and S2 Fig**). The CARD8 Q37A Q61A mutant was completely insensitive to
79 cleavage by SARS-CoV-2 3CL^{pro} (**Fig 1B**), whereas cleavage by the HIV-1^{pro} was unperturbed
80 by either mutant. Thus, CARD8 can be cleaved by SARS-CoV-2 3CL^{pro} at amino acid sequences
81 that mimic the coronavirus polyprotein cleavage site.

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83 For both NLRP1 and CARD8, N-terminal proteolytic cleavage can activate CASP1 in a
84 reconstituted inflammasome assay (10-12, 14, 24). Consistent with the prior observation that
85 CARD8 is endogenously expressed in some HEK293T cell lines (12), transfection of our



86

87 **Fig 1. 3CL^{pro} from SARS-CoV-2 and other coronaviruses cleaves and activates the human**
 88 **CARD8 inflammasome.** (A) A consensus betacoronavirus 3CL^{pro} cleavage motif (*upper panel*,
 89 Fig S1 and Methods) was used to predict two 3CL^{pro} cleavage sites (red triangles) within the
 90 disordered 'tripwire' N-terminus of human CARD8 (*lower panel*, green) near the described site

91 of HIV-1^{pro} cleavage (black triangle). Flanking residues and p-values of prediction for each site
92 (Q37 and Q61) are shown. (B) HEK293T cells were transfected with the indicated CARD8
93 construct in the presence ('+') or absence ('-') of indicated proteases. Active (SARS-2 3CL^{pro}) or
94 catalytically inactive (3CL^{pro} mutant) protease from SARS-CoV-2 was expressed as an HA-
95 tagged fusion construct (Fig S2). HIV-1^{pro} was expressed from an untagged gag-pol construct.
96 Triangles are as described in (A). (C-D) WT or *CARD8* KO HEK293T cells were transfected
97 with ('+') or without ('-') indicated constructs. Inflammasome activation was monitored by
98 immunoblotting for mature IL-1 β (p17) (C) or measuring culture supernatant levels of bioactive
99 IL-1 β using IL1R-expressing reporter cells (D). (E-F) *CARD8* KO HEK293T cells were co-
100 transfected with the indicated CARD8 and protease constructs and supernatant levels of
101 bioactive IL-1 β were measured by IL1R reporter assay. 3CL^{pro}s from the following viruses were
102 used: HCoV-229E (229E), HCoV-NL63 (NL63), HCoV-HKU1 (HKU1), SARS-CoV (SARS),
103 MERS-CoV (MERS), mouse hepatitis virus (MHV). (D-F) Individual values (n=3), averages,
104 and standard deviations shown are representative of experiments performed in triplicate. Data
105 were analyzed using two-way ANOVA with Šidák's post-test (D-E) or one-way ANOVA with
106 Tukey's post-test (F). *** = p<0.001, **** = p<0.0001, n.s. = not significant.
107

108 HEK293T cells with only CASP1, pro-IL-1 β , and SARS-CoV-2 3CL^{pro} resulted in robust
109 CASP1-dependent processing of pro-IL-1 β to mature bioactive IL-1 β (p17) as measured by
110 immunoblot or IL-1 β reporter assay (see **Methods**) (**Fig 1, C and D**). Inflammasome activation
111 was not observed in cells in *CARD8* knock out (KO) cells.

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113 For both NLRP1 and *CARD8*, N-terminal proteolytic cleavage can activate CASP1 in a
114 reconstituted inflammasome assay (10-12, 14, 24). Validating a prior observation that *CARD8* is
115 endogenously expressed in some HEK293T cell lines (12), transfection of our HEK293T cells
116 with CASP1, pro-IL-1 β , and SARS-CoV-2 3CL^{pro} resulted in robust CASP1-dependent
117 processing of pro-IL-1 β to mature bioactive IL-1 β (p17) as measured by immunoblot or IL-1 β
118 reporter assay (**Fig 1, C and D**). We found that inflammasome activation was *CARD8*-
119 dependent since we did not observe CASP1-processed IL-1 β in HEK293T *CARD8* knock out
120 (KO) cells. To confirm that SARS-CoV-2 3CL^{pro} cleavage of *CARD8* is responsible for
121 inflammasome activation, we complemented *CARD8* KO cells with WT *CARD8* or cleavage site
122 mutants. Complementation with WT *CARD8* rescued both HIV-1^{pro} and 3CL^{pro}-induced
123 inflammasome activation (**Fig 1E and S3 Fig**). In contrast, whereas *CARD8* cleavage site
124 mutants had no effect on HIV-1^{pro}-induced inflammasome activation, 3CL^{pro}-induced
125 inflammasome activation was reduced or abolished in *CARD8* KO cells complemented with
126 *CARD8* Q37A or *CARD8* Q37A Q61A, respectively (**Fig 1E and S3 Fig**). These results validate
127 that 3CL^{pro} site-specific cleavage is required for *CARD8* inflammasome activation. As expected,
128 3CL^{pro} inflammasome activation did not occur in *CARD8* KO cells complemented with the
129 *CARD8* FIIND auto-processing mutant S297A mutant (**S4 Fig**) (12, 25, 26). Taken together, our

130 results indicate that human CARD8 senses the proteolytic activity of the SARS-CoV-2 3CL^{pro},
131 which drives inflammasome activation via functional degradation.

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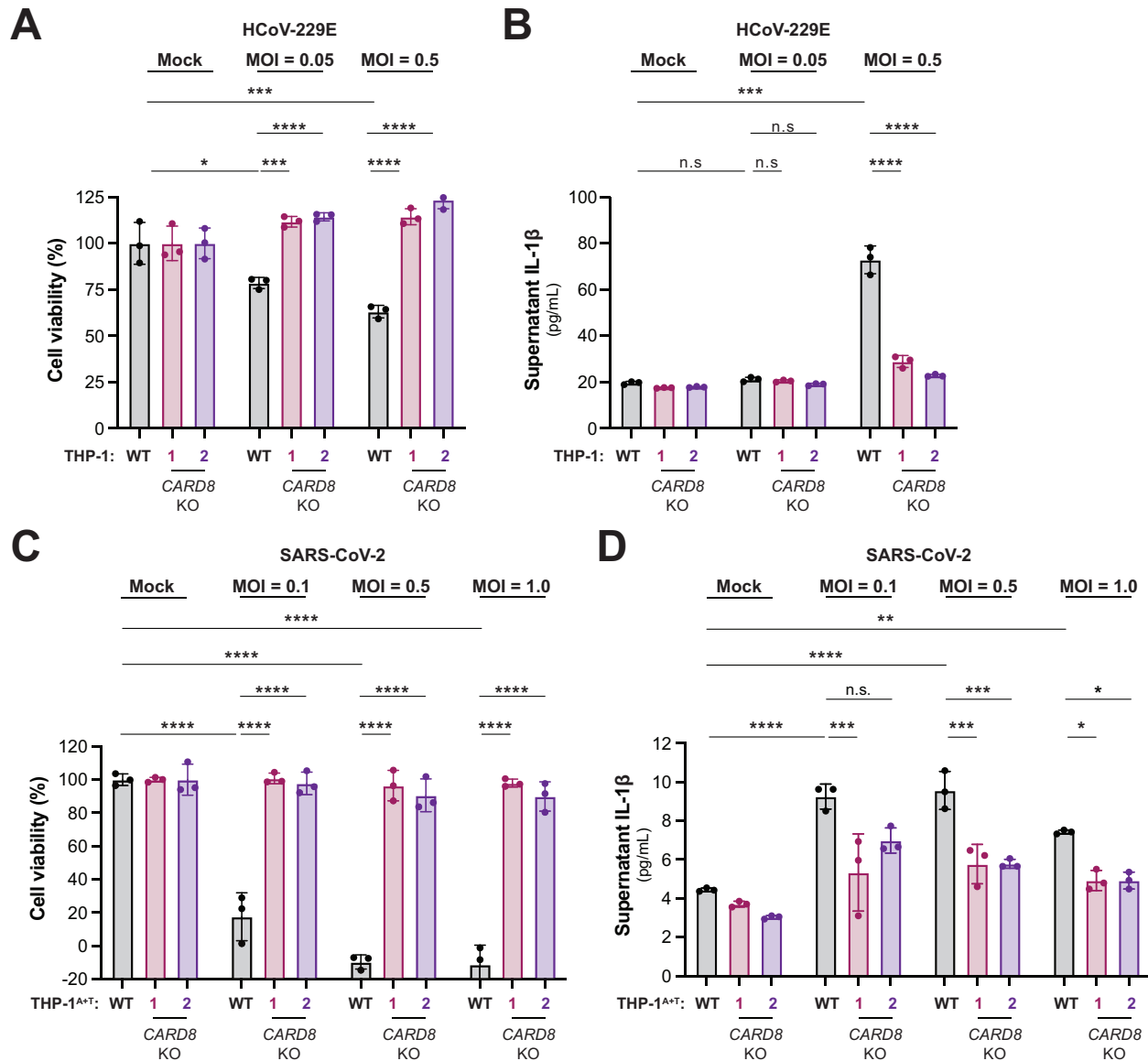
133 To test if proteases from other coronaviruses also cleave CARD8, we cloned 3CL^{proS} from other
134 human-relevant beta-coronaviruses (SARS-CoV (SARS), MERS-CoV (MERS), HCoV-HKU1
135 (HKU1), two human alpha-coronaviruses (HCoV-229E (229E) and HCoV-NL63 (NL63), and
136 the mouse beta-coronavirus murine hepatitis virus (MHV) (**S5 Fig**). Consistent with their
137 structural and cleavage motif similarity (27), we found that every tested 3CL^{pro} was able to
138 cleave and activate CARD8 in a site-specific manner (**Fig 1F and S6 Fig**). Thus, effector-
139 triggered immunity by human CARD8 is a conserved pathway for sensing of both endemic and
140 pandemic human coronaviruses.

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142 **Coronavirus infection activates the CARD8 inflammasome**

143 We next evaluated the consequences of CARD8 inflammasome activation by infecting the
144 human monocyte-like cell line THP-1 with HCoV-229E. We found that HCoV-229E infected
145 WT but not two independently-derived *CARD8* KO THP-1 cell lines underwent significant cell
146 death (**Fig 2A**) and release of IL-1 β (**Fig 2B**), a result similar to treatment with Val-boroPro
147 (VbP), which specifically activates the CARD8 inflammasome in myeloid and lymphoid
148 lineages (26, 28) (**S7 Fig**). SARS-CoV-2 infection (29, 30) or uptake via Fc γ R-mediated
149 antibody-dependent enhancement (31) by monocytes or macrophages induces inflammasome
150 activation. To determine if CARD8 senses and responds to SARS-CoV-2 infection in THP-1
151 cells, we engineered WT or *CARD8* KO THP-1 cells to express ACE2 and TMPRSS2 (THP-
152 1^{A+T}). As with HCoV-229E, we found that SARS-CoV-2 infection of THP-1^{A+T} WT but not

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Fig 2. Coronavirus infection activates the CARD8 inflammasome in THP-1 cells. WT, *CARD8* KO1, and *CARD8* KO2 THP-1 cells (A-B) or THP-1 cells expressing ACE2 and TMPRSS2 (THP-1^{T+A}) (C- D) were primed with 0.5 µg/mL Pam3CSK4 for 6h, followed by infection with the coronaviruses hCoV-229E (A-B) or SARS-CoV-2 (SARS-2) (C-D) at the indicated multiplicity of infection (MOI). 48h post-infection, cell viability (A, C) was measured using the Cell Titer Glo assay and IL-1β levels were measured using the IL1R reporter assay (B, D) as in Fig 1D. Data presented are representative of experiments performed at least twice. Data were analyzed using two-way ANOVA with Šidák's post-test: * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, n.s. = non-significant.

164 *CARD8* KO cells induced both cell death and IL-1 β release (**Fig 2, C and D**). Our results
165 demonstrate that *CARD8* is a bona fide innate immune sensor of viral infection.

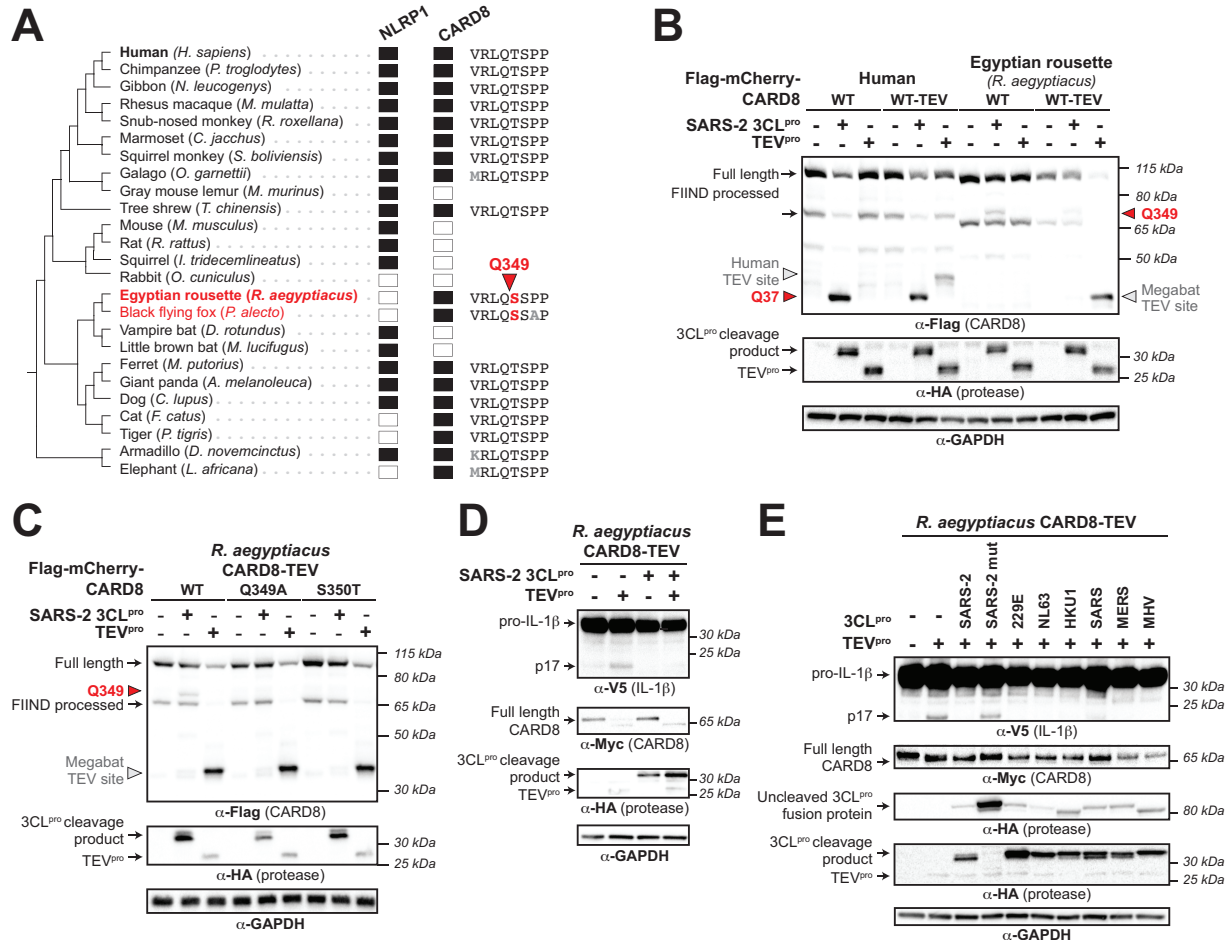
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167 **Inter- and intra-host diversity in *CARD8* impacts inflammatory responses to coronavirus**
168 **proteases**

169 Host-virus interactions, including those between viral proteases and host cleavage targets, are
170 often engaged in evolutionary arms races that shape the specificity of host-virus interactions (15,
171 32-34). Indeed, we have previously shown that the *CARD8* homolog and viral protease sensor,
172 *NLRP1*, has been duplicated and recurrently lost across mammalian evolution. *NLRP1* also has
173 strong signatures of positive selection in an N-terminal region of the protein that is cleaved by
174 pathogen-encoded proteases, which we refer to as the ‘tripwire’ region due to its role in virus
175 detection and subsequent inflammasome activation (11, 24). We thus predicted that *CARD8* may
176 have a similarly dynamic evolutionary history, and that host inter- and intraspecies variation
177 would underlie differences in *CARD8* cleavage and inflammasome activation by coronavirus
178 3CL^{pro}.

179

180 We first found that both *CARD8* and *NLRP1* are each present in only certain mammalian
181 lineages, consistent with their dynamic roles in host defense as opposed to dedicated
182 housekeeping functions (**Fig 3A**). For instance, we found that within the order *Chiroptera* (bats),
183 microbats retain a *NLRP1* ortholog but have lost *CARD8*, whereas megabats have lost *NLRP1*
184 and only encode *CARD8*. Because bats serve as main reservoir hosts of emerging coronaviruses
185 (35, 36), but is missing from microbats, we tested if the *CARD8* inflammasome could serve as a
186 sensor for 3CL^{pro} in the megabat species *Rousettus aegyptiacus*. Unlike human *CARD8*, the *R.*



187

188 **Fig 3. Megabat CARD8 is antagonized rather than activated by coronavirus 3CL^{pro}.** (A)

189 Presence (filled rectangle) or absence (empty rectangle) of predicted *NLRP1* or *CARD8*

190 orthologs in the indicated mammalian species. To the left is a species phylogeny. Megabat

191 species are indicated in red. To the right is an alignment of a predicted 3CL^{pro} cleavage site in

192 *Roussetus aegyptiacus* CARD8 (red triangle indicates site and number indicates residue

193 position). (B) Human CARD8 or *R. aegyptiacus* CARD8 was co-transfected with either SARS-

194 CoV-2 (SARS-2) 3CL^{pro} or protease from tobacco etch virus (TEV^{pro}). For human or *R.*

195 *aegyptiacus* CARD8 constructs labeled “WT-TEV”, a TEV^{pro} site was introduced into the N-

196 terminus. The red triangles and amino acid number indicates the sites of 3CL^{pro} cleavage in

197 human and *R. aegyptiacus* CARD8. The gray triangles indicate the sites of TEV protease

198 cleavage within each CARD8 WT-TEV. (C) Mapping of the 3CL^{pro} site within *R. aegyptiacus*

199 CARD8 was performed by transfecting the indicated point mutants with SARS-CoV-2 (SARS-2)

200 3CL^{pro} or TEV^{pro}. 3CL^{pro} and TEV^{pro} sites are marked by triangles as in (B). (D) *CARD8* KO

201 HEK293T cells were co-transfected with *R. aegyptiacus* IL-1 β and CASP1, along with the

202 indicated CARD8 and protease constructs. Presence of mature IL-1 β (p17) upon TEV^{pro} addition

203 indicates successful reconstitution of the *R. aegyptiacus* CARD8 inflammasome, whereas

204 absence of p17 upon SARS-2 3CL^{pro} indicates antagonism of the *R. aegyptiacus* CARD8

205 inflammasome. (E) *R. aegyptiacus* CARD8 inflammasome activation assays were performed as

206 in (D) with the indicated 3CL^{pro} constructs.

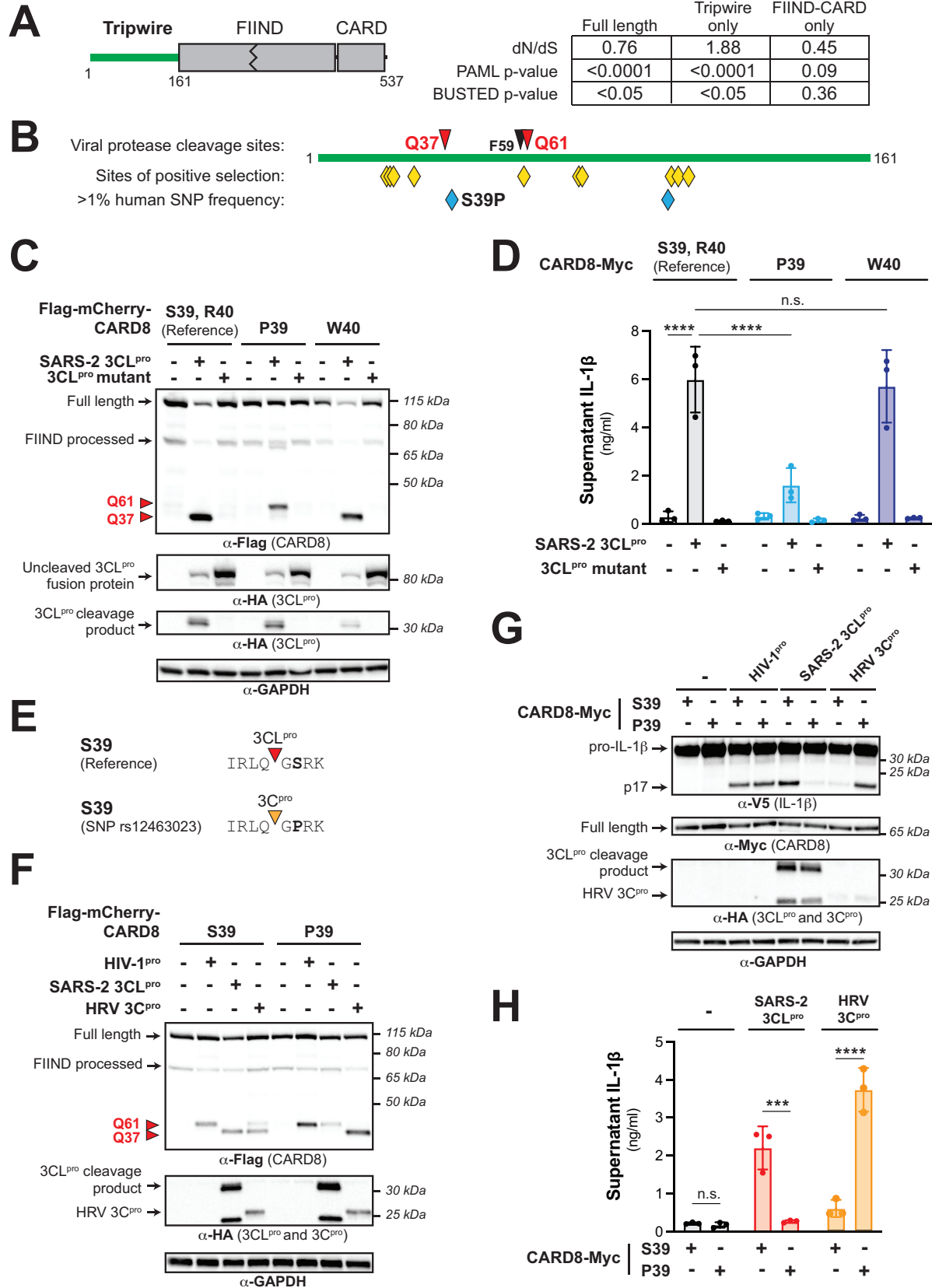
207 *aegyptiacus* CARD8 (CARD8_{Ra}) N-terminus lacks sites Q37 and Q61 and was not cleaved by
208 SARS-CoV-2 3CL^{pro}. We did, however, observe a cleavage product that matched a predicted
209 3CL^{pro} site at Q349 in CARD8_{Ra} downstream of the FIIND auto-processing site in the
210 inflammasome-forming C-terminus (**Fig 3, A and B**). Interestingly, megabats are the only
211 mammals with a serine in the P1' position of the cleavage site (**Fig 3A and S8 Fig**), which is
212 preferred for cleavage based on our computational model (**Fig 1A**). Indeed, a threonine in this
213 position (S350T), which is found in human CARD8 as well as most other mammals, prevents
214 cleavage of CARD8_{Ra} by SARS-CoV-2 3CL^{pro} (**Fig 3C and S8 Fig**). We next wished to test the
215 functional effect of 3CL^{pro} on the megabat CARD8 inflammasome. First, we determined if
216 functional degradation could activate megabat CARD8 by inserting a TEV^{pro} cleavage site into
217 the N-terminus of CARD8_{Ra}, permitting TEV^{pro} cleavage of CARD8_{Ra}-TEV but not WT
218 CARD8_{Ra}. When co-expressed with CASP1 and pro-IL-1 β from *R. aegyptiacus*, TEV^{pro}
219 cleavage of CARD8_{Ra}-TEV resulted in inflammasome activation, indicating that we can
220 reconstitute the *R. aegyptiacus* CARD8 inflammasome in human cells (**Fig 3D**). Using this
221 reconstitution system, we found that SARS-CoV-2 3CL^{pro} does not activate, and in fact
222 antagonizes TEV-mediated CARD8_{Ra} inflammasome activation (**Fig 3D**), similar to our previous
223 observations of viral proteases that antagonize the activation of the NLRP1 inflammasome (11).
224 We further found that all 3CL^{pro}s that we tested can instead prevent TEV^{pro}-mediated activation
225 of the CARD8_{Ra} inflammasome (**Fig 3E**). Together with the loss of CARD8 from many bat
226 species, these data provide a putative mechanism of disease tolerance that protects bats from
227 immunopathogenic effects of inflammasome activation.

228

229 We next focused on the evolution of human *CARD8*. Supporting a previous genome-wide study
230 (37), we found evidence that *CARD8* has evolved under recurrent positive selection in hominoids
231 and Old World monkeys, which we find is primarily driven by the N-terminal region of the
232 protein (**Fig 4A**). Codon-based analyses also show that positively selected sites are
233 predominantly found in the N-terminus, including a codon at position 60 that lies in the HIV-1^{pro}
234 site and the secondary coronavirus 3CL^{pro} site (**Fig 4B, S9 Fig, and S5 Table**). We thus infer
235 that, like NLRP1, the *CARD8* disordered N-terminus is a molecular ‘tripwire’ that is rapidly
236 evolving to mimic viral polyprotein sites and sense diverse viral proteases. We further analyzed
237 the human population for non-synonymous SNPs in *CARD8* (**Fig 4B**). Within the N-terminus,
238 we found several high frequency human SNPs, including a S39P variant that resides at the P2’
239 position within the 3CL^{pro} cleavage site and is present in >20% of all sampled African and
240 African American individuals (GnomAD v3.1.2 (38)) (**S9 Fig and S5 and S6 Tables**).
241 Strikingly, while *CARD8* S39 and P39 variants are similarly cleaved and activated by HIV-1^{pro},
242 the *CARD8* P39 variant exhibits reduced sensitivity to cleavage and activation by coronavirus
243 3CL^{pro}s (**Fig 4, C and D, and S10 Fig**). This is reinforced by our observation that a proline in the
244 P2’ position is never found in the >10,000 polyprotein cleavage sites we sampled from beta-
245 CoVs (**S1 Table**). In contrast, another human SNP (R40W) found in approximately 1 in every
246 2000 alleles (**S5 Table**), does not detectably affect *CARD8* cleavage in our assays (**Fig 4, C and**
247 **D**). These data suggest that standing genetic variation in human *CARD8* underlies differential
248 sensing and inflammasome responses to coronavirus infection.

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250



252 **Fig 4. Human polymorphism in CARD8 reduces sensing of coronavirus 3CL^{pro} while**
253 **increasing sensing of select picornavirus 3C^{pro}s.** (A) Evolutionary analyses of positive
254 selection were performed on full length *CARD8* (encoding residues 1-537), the disordered N-
255 terminal ‘tripwire’ region (encoding residues 1-161), and the FIIND-CARD region (encoding
256 residues 162-537). P-values from PAML and BUSTED analyses are shown, along with the
257 dN/dS value obtained from PAML. (B) Schematic of the *CARD8* ‘tripwire’ region. Red and
258 black triangles and amino acid numbers indicate sites of 3CL^{pro} and HIV-1^{pro} cleavage
259 respectively. Yellow diamonds indicate codons predicted to be evolving under positive selection
260 by at least one evolutionary analysis (table S4). Blue diamonds indicate high frequency (>1%
261 allele frequency) non-synonymous single nucleotide polymorphisms (SNPs) in humans (table S5
262 and S6). The position of the S39P substitution that results from SNP rs12463023 is shown. (C-D)
263 Reference human *CARD8* (S39, R40) or human *CARD8* variants (P39 or W40) were co-
264 expressed with the indicated protease construct and assayed for 3CL^{pro}-mediated cleavage (C) or
265 *CARD8* inflammasome activation (D). (E) Amino acid sequence surrounding the Q37 cleavage
266 site for S39 (reference) and P39 variants of human *CARD8*. Triangles mark sites of cleavage by
267 the indicated viral protease (F-H). Human *CARD8* S39 or *CARD8* P39 were transfected with the
268 indicated proteases and assayed for 3CL^{pro}-mediated cleavage (F) or *CARD8* inflammasome-
269 mediated maturation of IL-1 β (G) or the release of bioactive IL-1 β (H). HRV 3C^{pro} = human
270 rhinovirus 3C^{pro}. Individual values (n=3), averages, and standard deviations shown are
271 representative of experiments performed in triplicate. Data were analyzed using two-way
272 ANOVA with Šidák's post-test. *** = p<0.001, **** = p<0.0001, n.s. = not significant.

273 **A human SNP confers a specificity switch for CARD8 sensing of coronavirus 3CL^{pro} and**
274 **human rhinovirus 3C^{pro}**

275 Finally, we considered if the CARD8 P39 variant, in addition to affecting sensing of coronavirus
276 3CL^{pro}s, also alters recognition of other pathogens. We noticed that although a P2' proline is
277 disfavored in our model for 3CL^{pro} cleavage, a P2' proline is strongly preferred in our model for
278 enterovirus 3C^{pro} cleavage (11). Indeed, our 3C^{pro} motif search (11) identified a cleavage site in
279 CARD8 P39 but not CARD8 S39 (**Fig 4E and S11 Fig**). Validating these bioinformatic
280 predictions, cleavage assays with the 3C^{pro} from the respiratory picornavirus, human rhinovirus
281 (HRV), revealed that HRV 3C^{pro} cleavage of human CARD8 at site Q37 is considerably more
282 pronounced for the P39 variant than the S39 variant (**Fig 4F**). Likewise, we found that
283 inflammasome activation by HRV 3C^{pro} was nearly absent in HEK293T *CARD8* KO cells
284 complemented with CARD8 S39, whereas we observed robust inflammasome activation in cells
285 complemented with CARD8 P39 – the opposite sensitivity observed for SARS-CoV-2 3CL^{pro}
286 (**Fig 4, G and H**). Finally, we found that CARD8 variation also impacted cleavage by other
287 picornavirus 3C^{pro}s. For example, 3C^{pro}s from enterovirus D68 (EV68) and poliovirus (PV1) were
288 better sensed by CARD8 P39, whereas the Aichi virus 3C^{pro} was better sensed by the S39
289 CARD8 variant (**S12 Fig**). Thus, a single amino acid change in CARD8 functions as a viral-
290 specificity switch, underscoring the importance of pathogen-driven evolution in shaping
291 inflammasome responses.

292

293 **Discussion**

294 As is clear from the ongoing COVID-19 pandemic, understanding the molecular mechanisms
295 that drive viral sensing and inflammatory pathogenesis during infection remains key to

296 developing rationalized, host-directed treatments to support antiviral defense or quell severe
297 disease. CARD8 is expressed in airway epithelia (10, 39, 40) and other cell types, including
298 monocytes and T cells (26, 28, 41) that are physiologically relevant for respiratory coronaviruses
299 and picornaviruses (39, 40, 42), including SARS-CoV-2 (29-31). Our data showing that SARS-
300 CoV-2 infection activates the CARD8 inflammasome in THP-1 cells supports findings that
301 inflammasome activation contributes to severe COVID-19, and suggests that the CARD8
302 inflammasome in monocytes and macrophages contribute to inflammation in COVID-19
303 patients.

304

305 Based on our results and the finding that FcγR-mediated uptake of SARS-CoV-2 virions into
306 monocytes leads to abortive infection (31), we speculate that CARD8-dependent pyroptosis
307 contributes to the poor permissiveness of myeloid cells for SARS-CoV-2 (43), wherein the
308 myeloid compartment may not substantially contribute to viral load but likely impacts
309 immunopathology via inflammasome-driven inflammation (44, 45). Prior reports have proposed
310 a similar role for the NLRP3 inflammasome in myeloid cells (29-31). We favor a unifying model
311 in which CARD8-dependent GSDMD pore formation contributes to NLRP3 inflammasome
312 activation, which also offers an explanation for CARD8-dependent release of IL-1β (46).

313 Interestingly, the SARS-CoV-2 3CL^{pro} also cleaves and activates NLRP1 in airway epithelia (9).

314 This results in cell death via a non-canonical NLRP1>CASP8>CASP3>GSDME inflammasome
315 pathway, suggesting that the cellular context of inflammasome responses may uniquely shape
316 antiviral defense and/or inflammation. We also note that seasonal coronaviruses are capable of
317 activating CARD8, and we speculate that a multitude of variables such as cell tropism shape the
318 outcome of virus-induced inflammasome activation in antiviral immunity and pathogenesis.

319 Nevertheless, given the impact of SNPs on human CARD8 sensing of pathogenic viruses, it is
320 tempting to speculate that diminished CARD8 inflammasome activation may be a contributing
321 factor to variation in COVID-19 disease outcomes, and more generally for other human
322 pathogenic coronavirus and picornavirus infections. Further studies are required to establish this
323 connection.

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325 Taken together, our findings establish CARD8 as a rapidly evolving, polymorphic, innate
326 immune sensor of infection by positive-sense RNA viruses. We demonstrate that CARD8 has the
327 capacity to detect viral proteases from at least three viral families that include important human
328 pathogens: *Coronaviridae*, *Picornaviridae*, and *Retroviridae*. These findings also build on the
329 emerging concept that ETI is an important mechanism of pathogen recognition, including the use
330 of host mimicry of viral polyprotein cleavage motifs as an evolutionary strategy in the ongoing
331 arms race between host and viruses.

332

333 **Materials and Methods**

334 *Motif generation and search*

335 To build the betacoronavirus (betaCoV) 3CL^{pro} cleavage motif, 995 nonredundant betaCoV
336 polyprotein sequences were collected from the Viral Pathogen Resource (ViPR) (47) and aligned
337 with five well-annotated reference enteroviral polyprotein sequences from RefSeq (**S1 Fig, A**
338 **and B**). P1 and P1' of the annotated cleavage sites across the RefSeq sequences served as
339 reference points for putative cleavage sites across the 995 ViPR sequences (**S1 Table**). Four
340 amino acid residues upstream (P4-P1) and downstream (P1'-P4') of each cleavage site were
341 extracted from every MAFFT-aligned (48) polyprotein sequence, resulting in 1000 sets of

342 cleavage sites (RefSeq sites included) (**S1 Table**). Each set of cleavage sites representative of
343 each polyprotein was then concatenated (**S2 Table**). Next, duplicates were removed from the
344 concatenated cleavage sites (**S2 Table**). The remaining 60 nonredundant, concatenated cleavage
345 sites were then split into individual 8-mer cleavage sites and were aligned using MAFFT (48) to
346 generate Geneious-defined (49) sequence logo information at each aligned position (**S2 Table**).
347 Pseudo-counts to the position-specific scoring matrix were adjusted as described previously (11)
348 and a motif p-value cut-off of 0.00231 corresponding to detection of 99% of the initial
349 polyprotein cleavage sites was selected (**S1 FigC**).

350

351 *Sequence alignments and phylogenetic trees*

352 Complete polyprotein sequences from 60 betaCoVs with non-redundant cleavage sites (see
353 ‘Motif generation and search’ section above) were downloaded from ViPR. Sequences were
354 aligned using MAFFT (48) and a neighbor-joining phylogenetic tree was generated using
355 Geneious software (49).

356

357 *Evolutionary analyses*

358 For phylogenomic analyses of CARD8 and NLRP1 (**Fig 3A**), human CARD8 (accession
359 NP_001338711) and human NLRP1 (accession NP_127497.1) were used as BLASTP (50)
360 search queries against the indicated mammalian proteomes (**Fig 3A**). A species was determined
361 to have an ortholog if it had a protein with >50% sequence identity, >70% sequence coverage,
362 and was the bi-directional best hit to the indicated human protein. The species tree shown in **Fig**
363 **3A** is based on NCBI Common Tree
364 (<https://www.ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi>). To identify regions of

365 mammalian CARD8s that are orthologous to the 3CL^{pro} cleavage site (Q349) in *Rousettus*
366 *aegyptiacus* CARD8, a 50 amino acid region of *R. aegyptiacus* CARD8 that was centered on the
367 Q349 cleavage site was used as a BLASTP query against the entire RefSeq protein database with
368 a 60% sequence identity cut-off. A single CARD8 sequence from each species (**S8 Fig**) was
369 aligned using MAFFT (48) and trimmed to only include the eight amino acid spanning the
370 cleavage site.

371 For positive selection analyses, primate nucleotide sequences that aligned to human full
372 length CARD8 (Protein: NP_001338711, mRNA: NM_001351782.2) were downloaded from
373 NCBI and aligned using MAFFT (48). Only eight other primate sequences, only from hominoids
374 and Old World monkeys, were fully alignable to full length human CARD8 (sequence accessions
375 in table S3). Maximum likelihood (ML) tests were performed with codeml in the PAML
376 software suite (51) or using BUSTED (52) on the DataMonkey (53) server. For PAML, aligned
377 sequences were subjected to ML tests using NS sites models disallowing (M7) or allowing (M8)
378 positive selection. The p-value reported is the result of a chi-squared test on twice the difference
379 of the log likelihood (lnL) values between the two models using two degrees of freedom. We
380 confirmed convergence of lnL values by performing each analysis using two starting omega
381 (dN/dS) values (0.4 and 1.5). Results are reported from analyses using the F61 codon frequency
382 model. Analyses with the F3x4 model gave similar results. For evolutionary analyses of regions
383 of CARD8, the full-length alignment was truncated to only include codons 1-161 ('tripwire'
384 region) or 162-537 (FIIND-CARD region) and PAML or BUSTED analyses were performed as
385 described above.

386 We used three independent methods to estimate individual codons within CARD8 that
387 have been subject to positive selection (**S4 Table**). PAML was used to identify positively

388 selected codons with a posterior probability greater than 0.90 using a Bayes Empirical Bayes
389 (BEB) analysis and the F61 or F3x4 codon frequency models. The same CARD8 alignment was
390 also used as input for FEL (54) and FUBAR (55) using the DataMonkey (53) server. In both
391 cases, default parameters were used and codons with a signature of positive selection with a p-
392 value of <0.1 are reported. In all cases, codon numbers correspond to the amino acid position and
393 residue in human CARD8 (NCBI accession NP_001338711).

394

395 *Plasmids and constructs*

396 Megabat CARD8, CASP1, IL-1 β , and all 3CL^{pro} sequences were ordered as either gBlocks
397 (Integrated DNA Technologies, San Diego, CA) or Twist Gene Fragments (Twist Biosciences,
398 South San Francisco, CA). All sequences are found in **S7 Table**. Vectors containing the coding
399 sequences of human CARD8, ASC, human CASP1, human IL-1 β -V5, and TEV^{pro} were
400 previously described (24). Vector psPAX2 containing the untagged coding sequence for HIV-1
401 gag-pol was a gift from Didier Trono (Addgene plasmid # 12260).

402 For CARD8 cleavage assays, the coding sequences of human CARD8 (NCBI accession
403 NP_001171829.1), human CARD8 mutants (Q37A, Q37A Q61A, S39P, R40W), human CARD8
404 TEV, *Rousettus aegyptiacus* (megabat) CARD8 (NCBI accession XP_016010896), and megabat
405 CARD8 TEV were cloned into the pcDNA5/FRT/TO backbone (Invitrogen, Carlsbad, CA) with
406 an N-terminal 3xFlag-mCherry tag. For CARD8 activation, the same sequences were cloned into
407 the pQCXIP vector backbone (Takara Bio, Mountain View, CA) with a C-terminal Myc tag.
408 Megabat CASP1 (NCBI accession KAF6464288) and megabat IL-1 β (NCBI accession
409 KAF6447073), also from *Rousettus aegyptiacus*, were cloned in the same vector as their
410 respective human orthologues. 3CL^{pro} sequences were cloned with an N-terminal HA tag into the

411 QCXIP vector backbone, flanked by polyprotein cleavage sites fused to N-terminal eGFP and C-
412 terminal mCherry (**S2 Fig**). 3C^{pro} constructs were described previously (*11*).

413 Single point mutations were made using overlapping stitch PCR. All plasmid stocks were
414 sequenced across the entire inserted region to verify that no mutations were introduced during
415 the cloning process. The primers used for cloning are described in **S7 Table**.

416

417 *Cell culture and transient transfection*

418 All cell lines (HEK293T, HEK-Blue-IL-1 β) are routinely tested for mycoplasma by PCR kit
419 (ATCC, Manassas, VA) and kept a low passage number to maintain less than one year since
420 purchase, acquisition or generation. HEK293T cells were obtained from ATCC (catalog # CRL-
421 3216) and HEK-Blue-IL-1 β cells were obtained from Invivogen (catalog # hkb-il1b) and all lines
422 were verified by those sources, and were grown in complete media containing DMEM (Gibco,
423 Carlsbad, CA), 10% FBS, and appropriate antibiotics (Gibco, Carlsbad, CA). THP-1 cells were
424 purchased from ATCC, and grown in complete media containing RPMI (Gibco, Carlsbad, CA)
425 10% FBS, and 1% L-glutamine. For transient transfections, HEK293T cells were seeded the day
426 prior to transfection in a 24-well plate (Genesee, El Cajon, CA) with 500 μ l complete media.
427 Cells were transiently transfected with 500 ng of total DNA and 1.5 μ l of Transit X2 (Mirus Bio,
428 Madison, WI) following the manufacturer's protocol. HEK-Blue IL-1 β reporter cells (Invivogen,
429 San Diego, CA) were grown and assayed in 96-well plates (Genesee, El Cajon, CA).

430

431 *Generation of knockout and transgenic cell lines*

432 *CARD8* knockouts in HEK293T cells were generated similarly to *NLRP1* knockouts described in
433 (*11*). Briefly, lentivirus-like particles were made by transfecting HEK293T cells with the

434 plasmids psPAX2 (gift from Didier Trono, Addgene plasmid # 12260), pMD2.G (gift from
435 Didier Trono, Addgene plasmid # 12259), and either pLB-Cas9 (gift from Feng Zhang, Addgene
436 plasmid # 52962) (56) or plentiGuide-Puro, which was adapted for ligation-independent cloning
437 (gift from Moritz Gaidt) (57). Conditioned supernatant was harvested 48 and 72 hours post-
438 transfection and used for spinfection of HEK293T cells at 1200 x g for 90 minutes at 32°C.
439 Forty-eight hours post-spinfection, cells with stable expression of Cas9 were selected in media
440 containing 100 µg/ml blasticidin. Blasticidin-resistant cells were then transduced with sgRNA-
441 encoding lentivirus-like particles, and selected in media containing 0.5 µg/ml puromycin. Cells
442 resistant to blasticidin and puromycin were single cell cloned by limiting dilution in 96-well
443 plates, and confirmed as knockouts by Sanger sequencing. *CARD8* knockout THP-1 cells were
444 generated as described previously (58). Briefly, a *CARD8* specific sgRNA was designed using
445 CHOPCHOP (59), and cloned into a plasmid containing U6-sgRNA-CMV-mCherry-T2A-Cas9
446 using ligation-independent cloning. THP-1 cells were electroporated using the BioRad
447 GenePulser Xcell. After 24 h, mCherry-positive cells were sorted and plated for cloning by
448 limiting dilution. Monoclonal lines were validated as knockouts by deep sequencing and
449 OutKnocker analysis, as described previously (60, 61). Knockout lines were further validated by
450 immunoblot and functional assays. sgRNA used to generate knockouts are described in **S7**
451 **Table**. To make THP-1 cells susceptible to SARS-CoV-2 infection(62), ACE2 and TMPRSS2
452 expressing THP-1 cells were made using the same lentiviral transduction protocol as described
453 above, but using the transfer plasmid PpWIP-IRES-Bla-AK-ACE2-IRES-TMPRSS2 (gift from
454 Sonja Best). THP-1 cells were selected with 10 µg/ml blasticidin.

455

456 *THP-1 treatments and viral infections*

457 50,000-100,000 THP-1 cells per well in 96-well round bottom plates in 50 μ l OptiMEM
458 containing 500 ng/mL Pam3CSK4 for 6h, followed by treatment with Val-boroPro (10 μ M) or
459 infection with the coronaviruses hCoV-229E (BEI NR-52726) or SARS-CoV-2 (USA/WA-
460 1/2020, a gift from Dr. Ralph Baric) at indicated multiplicities of infection (MOIs). 48h post-
461 treatment or infection, supernatants were harvested for the detection of IL-1 β (see below). Cells
462 were transferred to a white-walled 96-well assay plate and mixed with an equal volume of Cell
463 Titer Glo reagent (Promega). Measurements for fluorescence at 544-15 nm (excitation), 620-20
464 nm (emission) or luminescence at 555-70 (emission) were taken following incubation at room
465 temperature, rocking for 10min.

466

467 *CARD8 cleavage assays*

468 100 ng of epitope-tagged human CARD8 (WT, Q37A, Q37A Q61A, S39P, R40W), human
469 CARD8 TEV, megabat CARD8 WT or megabat CARD8 TEV was co-transfected with either
470 HA-tagged QCXIP empty vector ('-'), 250 ng of TEV^{pro}, 250 ng of untagged HIV-1^{pro} (HIV-1
471 gag-pol carrying HIV-1 protease activity), 5 ng of HA-tagged 3CL^{pro}, or 250 ng of HA-tagged
472 3C^{pro}-encoding constructs. Twenty-four hours post-transfection, the cells were harvested, lysed
473 in 1x NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA) containing 5% β -mercaptoethanol
474 (Fisher Scientific, Pittsburg, PA) and immunoblotted with antibodies described in **S8 Table**.

475

476 *CARD8 activity assays*

477 To reconstitute the human CARD8 inflammasome, 100 ng of human CASP1 and 50 ng of human
478 IL-1 β -V5 were co-transfected with 50 ng of either HA-tagged QCXIP empty vector, wild-type or
479 mutant pQCXIP-CARD8-Myc constructs in *CARD8* KO HEK293T cells. To reconstitute the

480 megabat CARD8 inflammasome, 10 ng of megabat CASP1 and 50 ng of megabat IL-1 β -V5
481 were co-transfected with 2 ng megabat CARD8 constructs in *CARD8* KO HEK293T cells. These
482 cells were further co-transfected with either empty vector ('-'), 250 ng of TEV^{pro}, 100 ng of
483 untagged HIV-1 gag-pol (with HIV-1 protease activity), 5 ng of HA-tagged 3CL^{pro}, 100 ng of
484 HA-tagged enteroviral 3C^{pro}, or 20 ng of HA-tagged non-enteroviral 3C^{pro}-encoding constructs.
485 Twenty-four hours post-transfection, cells were harvested and lysed in 1x NuPAGE LDS sample
486 buffer containing 5% β -mercaptoethanol and immunoblotted with antibodies described in **S8**
487 **Table** or culture media was harvested for quantification of IL-1 β levels by HEK-Blue assays (see
488 below). Appearance of the mature p17 band of IL-1 β indicates successful assembly and
489 activation of the inflammasome.

490

491 *HEK-Blue IL-1 β assay*

492 To quantify the levels of bioactive IL-1 β released from cells, we employed HEK-Blue IL-1 β
493 reporter cells (Invivogen, San Diego, CA). In these cells, binding to IL-1 β to the surface receptor
494 IL-1R1 results in the downstream activation of NF- κ B and subsequent production of secreted
495 embryonic alkaline phosphatase (SEAP) in a dose-dependent manner (*11*). SEAP levels are
496 detected using a colorimetric substrate assay, QUANTI-Blue (Invivogen, San Diego, CA) by
497 measuring an increase in absorbance at OD655.

498 Culture supernatant from inflammasome-reconstituted HEK293T cells or HEK293T
499 *CARD8* KO cells that had been transfected with 3CL pro was added to HEK-Blue IL-1 β reporter
500 cells plated in 96-well format in a total volume of 200 μ l per well. On the same plate, serial
501 dilutions of recombinant human IL-1 β (Invivogen, San Diego, CA) were added to generate a
502 standard curve for each assay. Twenty-four hours later, SEAP levels were assayed by taking 20

503 μ l of the supernatant from HEK-Blue IL-1 β reporter cells and adding to 180 μ l of QUANTI-Blue
504 colorimetric substrate following the manufacturer's protocol. After incubation at 37°C for 30–60
505 min, absorbance at OD655 was measured on a BioTek Cytation five plate reader (BioTek
506 Instruments, Winooski, VT) and absolute levels of IL-1 β were calculated relative to the standard
507 curve. All assays, beginning with independent transfections or infections, were performed in
508 triplicate.

509

510 *Immunoblotting and antibodies*

511 Harvested cell pellets were washed with 1X PBS, and lysed with 1x NuPAGE LDS sample
512 buffer containing 5% β -mercaptoethanol at 98C for 10 min. The lysed samples were spun down
513 at 15000 RPM for two minutes, followed by loading into a 4–12% Bis-Tris SDS-PAGE gel (Life
514 Technologies, San Diego, CA) with 1X MOPS buffer (Life Technologies, San Diego, CA) and
515 wet transfer onto a nitrocellulose membrane (Life Technologies, San Diego, CA). Membranes
516 were blocked with PBS-T containing 5% bovine serum albumin (BSA) (Spectrum, New
517 Brunswick, NJ), followed by incubation with primary antibodies for V5 (IL-1 β), FLAG
518 (mCherry-fused CARD8 for protease assays), Myc (CARD8-Myc for activation assays), HA
519 (viral protease), or GAPDH. Membranes were rinsed three times in PBS-T then incubated with
520 the appropriate HRP-conjugated secondary antibodies. Membranes were rinsed again three times
521 in PBS-T and developed with SuperSignal West Pico PLUS Chemiluminescent Substrate
522 (Thermo Fisher Scientific, Carlsbad, CA). The specifications, source, and clone info for
523 antibodies are described in **S8 Table**.

524

525

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530

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667

668 **Competing interests**

669 Authors declare that they have no competing interests.

670

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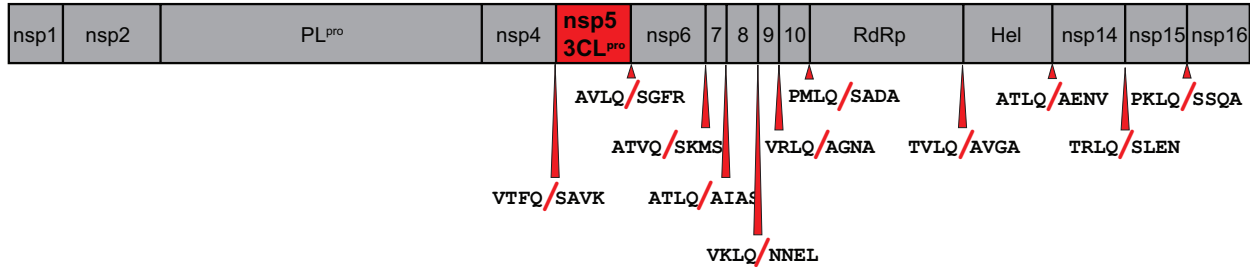
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684 Investigator of the Howard Hughes Medical Institute (REV)
685

686 **Author contributions:**

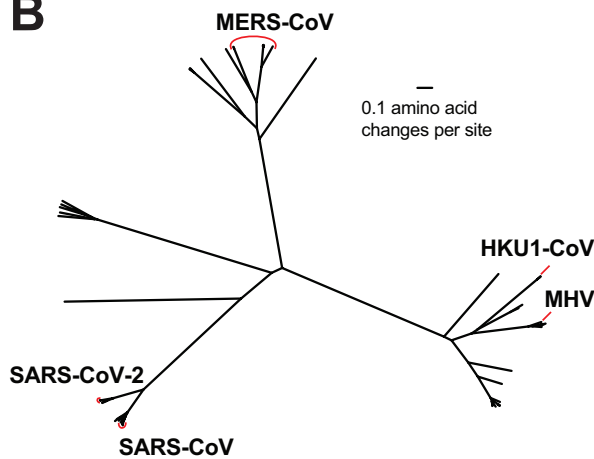
687 Conceptualization: BVT, PSM, MDD
688 Methodology: BVT, NSG, JK, APR, EAT, PSM, MDD
689 Investigation: BVT, RA, NSG, JK, APR, LKC, CMB, EAT, EJF, PSM, MDD
690 Visualization: BVT, NSG, PSM, MDD
691 Funding acquisition: REV, PSM, MDD
692 Project administration: REV, JLH, RS, PSM, MDD
693 Supervision: REV, JLH, RS, PSM, MDD
694 Writing – original draft: BVT, PSM, MDD
695 Writing – review & editing: All authors

696 **Supporting Information**

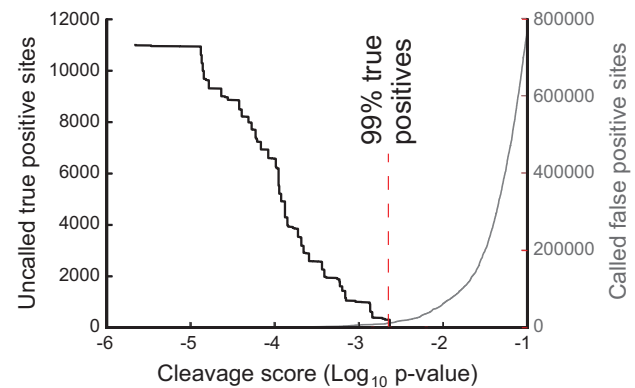
A



B



C



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698

S1 Fig. Motif generation of coronaviral 3CL^{pro} polyprotein cleavage.

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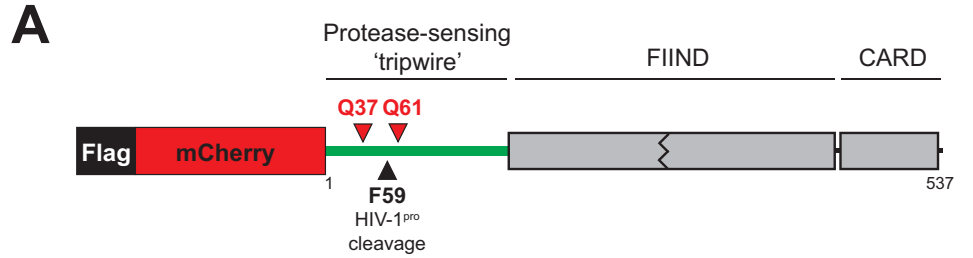
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(A) Schematic of 3CL^{pro} cleavage sites (11 red triangles) within the polyprotein of SARS-CoV-2 (SARS-2), the causative agent of COVID-19. Shown are four amino acids flanking each side of the cleavage site within the polyprotein. (B) Phylogenetic tree of 60 coronavirus polyprotein coding sequences depicting the betacoronaviruses sampled in this study with human relevant coronaviruses labeled (table S2). (C) Training set data used to determine the motif search threshold for FIMO (table S1). The X-axis represents a log₁₀ of the p-value reported by FIMO as an indicator for the strength of the cleavage motif hit (cleavage score). (Left) The Y-axis depicts the number of uncalled true positives, or motif hits that overlap with the initial set of 8mer polyprotein cleavage sites used to generate the motif, in the training set of coronavirus polyprotein sequences (black line). (Right) The Y-axis depicts the number of called false positive sites, or any motif hits found in the polyprotein that are not known to be cleaved by 3CL^{pro}, in the training set of coronaviral polyprotein sequences (gray). A red vertical dashed line indicates the threshold that captures 99% of true positive polyprotein hits (p-value = 0.00231).



Predicted protein products

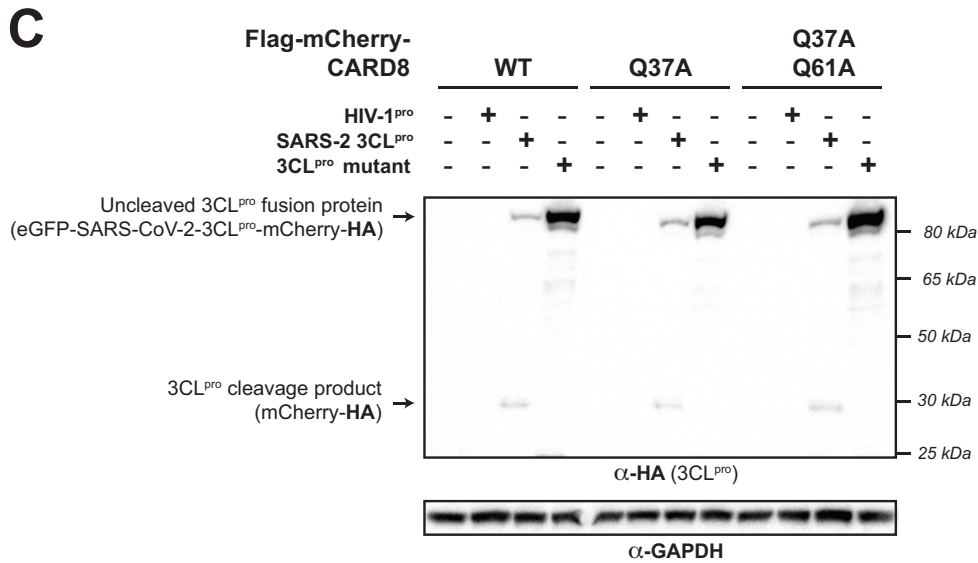
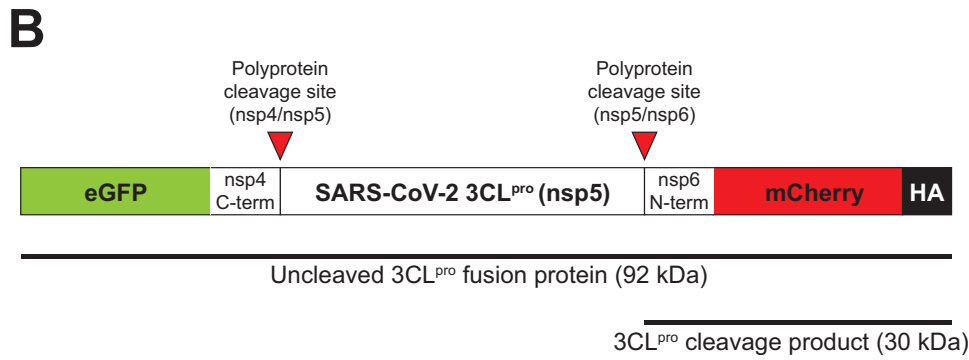
Full length CARD8 (92 kDa)

FIIND processed CARD8 (63 kDa)

3CL^{pro} cleaved CARD8 at site Q37 (34 kDa)

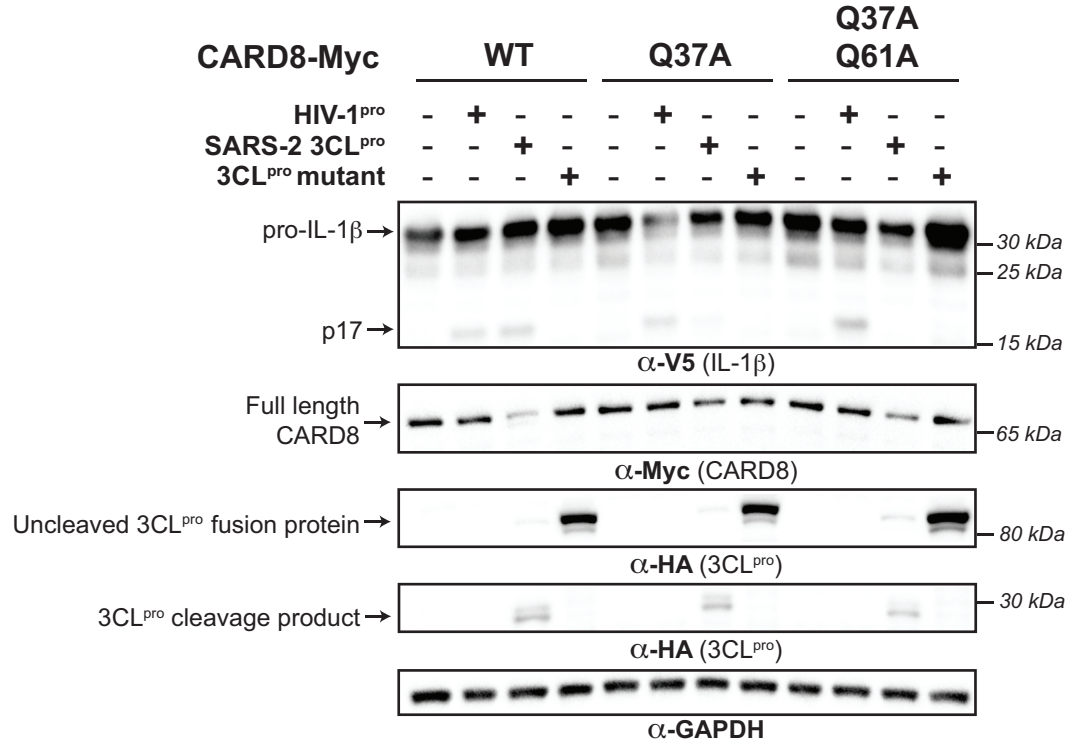
HIV-1^{pro} cleaved CARD8 at site F59 (37 kDa)

3CL^{pro} cleaved CARD8 at site Q61 (37 kDa)



713 **S2 Fig. Schematic of CARD8 and 3CL^{pro} expression constructs for cleavage assays and**
714 **immunoblot depicting HA-tagged SARS-2 3CL^{pro} and SARS-2 3CL^{pro} mutant.**

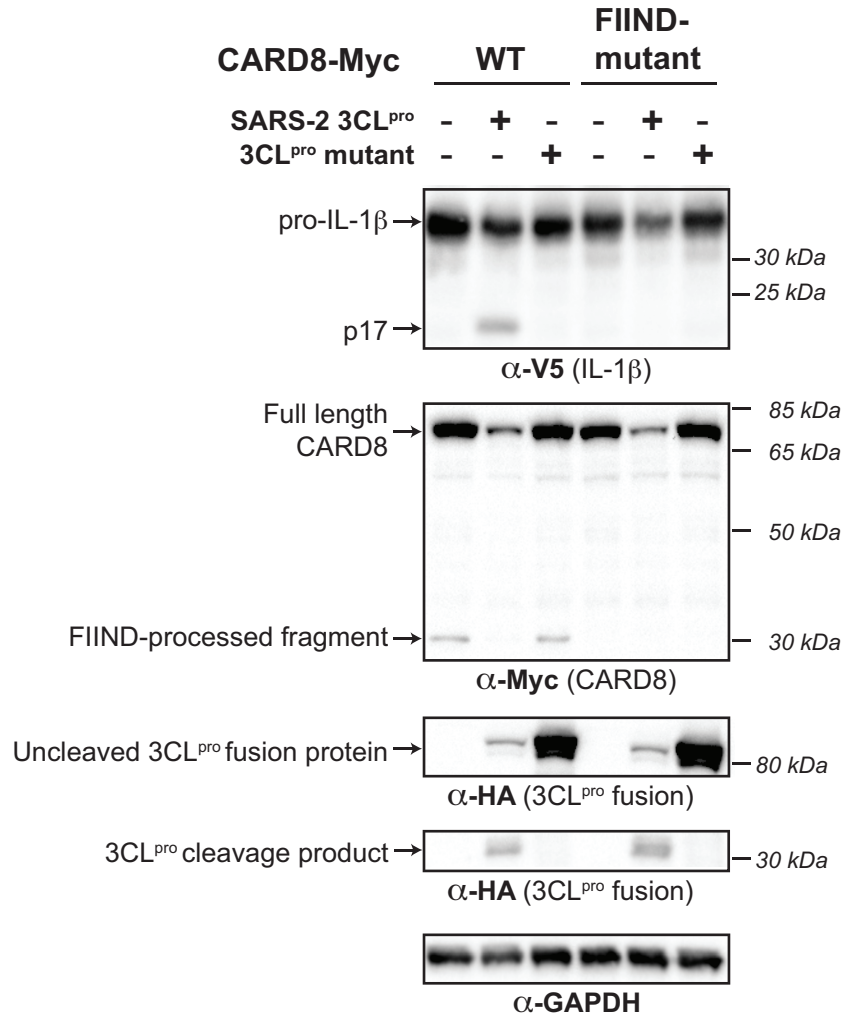
715 (A) Schematic of the expression constructs used for CARD8 cleavage assays. Full length
716 CARD8 was fused to a 3xFlag-mCherry domain to increase the stability and our ability to detect
717 viral protease cleavage products in the N-terminus of CARD8. Below are shown the expected
718 sizes of full length and FIIND processed CARD8, as well as the expected sizes that would result
719 from viral protease cleavage at the indicated sites. (B) Schematic of the expression constructs
720 used for 3CL^{pro}s (nsp5s). A region of the viral polyprotein spanning the C-terminal nine residues
721 of nsp4 through the N-terminal nine residues of nsp6 was cloned between eGFP and mCherry-
722 HA. Inactive protease is expressed as a full-length fusion protein (92 kDa predicted molecular
723 weight). Active 3CL^{pro} cleaves at the indicated polyprotein cleavage sites (red triangles),
724 liberating the active protease from the construct and resulting in an HA-tagged mCherry product
725 (30 kDa predicted molecular weight). (C) An expanded view of the anti-HA-stained immunoblot
726 shown in Figure 1B highlighting uncleaved (Uncleaved 3CL^{pro} fusion protein) and cleaved
727 (3CL^{pro} cleavage product) HA-tagged protein products.



728

729 **S3 Fig. CARD8 cleavage by viral proteases results in inflammasome activation and IL-1β**
 730 **maturation.**

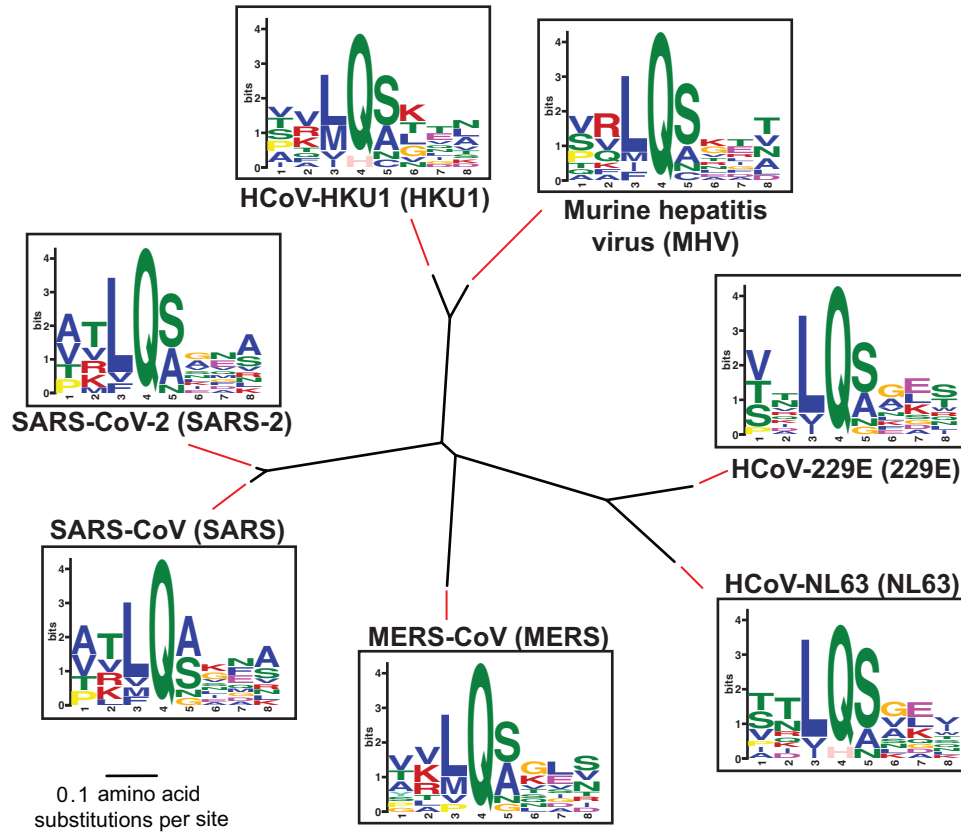
731 CARD8 inflammasome assay. CARD8 KO HEK293T cells were co-transfected using the
 732 indicated Myc-tagged CARD8 plasmid constructs, V5-IL-1β, CASP1, and HA-tagged protease
 733 constructs (SARS-CoV-2 3CL^{pro} (SARS-2 3CL^{pro}), SARS-CoV-2 3CL^{pro} catalytic mutant
 734 C145A (3CL^{pro} mutant), HIV-1 gag-pol (HIV-1^{pro}), or empty vector (-)). Appearance of a mature
 735 bioactive IL-1β (p17) indicates inflammasome activation.



736

737 **S4 Fig. 3CL^{pro}-mediated activation of the human CARD8 inflammasome depends on**
738 **FIIND autoprocessing.**

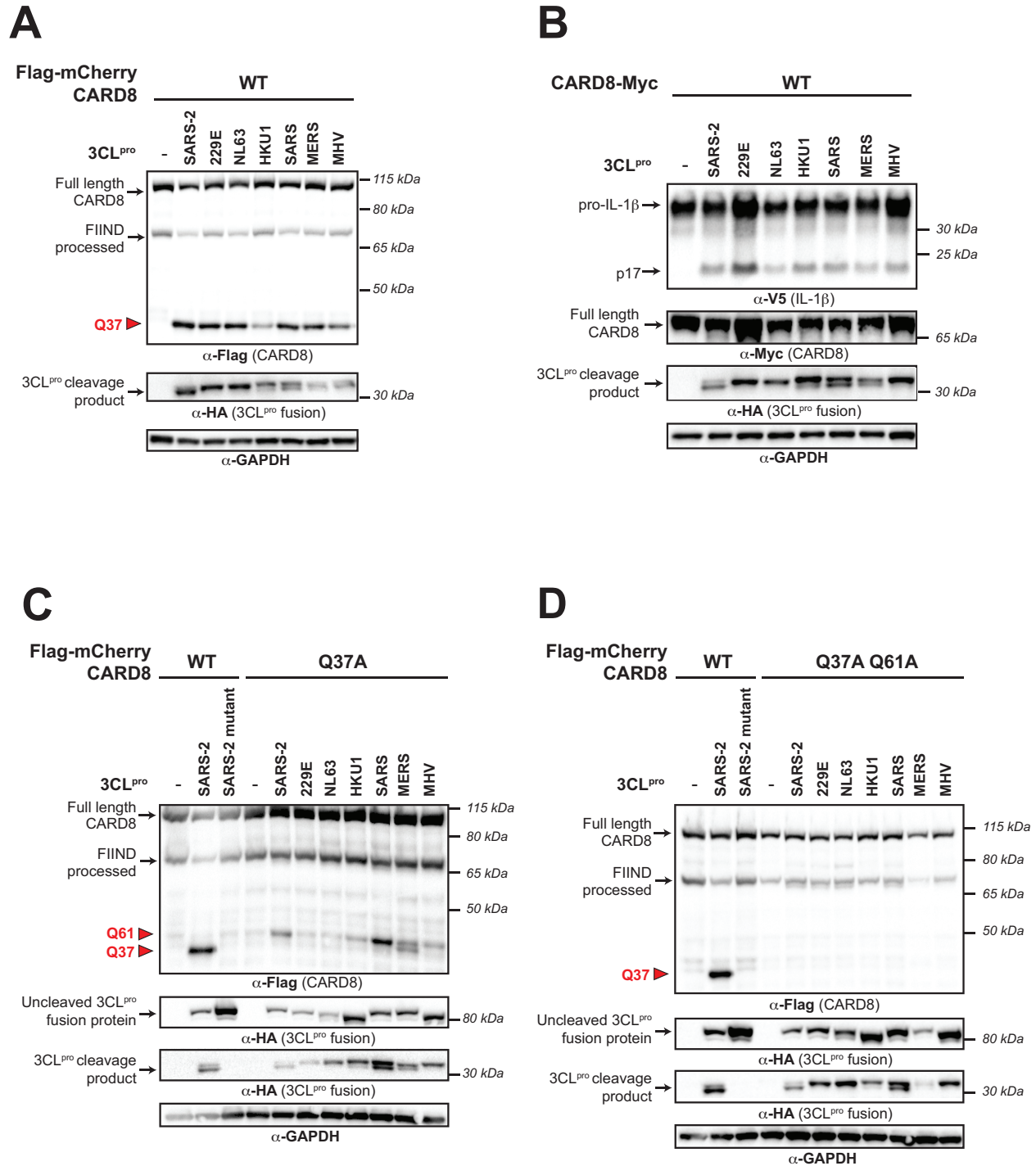
739 CARD8 inflammasome activation assay depicting loss of CARD8 activation with a FIIND
740 autoprocessing mutant (S297A).



741

742 **S5 Fig. 3CL^{pro}s used in this study demonstrate similar polyprotein cleavage.**

743 Phylogenetic tree of 3CL^{pro} protein sequences used in this study from the indicated
744 coronaviruses. Shown next to the virus name is the sequence motif generated from the 3CL^{pro}
745 polyprotein cleavage sites from that specific virus.

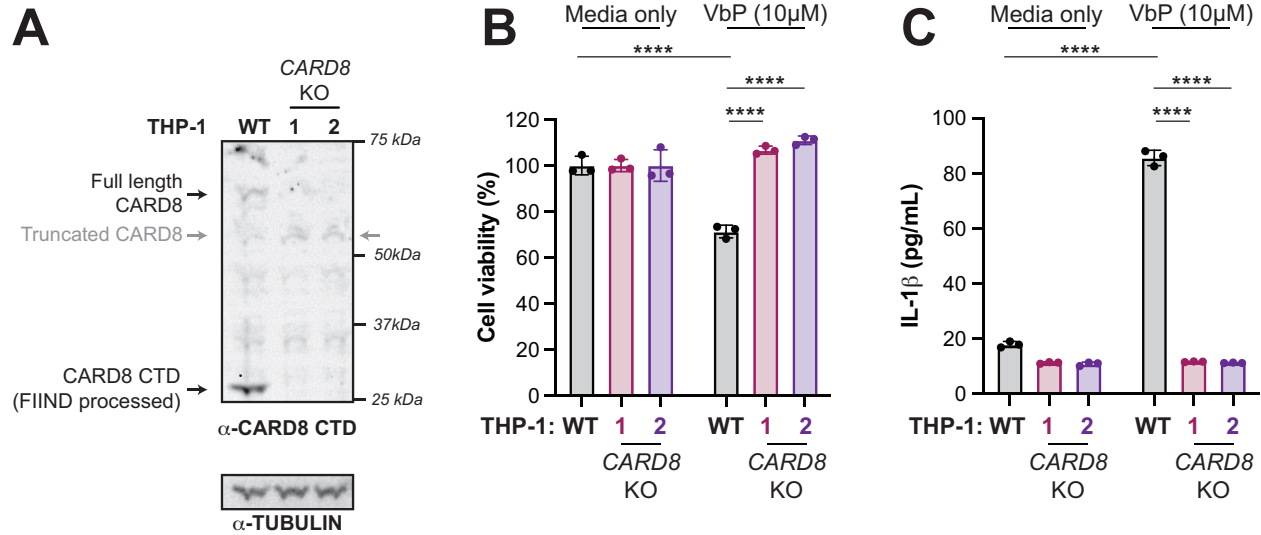


746

747 **S6 Fig. Site-specific CARD8 cleavage and inflammasome activation by diverse coronavirus**
748 **3CL^{pro}s.**

749 (A) Cleavage assay depicting cleavage of human CARD8 by the indicated 3CL^{pro}s from diverse
750 coronaviruses (SARS-CoV-2 (SARS-2), HCoV-229E (229E), HCoV-NL63 (NL63), HCoV-
751 HKU1 (HKU1), SARS-CoV (SARS), MERS-CoV (MERS), and murine hepatitis virus (MHV)).
752 (B) CARD8 inflammasome activation assay with human CARD8 WT and the indicated 3CL^{pro}.


753 (C-D) Cleavage assays mapping the cleavage specificity of diverse 3CL^{pro}s. Indicated proteases
754 were co-transfected with WT and Q37A (C) or Q37A Q61A (D).



755

756 **S7 Fig. Validation of *CARD8* KO THP-1 cells.**

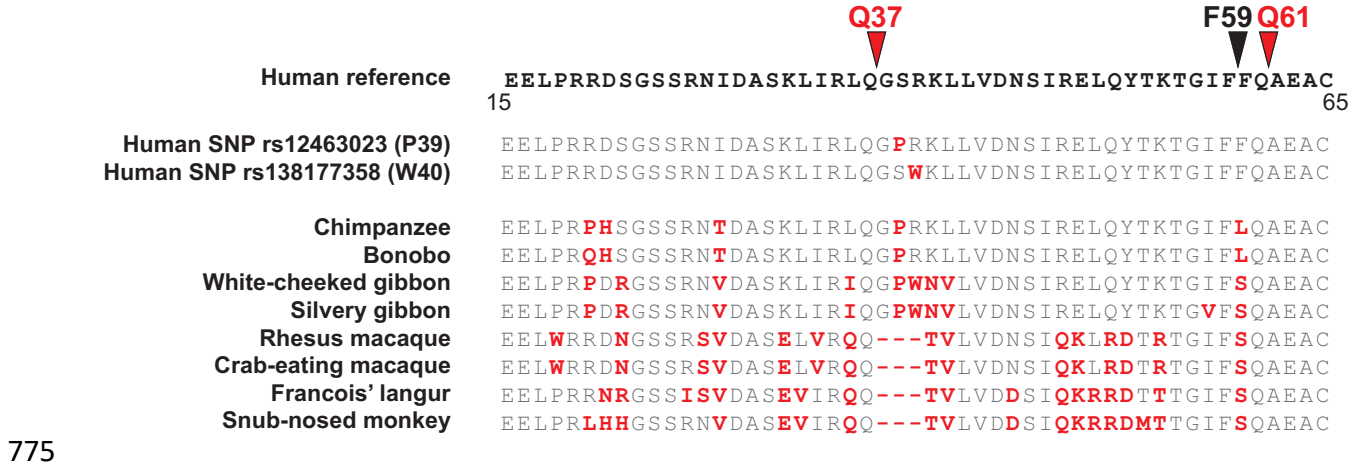
757 (A) Immunoblot of wildtype (WT) or *CARD8* knockout (KO) 1 and KO2 THP-1 cells. The
 758 sgRNA used to edit *CARD8* results in a truncated *CARD8* (grey arrow) that removes the C-
 759 terminal domain (CTD), including the CARD. (B-C) Indicated THP-1 cells were primed with 0.5
 760 µg/mL Pam3CSK4 for 6h, followed by treatment with 10 µM Val-boroPro (VbP) or media only.
 761 Cell viability (B) was measured 48h post-treatment via the Cell Titer Glo assay and IL-1β levels
 762 (C) were measured from cell supernatants using the IL1R reporter assay as in **Fig 1D** (see
 763 **Methods**). Data presented are representative of experiments performed in triplicate. Data were
 764 analyzed using two-way ANOVA with Šidák's post-test: $p < 0.0001$.

	Q349
Rousettus aegyptiacus (XP_016010887.2)	▼ VRLQSSPP
Mammalian consensus sequence	
Dasyopus novemcinctus (XP_004455135.1)	VRLQTSPP
Choloepus didactylus (XP_037676345.1)	VRLQTSPP
Orycteropus afer afer (XP_007941216.2)	VRLQTSPP
Echinops telfairi (XP_045148583.1)	VRLQTSPP
Elephas maximus indicus (XP_049756664.1)	VRLQTSPP
Trichechus manatus latirostris (XP_023591965.1)	VRLQTSPP
Loxodonta africana (XP_023398968.1)	VRLQTSPP
Tupaia chinensis (XP_006142263.2)	VRLQTSPP
Otolemur garnettii (XP_023363700.1)	VRLQTSPP
Carlito syrichta (XP_008065260.1)	VRLQTSPP
Callithrix jacchus (XP_008986567.2)	VRLQTSPP
Aotus nancymae (XP_021524182.1)	VRLQTSPP
Saimiri boliviensis boliviensis (XP_003940505.2)	VRLQTSPP
Cebus imitator (XP_017355929.1)	VRLQTSPP
Sapajus apella (XP_032126901.1)	VRLQTSPP
Pongo abelii (NP_001125643.1)	VRLQTSPP
Nomascus leucogenys (XP_030676824.1)	VRLQTSPP
Hylobates moloch (XP_032025189.1)	VRLQTSPP
Homo sapiens (NP_001171829.1)	VRLQTSPP
Gorilla gorilla gorilla (XP_030860007.1)	VRLQTSPP
Pan paniscus (XP_034801359.1)	VRLQTSPP
Pan troglodytes (XP_009434234.2)	VRLQTSPP
Rhinopithecus bieti (XP_017744314.1)	VRLQTSPP
Rhinopithecus roxellana (XP_030798493.1)	VRLQTSPP
Macaca mulatta (XP_028694412.1)	VRLQTSPP
Cercocebus atys (XP_011936103.1)	VRLQTSPP
Papio anubis (XP_031516220.1)	VRLQTSXP
Theropithecus gelada (XP_025223748.1)	VRLQTSPP
Papio anubis (XP_031514956.1)	VRLQTSPP
Macaca fascicularis (XP_045236836.1)	VRLQTSPP
Macaca nemestrina (XP_011734426.1)	VRLQTSPP
Chlorocebus sabaeus (XP_037847268.1)	VRLQTSPP
Mandrillus leucophaeus (XP_011831805.1)	VRLQTSPP
Colobus angolensis palliatus (XP_011801863.1)	VRLQTSPP
Trachypithecus francoisi (XP_033079741.1)	VRLQTSPP
Ceratottherium simum simum (XP_014650570.1)	VRLQTSPP
Rousettus aegyptiacus (XP_016010887.2)	VRLQTSPP
Pteropus alecto (XP_006905198.1)	VRLQSSPP
Pteropus vampyrus (XP_011376482.1)	VRLQSSPP
Pteropus giganteus (XP_039724536.1)	VRLQSSPP
Hyaena hyaena (XP_039104475.1)	VRLQTSPP
Puma concolor (XP_025770087.1)	VRLQTSPP
Leopardus geoffroyi (XP_045296688.1)	VRLQTSPP
Felis catus (XP_006941207.3)	VRLQTSPP
Prionailurus bengalensis (XP_043456356.1)	VRLQTSPP
Prionailurus viverrinus (XP_047693116.1)	VRLQTSPP
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Lynx rufus (XP_046933669.1)	VRLQTSPP
Puma yagouaroundi (XP_040313498.1)	VRLQTSPP
Acinonyx jubatus (XP_014938520.1)	VRLQTSPP
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Panthera uncia (XP_049478262.1)	VRLQTSPP
Panthera leo (XP_042775368.1)	VRLQTSPP
Panthera pardus (XP_019281377.1)	VRLQTSPP
Vulpes vulpes (XP_025869883.1)	VRLQTSPP
Canis lupus familiaris (XP_038316790.1)	VRLQTSPP
Canis lupus dingo (XP_025280236.1)	VRLQTSPP
Odobenus rosmarus divergens (XP_004401075.2)	VRLQTSPP
Callorhinus ursinus (XP_025705476.1)	VRLQTSPP
Zalophus californianus (XP_027475647.1)	VRLQTSPP
Eumetopias jubatus (XP_027943719.1)	VRLQTSPP
Halichoerus grypus (XP_035955734.1)	VRLQTSPP
Phoca vitulina (XP_032244836.1)	VRLQTSPP
Leptonychotes weddellii (XP_030897212.1)	VRLQTSPP
Neomonachus schauinslandi (XP_021536893.1)	VRLQTSPP
Mirounga angustirostris (XP_045746402.1)	VRLQTSPP
Mirounga leonina (XP_034842787.1)	VRLQTSPP
Ailuropoda melanoleuca (XP_002917973.3)	VRLQTSPP
Ursus arctos (XP_026337717.1)	VRLQTSPP
Ursus maritimus (XP_008682721.1)	VRLQTSPP
Ursus americanus (XP_045628234.1)	VRLQTSPP
Meles meles (XP_045843773.1)	VRLQTSPP
Neogale vison (XP_044116462.1)	VRLQTSPP
Mustela erminea (XP_032179857.1)	VRLQTSPP
Mustela putorius fuRo (XP_004767506.1)	VRLQTSPP
Lontra canadensis (XP_032694832.1)	VRLQTSPP
Lutra lutra (XP_047567415.1)	VRLQTSPP
Enhydra lutris kenyoni (XP_022379885.1)	VRLQTSPP

Megabats

766 **S8 Fig. A coronavirus 3CL^{pro} cleavage site in the CARD8 C-terminus is unique to**
767 **megabats.**

768 An alignment of protein sequences homologous to the coronavirus 3CL^{pro} cleavage site in
769 megabat (*Rousettus aegyptiacus*) CARD8 is shown for indicated species (scientific name,
770 accession number). Amino acid numbering is based on *R. aegyptiacus* CARD8. Changes relative
771 to the consensus, depicted as a sequence logo at the top of the alignment) are highlighted. Four
772 species of megabats are indicated, and are unique in having a serine in the P1' position of the
773 cleavage site (highlighted in red). Most other species have a threonine in this position, which
774 makes the protein uncleavable at this site (**Fig 3E**).

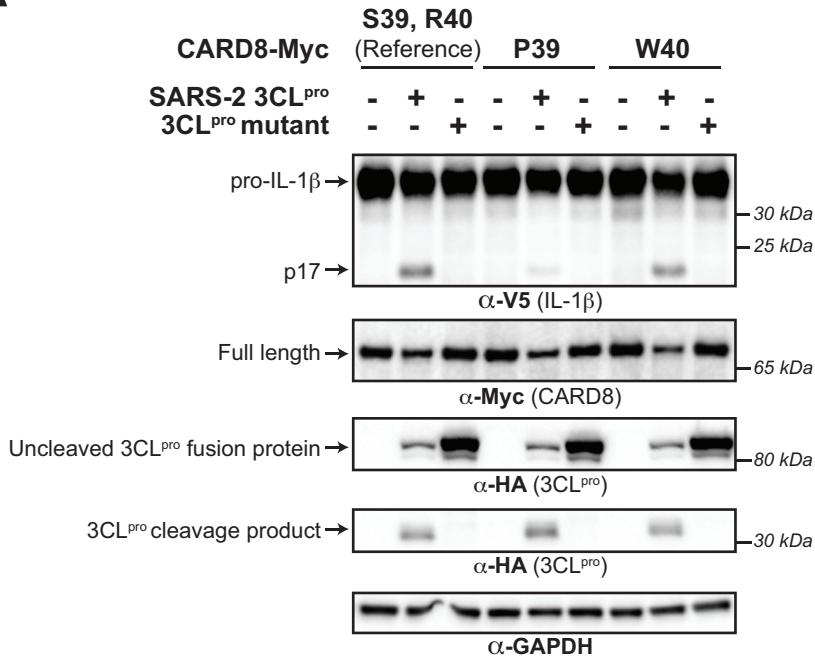


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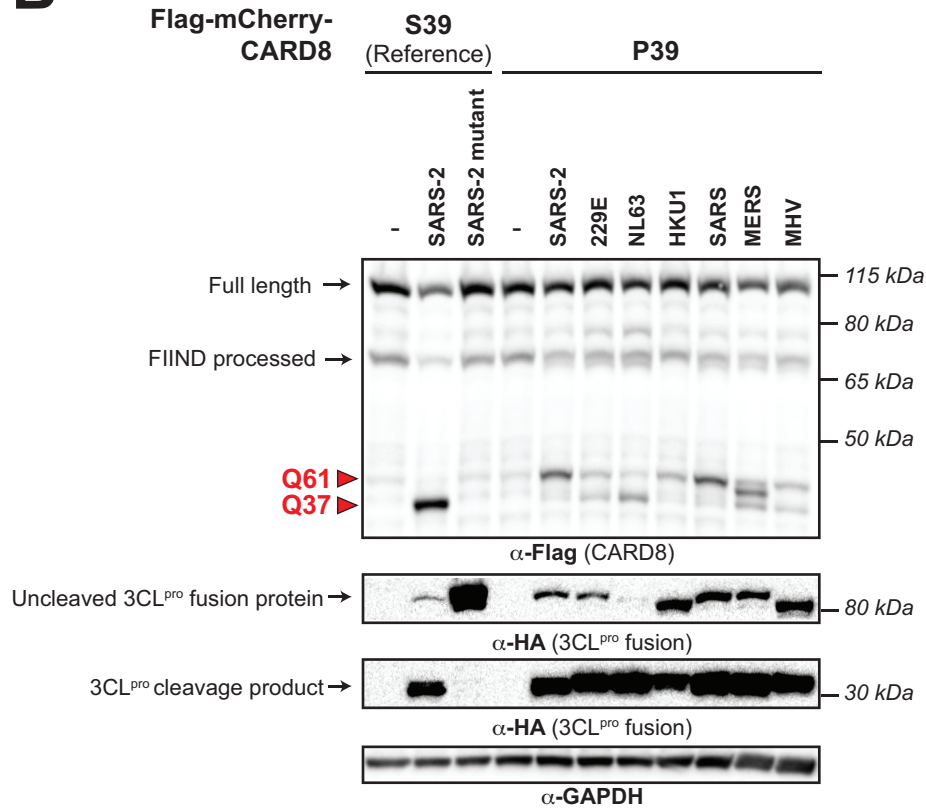
776 **S9 Fig. Rapid evolution and human polymorphism in the ‘tripwire’ region of CARD8 that**
 777 **is targeted by viral proteases.**

778 An alignment of the CARD8 N-terminus (amino acids 15-65, where amino acid numbering is
 779 based on human CARD8) or ‘tripwire’ region from humans and selected non-human primates.
 780 Human non-synonymous single nucleotide polymorphism (SNP) that encode CARD8 P39 and
 781 W40 are shown. Differences relative to the human reference protein sequence (accession
 782 NP_001338711) are indicated in red font. Coronavirus 3CL^{pro} (Q37 and Q61; red arrows) and
 783 HIV-1^{pro} cleavages sites in CARD8 are shown. ‘-’ = indel.

A



B



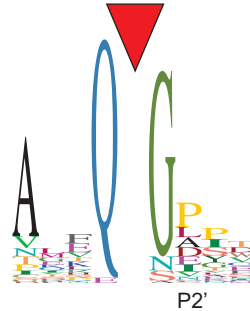
784

785 **S10 Fig. The human CARD8 S39P variant has reduced sensitivity to coronavirus 3CL^{pro}**
786 **cleavage and inflammasome activation.**

787 (A) CARD8 inflammasome activation assay, where inflammasome activation is measured by
788 CASP1-dependent processing of pro-IL-1 β to p17. *CARD8* knockout HEK293T cells were co-
789 transfected with Myc-tagged constructs encoding the reference allele of human CARD8 (protein
790 accession NP_001338711, mRNA accession NM_001351782.2) or the human CARD8 non-
791 synonymous single nucleotide polymorphism (SNP) CARD8-P39 (rs12463023) or CARD8-W40
792 (rs138177358) variants and the HA-tagged SARS-CoV-2 (SARS-2) 3CL^{pro}. (B) Comparison of
793 CARD8 S39 and CARD8 P39 cleavage by diverse coronavirus 3CL^{pro}s. *CARD8* knockout
794 HEK293T cells were co-transfected using the indicated Flag-tagged mCherry-CARD8 fusion
795 constructs with HA-tagged protease constructs (empty vector ('-'), SARS-2 catalytically inactive
796 mutant (3CL^{pro} mutant) or active 3CL^{pro} from SARS-2, 229E, NL63, HKU1, SARS, MERS, or
797 MHV. Red triangles indicate cleavage sites 3CL^{pro}.
798

799

Consensus 3C^{pro}
cleavage site

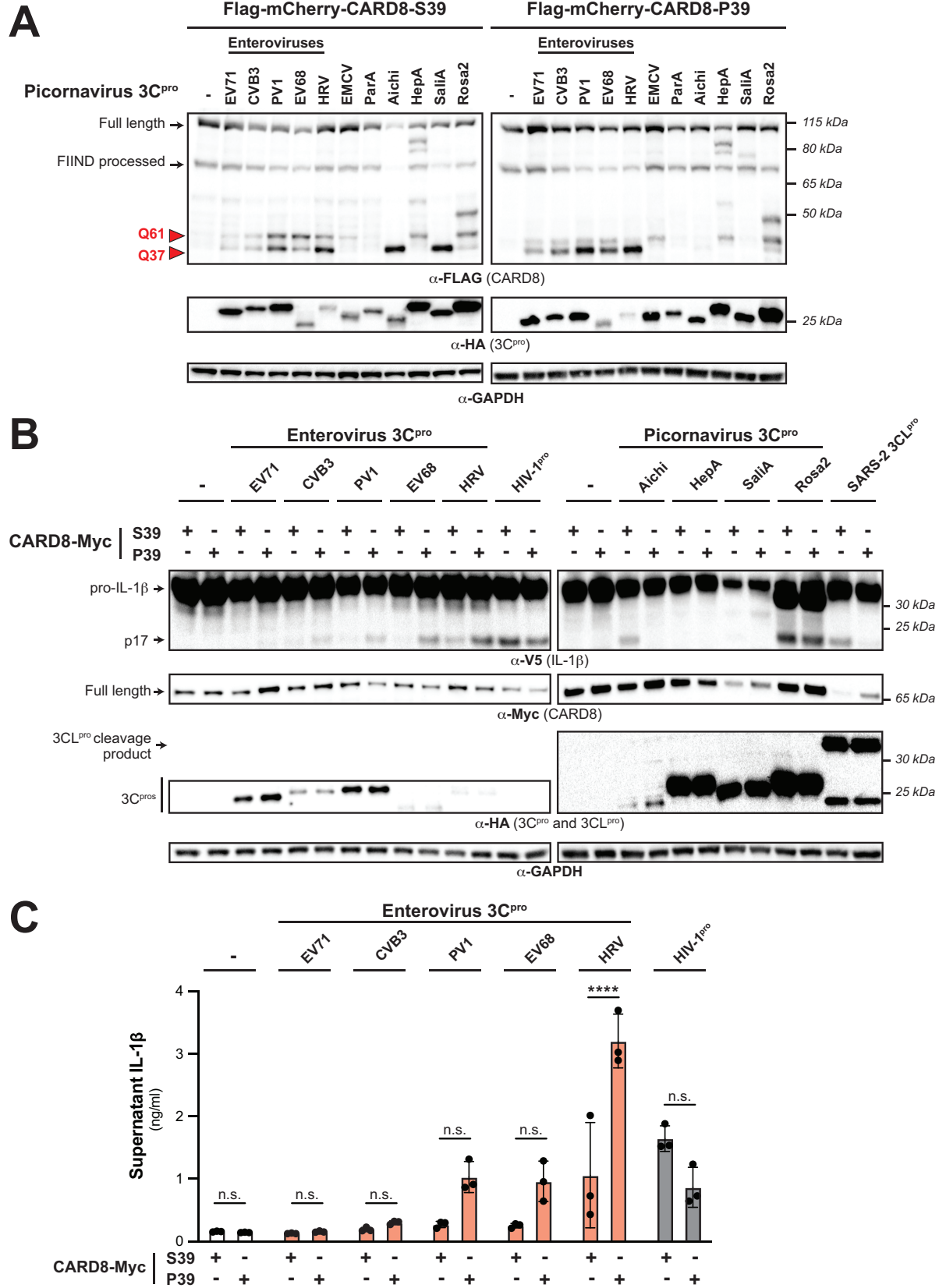


					p-val	
		34	IRLQ	GSRK	41	not detected
Human reference (S39)					
SNP rs12463023 (P39)		IRLQ	G P RK	0.00108

800

801 **S11 Fig. A human SNP is predicted to result in a CARD8 protein that is cleavable by**
802 **picornavirus 3C^{pro}s.**

803 A previously generated 3C^{pro} consensus cleavage motif for enteroviruses (a genus within
804 picornaviruses), in which a proline is most common in the P2' position, is shown (11). Using this
805 motif to search the human reference CARD8 protein sequence, which contains a serine at residue
806 39 (S39), does not generate a predicted cleavage site at Q37. However, a human SNP
807 (rs12463023) results in a proline at residue 39 (P39), which generates a predicted 3C^{pro} cleavage
808 site.



810 **S12 Fig. The CARD8 P39 variant is variably susceptible to picornavirus 3C^{pro}s.**

811 (A) WT HEK293T cells were co-transfected using the indicated Flag-tagged mCherry-CARD8
812 fusion plasmid constructs with V5-IL-1 β , CASP1, and HA-tagged 3C^{pro} constructs or empty
813 vector ('-'). 3C^{pro}s from the following viruses were used: enterovirus A71 (EV71),
814 coxsackievirus B3 (CVB3), poliovirus 1 (PV1), enterovirus D68 (EV68), human rhinovirus A
815 (HRV), encephalomyocarditis virus (EMCV), human parechovirus A (ParA), Aichi virus
816 (Aichi), hepatitis A virus (HepA), human salivirus A (SaliA), and human rosavirus 2 (Rosa2).
817 Red arrows denote CARD8 fragments resulting from 3C^{pro} cleavage at indicated sites. (B)
818 CARD8 inflammasome activation assay, where inflammasome activation is measured by
819 CASP1-dependent processing of pro-IL-1 β to p17. CARD8 knockout HEK293T cells were co-
820 transfected with Myc-tagged constructs encoding the reference allele of human CARD8 (protein
821 accession NP_001338711, mRNA accession NM_001351782.2) or the human CARD8 non-
822 synonymous single nucleotide polymorphism (SNP) CARD8-P39 (rs12463023) and the
823 indicated 3C^{pro}, 3CL^{pro}, or HIV-1^{pro} constructs, or empty vector ('-'). (C) IL-1 β assay, which
824 measures the release of bioactive IL-1 β in the culture supernatant of cells transfected as
825 described in (B). Individual values (n=3), averages, and standard deviations shown are
826 representative of experiments performed in triplicate. Data were analyzed using one-way
827 ANOVA with Tukey's post-test. n.s. = not significant. **** = p<0.0001.
828

- 829 **S1 Table. VIPR and RefSeq betacoronaviral polyproteins with 3CL cleavage site**
830 **concatenations.**
- 831 **S2 Table. Betacoronaviral polyproteins with unique 8mer 3CL cleavage site**
832 **concatenations.**
- 833 **S3 Table. Accession numbers of CARD8 sequences used for evolutionarily analyses.**
- 834 **S4 Table. Codon positions in full length CARD8 from hominoids and Old World monkeys**
835 **predicted to be evolving under recurrent positive selection by PAML, FUBAR, and FEL**
836 **analyses.**
- 837 **S5 Table. CARD8 missense variants (>100 allele counts) mapped to GRCh38 reported in**
838 **gnomAD v3.1.2.**
- 839 **S6 Table. CARD8 missense variants (>100 allele counts) mapped to GRCh38 reported in**
840 **gnomAD v3.1.2 represented by population with the highest allele frequency.**
- 841 **S7 Table. Primers, gBlocks, Twist fragments, and sgRNA.**
- 842 **S8 Table. List of antibody specifications.**