

1 **Positive selection analyses identify a single WWE**  
2 **domain residue that shapes ZAP into a super**  
3 **restriction factor**

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## 15 **Abstract**

16 The host interferon pathway upregulates intrinsic restriction factors in response to viral infection.  
17 Many of them block a diverse range of viruses, suggesting that their antiviral functions might have  
18 been shaped by multiple viral families during evolution. Virus-host conflicts have led to the rapid  
19 adaptation of viral and host proteins at their interaction hotspots. Hence, we can use evolutionary  
20 genetic analyses to elucidate antiviral mechanisms and domain functions of restriction factors.  
21 Zinc finger antiviral protein (ZAP) is a restriction factor against RNA viruses such as alphaviruses,  
22 in addition to other RNA, retro-, and DNA viruses, yet its precise antiviral mechanism is not fully  
23 characterized. Previously, an analysis of 13 primate ZAP identified 3 positively selected residues  
24 in the poly(ADP-ribose) polymerase-like domain. However, selective pressure from ancient  
25 alphaviruses and others likely drove ZAP adaptation in a wider representation of mammals. We  
26 performed positive selection analyses in 261 mammalian ZAP using more robust methods with  
27 complementary strengths and identified 7 positively selected sites in all domains of the protein.  
28 We generated ZAP inducible cell lines in which the positively selected residues of ZAP are  
29 mutated and tested their effects on alphavirus replication and known ZAP activities. Interestingly,  
30 the mutant in the second WWE domain of ZAP (N658A) is dramatically better than wild-type ZAP  
31 at blocking replication of Sindbis virus and other ZAP-sensitive alphaviruses due to enhanced  
32 viral translation inhibition. The N658A mutant inhabits the space surrounding the previously  
33 reported poly(ADP-ribose) (PAR) binding pocket, but surprisingly has reduced binding to PAR. In  
34 summary, the second WWE domain is critical for engineering a super restrictor ZAP and  
35 fluctuations in PAR binding modulate ZAP antiviral activity. Our study has the potential to unravel  
36 the role of ADP-ribosylation in the host innate immune defense and viral evolutionary strategies  
37 that antagonize this post-translational modification.

## 38 **Author summary**

39 Host proteins and viral proteins that encounter one another are locked in a perpetual genetic arms  
40 race. In this evolutionary race, a mutation that confers a survival advantage will become more  
41 frequent in the population. By looking at the sequences of genes that are known to have antiviral  
42 roles in mammals, we can identify the exact sites where a host and viral protein have interacted  
43 and gain insight into how an antiviral protein works. Here, we identified these sites in zinc finger  
44 antiviral protein (ZAP), a host protein that blocks many different viruses. We found that changing  
45 one of the sites from the original amino acid to another dramatically improves ZAP's antiviral  
46 activity against Sindbis virus, an alphavirus, due to improved inhibition of viral translation. Our  
47 mutation is also better at inhibiting other members in the *Alphavirus* genus. We observed that our  
48 mutant ZAP has reduced ability to bind poly(ADP-ribose), a post-translational modification that is  
49 targeted by alphaviruses for productive infection. Our findings help us better understand how  
50 viruses have shaped the evolution of broad-spectrum host antiviral proteins, with great  
51 implications for the engineering of super restriction factors.

## 52 **Introduction**

53 Viral and host proteins are constantly engaging in genetic conflicts that create selective pressures  
54 on the other side to evolve. In a host innate immune protein, an advantageous mutation that  
55 successfully maintains recognition of a viral protein or evades a viral antagonist will rise in  
56 frequency, a phenomenon called positive selection. The amino acid sites on which positive  
57 selection have acted can be identified by bioinformatic approaches when the non-synonymous  
58 substitution rate is estimated to exceed the synonymous substitution rate (1,2). The signatures of  
59 positive selection on a protein can inform us about historical interaction hotspots between the host  
60 and virus (3), as well as highlight sites that have important antiviral roles in winning the host-virus  
61 arms race.

62 Signatures of positive selection are especially prevalent in host interferon (IFN)-stimulated genes  
63 (ISGs) that are induced to counteract viral infections (3). One of these ISGs is zinc finger antiviral  
64 protein (ZAP), also known as poly(ADP-ribose) polymerase 13 (PARP13) (4). ZAP inhibits a  
65 diverse range of virus genera, yet its antiviral activity can be specific to particular members in a  
66 genus, suggesting viral evasion or antagonism of ZAP inhibition (5,6). For example, ZAP blocks  
67 many species of mosquito-borne alphaviruses to varying degrees, where Sindbis virus (SINV)  
68 and Ross River virus (RRV) are more sensitive than o'nyong'nyong virus (ONNV) and  
69 chikungunya virus (CHIKV) vaccine strain 181/clone 25 (7,8). Alphaviruses have a positive-sense  
70 RNA genome, which can be immediately translated into viral proteins by host ribosomes upon  
71 entry into the host cell (9,10). The viral proteins then replicate the viral genome, leading to the  
72 production of structural proteins and the assembly of mature virus particles. It is in the early stages  
73 of infection that ZAP acts to prevent the translation of alphaviral RNA by synergizing with the host  
74 E3 ubiquitin ligase, tripartite motif containing 25 (TRIM25) (11,12).

75 ZAP has two major splice isoforms, ZAPS (short) and ZAPL (long), with distinct antiviral and  
76 immunomodulatory activities (7,13–15). Recently discovered isoforms, ZAPM (medium) and  
77 ZAPXL (extralong), resemble the antiviral activities of ZAPS and ZAPL, respectively (7). The N-  
78 terminus of ZAP contains four zinc fingers (ZnFs) that bind RNA. It is followed by a fifth ZnF and  
79 two WWE domains, named for its motif containing tryptophan, tryptophan, and glutamic acid. The  
80 ADP-ribose-binding ability of the second WWE domain (WWE2) has only been recently  
81 discovered (16,17). At the C-terminus, there is a PARP-like domain. Despite being one of the 17  
82 PARPs, ZAP is the only PARP with a PARP-like domain that is catalytically inactive and cannot  
83 ADP-ribosylate substrates (18,19), but confers more antiviral activity on the longer isoforms  
84 (7,15,20,21). Even though the RNA binding activity of ZAP has been extensively studied, how the  
85 other domains contribute to ZAP antiviral activity are not well characterized.

86 While ZAP has been shown to be positively selected (15,22), there are outstanding questions  
87 about the antiviral mechanism of ZAP and how its cellular functions contribute to viral inhibition.  
88 A previous study performed positive selection analysis on ZAP sequences from 13 primate  
89 species and found 3 positively selected sites, all in the PARP-like domain. We aimed to expand  
90 upon this study because ZAP is effective against diverse groups of viruses including but not  
91 limited to lentiviruses. ZAP has a long-standing history of host-virus interactions and likely arose  
92 from a gene duplication event after the divergence of tetrapods (23). Assuming that at least some  
93 of the positively selected sites are driven by the ancestors of extant ZAP-sensitive viruses (e.g.  
94 alphaviruses, flaviviruses, coronaviruses, etc.), we would expect to detect positive selection  
95 signals from a broader range of mammals which these viruses tend to infect.

96 Here, we performed positive selection analyses on 261 mammalian ZAP sequences using four  
97 complementary and sophisticated models that make more realistic assumptions about the  
98 substitution rates. We identified 7 residues that are positively selected in ZAP, most of which are  
99 outside the PARP-like domain. We mutated each positively selected site and found that one  
100 mutant in the WWE2 (N658A) has antiviral activity that is almost 10 times stronger than wild-type  
101 (WT) ZAP against SINV, creating a super restrictor that is more antiviral than any versions of ZAP  
102 that were previously reported. The N658A mutant is more efficient than ZAPL WT at inhibiting  
103 virion production of SINV and replication of a panel of alphaviruses in a manner that is dependent  
104 on viral translation suppression. Interestingly, mutation of both positively selected sites in the  
105 WWE2 that form a potential interaction surface does not further increase the antiviral activity of  
106 ZAP.

107 We then investigated the role of viral RNA binding, TRIM25 interaction, IFN response, and  
108 poly(ADP-ribose) (PAR) binding in mediating the activity of a super restrictor ZAP. We found that  
109 the superior antiviral activity of the N658A mutant can be attributed to changes in PAR binding by  
110 the ZAPL mutant. We mutated site 658 to orthologous residues found in other mammalian species

111 and observed that all of them improved ZAP inhibition against SINV. This surprising finding  
112 suggests that evolutionary forces did not steer human ZAP to be the most antiviral, at least not  
113 against alphaviruses. By taking into account the history of host-virus conflicts, positive selection  
114 analyses allow us to identify specific sites with high impact on the effectiveness of the host antiviral  
115 program, providing a blueprint for generating super restriction factors.

## 116 **Results**

### 117 **ZAP is positively selected throughout mammalian evolution at novel sites**

118 We used the longest isoform of ZAP, ZAPXL, to curate and align 261 high quality mammalian  
119 orthologs. We ran 4 positive selection tests with complementary strengths on the alignment of  
120 mammalian ZAP sequences: Fixed Effects Likelihood (FEL); Mixed Effects Model of Evolution  
121 (MEME); Fast, Unconstrained Bayesian AppRoximation (FUBAR); and the Bayesian mutation-  
122 selection model by Rodrigue *et al* (24–27). FEL does not make assumptions about the distribution  
123 of selection parameters over sites but assigns independent nonsynonymous and synonymous  
124 rates to each site. MEME accounts for the fact that positive selection occurs episodically, rather  
125 than remaining constant over time. FUBAR improves upon random effect likelihood models (28)  
126 by implementing more parametrically complex models. Rodrigue *et al.*'s method is the first  
127 Bayesian mutation-selection model, offering higher sensitivity.

128 To validate the robustness of our tests, we ran the 13 primate ZAP sequences from the study by  
129 Kerns *et al.* and were able to replicate the 3 positively selected sites previously identified. Using  
130 the 261 mammalian ZAP, we identified 7 positively selected sites that are shared by all 4 tests  
131 (S1A Fig) and mapped them to human ZAP isoforms (S1B Fig). For consistency, the positively  
132 selected sites are numbered in the context of ZAPL and ZAPS, which are the more well studied  
133 isoforms with antiviral activities similar to ZAPXL and ZAPM, respectively. The positively selected  
134 sites we identified are concentrated in specific regions spanning across the ZAP gene (Fig 1A).  
135 Two of these sites are within the first 254 amino acids of the protein, which comprise the RNA

136 binding domain that is necessary for ZAP recognition and inhibition of viral RNA. These residues,  
137 Q28 and C38, are relatively close to each other but are positioned opposite the RNA binding  
138 groove, with both of their side chains pointing away from the rest of the structure (29) (Fig 1B).  
139 We had not expected any sites to be in the RNA binding domain because RNA binding is an  
140 essential function of ZAP. However, the identification of these two sites raises the possibility that  
141 viral proteins can interact with ZAP at a different location in its N-terminal region without interfering  
142 with binding to viral RNA.

143 More than half of the positively selected sites are in the central domain, 3 of which are tightly  
144 clustered in the WWE2, which has only recently been found in ZAP to bind PAR. When mapped  
145 to the available crystal structure of the central region consisting of the fifth zinc finger and the two  
146 WWE domains (16,17), two of the sites, N658 and A672, are next to the PAR binding pocket and  
147 face outward, supporting that they are at the interface of host-virus interactions (Fig 1C). Taken  
148 together, our positive selection analyses demonstrate that ZAP has been rapidly evolving not just  
149 during primate evolution, but also during mammalian evolution. These novel positively selected  
150 residues in ZAP are found in all domains of ZAP, suggesting that ancient viruses have likely  
151 targeted and antagonized ZAP at distinct sites.

### 152 **Most of the positively selected sites affect ZAP antiviral phenotype against SINV**

153 To probe the effect of the positively selected sites, we mutated each site from the WT amino acid  
154 in humans to alanine because alanine is chemically inert and would not dramatically change the  
155 secondary structure of the protein (30). In the case where the WT amino acid is alanine, we  
156 mutated it to valine, the next closest amino acid. We cloned either WT or mutant ZAPS and ZAPL  
157 with an N-terminal FLAG tag into the ePiggyBac (ePB) transposon system and generated stable  
158 cell lines in ZAP knockout (KO) HEK293T cells (31,32). We tested the mutants in the ZAPS and  
159 ZAPL background because ZAPS and ZAPL are most commonly studied and have comparable  
160 antiviral activities to ZAPM and ZAPXL, respectively.

161 Almost all the mutant cell lines have robust ZAP expression when induced by doxycycline, with  
162 the exception of ZAPS Q28A which appears to have a truncation at the C-terminus, as it is still  
163 able to be detected by the N-terminal FLAG tag (Fig 2A). Since our candidate sites are positively  
164 selected throughout mammalian evolution, we chose to test their antiviral activity against  
165 alphaviruses, whose primary hosts are mammals such as primates, horses, and rodents (33). We  
166 first infected the ZAP cell lines with SINV, a prototype alphavirus that is susceptible to ZAP  
167 inhibition.

168 We infected ZAPS and ZAPL WT and mutant cell lines with a luciferase-expressing SINV reporter  
169 virus. Despite differences in absolute fold inhibitions between independent experiments featuring  
170 ZAPS and ZAPL mutants, we found that ZAPL WT is invariably more antiviral than ZAPS WT,  
171 consistent with previous reports (7,15). While a couple of mutants are considerably less antiviral  
172 than the corresponding WT ZAP (ZAPS Q28A and C38A; ZAPL Q28A and H551A), most mutants  
173 have enhanced antiviral activity, as evidenced by a greater fold inhibition than the corresponding  
174 WT ZAP (ZAPS mutants ranging from 16- to 123-fold vs. 12-fold for ZAPS WT; and ZAPL mutants  
175 ranging from 103- to 580-fold vs. 78-fold for ZAPL WT). These results suggest that most PS sites  
176 are not evolutionarily optimized for anti-SINV activity in humans and *could* be enhanced (Fig 2B  
177 & Fig 2D). Notably, the N658A mutant located in the WWE2 shows a significant improvement in  
178 ZAP antiviral activity (10 times better than ZAPS WT and 7 times better than ZAPL WT). In  
179 addition, some mutants displayed isoform-specific effects. For instance, ZAPL C38A is more  
180 antiviral than ZAPL WT, but its ZAPS counterpart is less antiviral than ZAPS WT. These results  
181 suggest that altering the WT amino acid at these positively selected sites *a posteriori* changes  
182 the antiviral activity of ZAP against SINV—many stronger than before—and that adaptations at  
183 these sites have important functional consequences.

184 Since both sites 658 and 672 are located in the WWE2 and flank the PAR binding pocket in the  
185 crystal structure (Fig 1A & 1C), we wondered if the two sites constitute a critical host-virus



186 interface in concert, as is the case with TRIM5 $\alpha$  (34). We generated the double mutant  
187 N658A/A672V (NA) in the same ZAP KO ePB system and assessed its ability to restrict SINV  
188 replication. Both ZAPS and ZAPL NA double mutants are as stably expressed as the single  
189 mutants (Fig 3A & 3C). To our surprise, the antiviral activity of the ZAPS NA double mutant is not  
190 an intermediate between ZAPS N658A (33x) and A672V (5x); rather, it reduces the antiviral  
191 activity of N658A to that of ZAPS WT and A672V (Fig 3B, 4 to 5x), suggesting that A672V has a  
192 dominant negative effect on N658A in ZAPS. The ZAPL NA double mutant increases antiviral  
193 activity against SINV replication by less than 2-fold (103x vs. 58x for ZAPL WT). However, it does  
194 not approach the strength of ZAPL N658A (224x) and A672V (225x) (Fig 3D). The differential  
195 antiviral activity of the A672V single mutant and the NA double mutant in ZAPS and ZAPL again  
196 highlights isoform specificity at particular sites. Together, the WWE2 mutations in combination  
197 lessen the increase in antiviral activity we observed with the single N658A mutation in both ZAPS  
198 and ZAPL backgrounds, suggesting that these mutations may not act as a single protein  
199 interaction surface.

#### 200 **The ZAPL N658A mutant blocks the early steps of alphaviral infection more effectively**

201 We were interested by the superior antiviral activity of the N658A mutant alone and focused on  
202 the ZAPL isoform to study the mutant in the presence of all domains of ZAP, including the PARP-  
203 like domain. We wanted to determine whether the effects on viral replication impact the overall  
204 virion production. We infected ZAPL WT or N658A cells with SINV and collected the cell  
205 supernatant containing mature and released virions at 0, 6, 12, 24, and 36 hours post-infection  
206 (h.p.i.). We determined the viral titer on BHK-21 cells via plaque assay. We found that both ZAPL  
207 WT and N658A significantly inhibited SINV virion production, but at 24 h.p.i., ZAPL N658A is  
208 about 4-fold more inhibitory (Fig 4A, 11x vs. 40x), consistent with the phenotype we observed  
209 with viral replication.

210 Next, we sought to determine the stage in the viral life cycle at which the ZAPL N658A mutant  
211 acts. Because ZAP is known to act by blocking alphaviral RNA translation, we tested the positively  
212 selected ZAP mutant N658A against a temperature-sensitive replication-deficient SINV luciferase  
213 reporter virus that cannot replicate at 40°C (35). We infected ZAP WT and N658A cell lines with  
214 the replication-deficient virus at the non-permissive temperature and found that the N658A mutant  
215 is better at blocking SINV RNA translation (Fig 4B). Our finding supports that the superior antiviral  
216 activity of the N658A mutant is likely due to an enhanced block at the step of incoming viral RNA  
217 translation.

218 Since we hypothesized that the positive selection of ZAP may be driven by ancient alphavirus-  
219 like viruses, we tested whether the N658A mutant also inhibits other alphaviruses better. We  
220 infected the ZAPL WT or N658A cell line with GFP-expressing SINV, RRV, ONNV, CHIKV vaccine  
221 strain 181/clone 25, and VEEV. Alphaviruses known to be more sensitive to ZAP inhibition are  
222 more inhibited by the N658A mutant (Fig 5A, 5x vs. 38x against SINV; Fig 5B, 24x vs. 132x against  
223 RRV), while the ones that are less sensitive were similarly resistant to both ZAPL WT and N658A  
224 (Fig 5C, 3.8x vs. 8.3x against ONNV; Fig 5E, 1.1x vs. 1.1x against VEEV). Interestingly, even  
225 though we previously observed that the non-reporter CHIKV vaccine strain is less susceptible to  
226 ZAP inhibition (7), we saw that both ZAPL WT and N658A dramatically inhibited GFP-expressing  
227 CHIKV vaccine strain, with the N658A mutant being more antiviral than WT (Fig 5D). Since the  
228 CHIKV strain we tested expresses the GFP reporter under the control of the viral subgenomic  
229 promoter, our results suggest that ZAP might inhibit step(s) at or prior to viral subgenomic mRNA  
230 expression.

231 **The improved antiviral activity of the N658A mutant is not due to changes in binding to**  
232 **SINV RNA, interaction with TRIM25, or increased activation of ISGs**

233 To determine the mechanism of the enhanced antiviral activity of the N658A mutant, we  
234 characterized the mutant in terms of known abilities of ZAP. Since ZAP is recognized as a sensor

235 of CpG-rich viral RNA, we wondered if N658A binds better to SINV genomic RNA than ZAPL WT  
236 does. We performed an *in vitro* RNA pulldown assay by incubating protein lysates from either the  
237 ZAPL WT or N658A cell line with equal amounts of biotinylated SINV genomic RNA. We pulled  
238 down the biotinylated viral RNA using streptavidin beads and probed for ZAP. We generated and  
239 tested a ZAP KO HEK293T cell line with inducible expression of a ZAPS C86A/Y96A mutant  
240 (ZAPS CY), which is deficient in RNA binding (36,37), as negative control. As expected, markedly  
241 less ZAPS CY is bound to equal amounts of SINV RNA compared to ZAPL WT. Equal amounts  
242 of ZAPL WT and ZAPL N658A are bound to SINV RNA (Fig 6A), suggesting that factors other  
243 than viral RNA binding may contribute to the enhanced antiviral activity of the mutant.

244 We then asked whether the N658A mutant changes ZAP's ability to interact with TRIM25, a host  
245 E3 ubiquitin ligase that is a requisite cofactor for ZAP's inhibition of viral RNA translation (11,12).  
246 We transfected 3XFLAG-ZAPL and myc-TRIM25 into ZAP KO HEK293T cells and performed a  
247 co-immunoprecipitation with FLAG beads. We found that ZAPL WT and N658A interact with  
248 TRIM25 similarly (Fig 6B), suggesting that the increased antiviral activity of the N658A mutant is  
249 not related to changes to its synergy with TRIM25.

250 We further evaluated whether increased IFN induction is responsible for the enhanced antiviral  
251 activity of the ZAPL N658A mutant. After treating ZAPL WT and N658A cell lines with poly(I:C), a  
252 double-stranded RNA mimic, to stimulate the IFN response, we performed quantitative PCR  
253 analysis of the mRNA levels of IFN- $\beta$  and IFIT1, a classical antiviral ISG. We found that poly(I:C)  
254 treatment upregulates IFN- $\beta$  and IFIT1 levels, and expression of ZAPL WT and N658A further  
255 augments the response (S2 Fig). Importantly, both IFN- $\beta$  and IFIT1 induction levels in ZAPL WT  
256 and N658A cell lines are similar upon stimulation (S2 Fig), ruling out a heightened IFN response  
257 as responsible for the N658A improved antiviral phenotype.

258 **The ZAPL N658A mutant has reduced binding to PAR**

259 Since RNA binding, TRIM25 interaction, and the IFN response do not appear to mediate the  
260 superior antiviral activity of ZAPL N658A, we decided to characterize the effects of the mutation  
261 on WWE domain function. The WWE2 in ZAP has recently been found to bind to PAR, an ability  
262 that enhances ZAP's antiviral function against a CpG-enriched HIV-1 (17). We wondered if  
263 mutating site 658, which is within the WWE2, changes ZAP's ability to bind to PAR. We performed  
264 a co-immunoprecipitation assay in which we pulled down ZAP and probed for PAR. PAR levels  
265 in the whole cell lysate are markedly lower in cells without ZAP induced (Fig 6C). Compared to  
266 ZAPL WT, ZAPL N658A binds to less PAR (Fig 6C). Altogether, these data suggest that an  
267 alanine mutation at site 658 negatively impacts ZAPL's ability to bind PAR, despite the site being  
268 outside of the PAR binding groove. The mutation might prevent an active PARP from accessing  
269 and PARylating ZAPL in an uninfected cell. Contrary to the Q668R mutation in the PAR binding  
270 pocket which diminishes ZAP PAR binding and anti-HIV activity (17), our N658A mutant is less  
271 proficient in binding PAR, but surprisingly more adept at restricting SINV.

272 **Asparagine is the predominant amino acid at site 658 in ZAP yet the least antiviral**

273 To further understand the requirements at site 658 for ZAP to become a super restrictor, we  
274 analyzed the amino acid distribution in our mammalian ZAP sequences. We observed that site  
275 28, one of the positively selected sites, displays an even distribution of amino acids (Fig 7A). In  
276 contrast, at site 658, asparagine is the most prevalent amino acid in our 261 mammalian ZAP  
277 sequences (68%, Fig 7A). In terms of the amino acid property, there is less variation at site 658  
278 than at site 28. Even though site 658 has rapidly evolved, polar amino acids seem to be favored  
279 by evolution. 80% of the mammals in our alignment have a polar amino acid at site 658: 177 out  
280 of the 261 mammals (68%) have asparagine and 32 (12%) have serine (Fig 7A). This is in stark  
281 contrast to Q28, where every amino acid property is present: 7% have a nonpolar amino acid  
282 (alanine, glycine); 38% have a polar amino acid (glutamine, asparagine, serine); 38% have a

283 negatively charged amino acid (aspartic acid, glutamic acid); and 23% have a positively charged  
284 amino acid (histidine, lysine, arginine) (Fig 7A & Fig 7C), demonstrating that site 28 is able to  
285 tolerate more flexibility in the chemical property of its amino acid. Sites that are not under positive  
286 selection, 657 and 659, show even less amino acid diversity (Fig 7B). Site 657 is dominated by a  
287 polar (glutamine) or positive (arginine) amino acid, and site 659 permits only nonpolar amino acids  
288 with an aromatic ring (tyrosine and phenylalanine).

289 To ascertain if a specific amino acid or a nonpolar property is required at site 658 to achieve better  
290 antiviral activity, we generated additional ZAPL N658 mutants by mutating the WT residue in  
291 humans, asparagine, to residues found in other mammalian species such as glycine (nonpolar;  
292 in African woodland thicklet rat), serine (polar uncharged; in California deer mouse), lysine  
293 (positive; in greater bamboo lemur), or aspartic acid (negative; in little brown bat). We infected  
294 cell lines with inducible expression of each of these ZAPL site 658 mutants with the same  
295 luciferase-expressing SINV and found that all of them exhibit higher antiviral activity (Fig 7C),  
296 including the polar N658S, although none attains the strength of alanine mutation. Taken together,  
297 these findings suggest that just mutating asparagine to another amino acid is sufficient to improve  
298 ZAP, but the ones with the best antiviral activities (N658A and N658D) are either nonexistent or  
299 rare in nature. Further studies are required to understand why positive selection has selected for  
300 a version of ZAP that does not maximize its antiviral activity.

## 301 **Discussion**

302 In this study, we sought other positively selected sites beyond the 3 previously identified in the  
303 PARP-like domain of ZAP and asked whether they have also been shaped into interaction  
304 interfaces during evolution. We identified 7 positively selected sites in total throughout mammalian  
305 evolution of ZAP, with only 1 residing in the PARP-like domain, supporting the notion that ZAP  
306 has been the target in more than one host-virus arms race. Notably, 4 of these positively selected  
307 sites are concentrated in the central region. We found that mutating each of these 7 positively

308 selected sites confers differential antiviral activities against SINV. Specifically, a mutation at the  
309 WWE2 (N658A) was almost 10 times better at inhibiting SINV and other Old World alphaviruses  
310 than WT ZAP. In line with a deep mutational scanning study of TRIM5 $\alpha$  (38), we observed that it  
311 is possible for a positively selected site to maintain strong antiviral activity when mutated to other  
312 amino acids.

313 Most analyses of positive selection in innate immune factors have focused on a subset of species.  
314 For example, using 17 primate TRIM5 $\alpha$  sequences, Sawyer *et al.* identified 5 residues under  
315 positive selection all within a 13-amino acid patch that is responsible for species specificity against  
316 lentiviruses (34). Enabled by the more comprehensive sequences and robust codon substitution  
317 models presently, we hypothesized that including more species would allow us to detect positive  
318 selection signatures in regions across the whole protein and provide a more well-rounded picture  
319 of antiviral effectors. Consistent with a study that identified distinct positively selected sites in  
320 SAMHD1 using different subsets of mammals (39), we found that positively selected sites in ZAP,  
321 while concentrated, are not just restricted to the PARP-like domain (15), but span the N-terminus,  
322 central region, and C-terminus. This reflects the highly diverse and long evolutionary history of  
323 ZAP, which arose during the emergence of tetrapods (23). Further positive selection analyses in  
324 subsets of mammals are required to confirm if each positively selected site or domain is driven  
325 by distinct viruses.

326 We found that mutating the N658 site in the WWE2 of ZAP results in a ZAP that has stronger anti-  
327 alphavirus function yet diminishes PAR binding ability. ADP-ribosylation may be a post-  
328 translational modification exploited by alphaviruses, as a productive alphaviral infection relies on  
329 the binding to and removal of ADP-ribose by the highly conserved alphaviral macrodomains  
330 encoded by nonstructural protein 3 (40–43). It is possible that having less PAR bound to ZAPL  
331 N658A minimizes interaction between the SINV macrodomain and ZAP, thus allowing ZAP to  
332 evade recognition by a viral antagonist. Alternatively, decreased PAR binding to the ZAPL N658A

333 mutant may also be a way to reduce PAR-dependent ubiquitination of ZAP to prevent ZAP  
334 degradation (44). Further studies are required to understand how macrodomains, PARylation,  
335 and/or ubiquitination play a role in virus infection.

336 Why has evolution selected for an amino acid at site 658 that makes a less antiviral version of  
337 ZAP in humans? One possibility is that having a stronger antiviral activity incurs a fitness cost on  
338 the host cell by interfering with non-immune-related cellular functions of ZAP. In cells not infected  
339 by a virus, PAR was bound to ZAP; when cells were treated with arsenite to induce stress granule  
340 formation, the amount of PAR on ZAP increased and miRNA-mediated silencing decreased (45).  
341 While the direct mRNA targets bound by ZAP and the miRNA complex remain mostly unknown,  
342 ZAP is implicated in the regulation of host transcripts in a non-viral context. A recent RNA-seq  
343 analysis also discovered that ZAPS and ZAPL bind to host mRNAs involved in the unfolded  
344 protein response and the epithelial-mesenchymal transition (46). Indeed, many genes that show  
345 strong signatures of positive selection participate in both the proper functioning of the cell and the  
346 host-virus conflict. An example gene is the Niemann-Pick C1 protein, which is an intracellular  
347 cholesterol transporter and a viral receptor for filoviruses (47,48). It would be interesting to explore  
348 if any intrinsic cellular functions of ZAP are affected by the more antiviral N658A mutation.

349 ZAP is a broad-spectrum antiviral protein that is effective against members from a wide range of  
350 virus families. Therefore, it is possible that some of our positively selected sites did not have a  
351 dramatically better antiviral effect compared to WT ZAP because the selection at these other sites  
352 were driven by ancient viruses that were not alphavirus-like. We wonder how our other positive  
353 selection mutants would behave against other viruses that infect mammals as their primary  
354 reservoir hosts. For instance, alphaviruses and flaviviruses share similar transmission cycles  
355 where they circulate between wild mammals and domestic mammalian dead-end hosts.  
356 Coronaviruses also commonly exploit mammals as hosts, such as camels for MERS and bats for  
357 SARS-CoV-1. If flavivirus- or coronavirus-like viruses drove the positive selection of ZAP, we

358 expect to see a greater impact on its antiviral activity when ZAP mutants are tested against those  
359 viruses. Alternatively, viruses that are not susceptible to the increased antiviral activity of the  
360 N658A mutant might encode viral antagonists of ZAP. Notably, we saw that there was no  
361 difference in the ability of ZAPL WT and N658A to inhibit VEEV. It is possible that VEEV encodes  
362 a viral antagonist that can still recognize ZAP despite the mutation and thus is impervious to any  
363 improvement in ZAP's antiviral activity. To determine whether the positively selected sites form  
364 an exclusive interaction interface, future studies should test more viruses from different families.  
365 Our study is one of the first that look at positive selection of a broad-spectrum antiviral protein in  
366 a comprehensive and diverse group of mammals. By understanding what makes a super-  
367 restrictor and the host cell constraints, we can design better antiviral therapeutics that have the  
368 potential to outrun the virus in the host-virus arms race.

## 369 **Author contributions**

370 S.H. and M.M.H.L. conceptualized and designed the study. S.H. and J.G. performed the  
371 experiments and analyzed the data. L.N. generated the ePB 3XFLAG ZAPS/L WT single cell  
372 clones and provided technical guidance on the RNA binding assay. D.E. curated the alignment of  
373 mammalian ZAP, generated the phylogeny tree, and provided expertise in bioinformatics. S.H.  
374 wrote the first draft of the manuscript with substantial help from J.G. M.M.H.L. provided critical  
375 feedback and S.H. edited subsequent drafts. All authors contributed to manuscript revision, read,  
376 and approved the submitted version.

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## 391 **Materials and methods**

### 392 **Cell culture**

393 HEK293T (parental and ZAP knockout) cells were gifts from Dr. Akinori Takaoka at Hokkaido  
394 University (32) and maintained in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher  
395 Scientific, Waltham, MA) with 10% fetal bovine serum (FBS; Avantor Seradigm, Radnor, PA).  
396 Baby hamster kidney 21 cells (BHK-21; American Type Culture Collection, Manassas, VA) cells  
397 were maintained in Minimal Essential Media (Thermo Fisher Scientific) with 7.5% FBS. 0.1mg/mL  
398 poly-L-lysine hydrobromide (Millipore Sigma, Darmstadt, Germany) and water were used to coat  
399 cell culture dishes when thawing or seeding each cell line to promote cell adhesion and recovery.

### 400 **Plasmid**

401 WT or mutant ZAP was cloned into the plasmid pcDNA3.1-3XFLAG (gift from Dr. Oliver Fregoso,  
402 University of California, Los Angeles) as previously described (37). 3XFLAG-ZAPS and -ZAPL  
403 were amplified from the pcDNA3.1-3XFLAG plasmids using primers to add ClaI and NotI  
404 restriction sites for ligation into the ePB vector (gift from Dr. Ali Brivanlou, Rockefeller University)

405 (31). Full-length TRIM25 (gift from Dr. Jae U. Jung at Cleveland Clinic Lerner Research Institute)  
406 (49) was cloned into pcDNA3.1-myc as previously described (50). ZAP positive selection mutants  
407 were generated by the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA) or  
408 synthesized as a gene block (Twist Bioscience, South San Francisco, CA) with ClaI and NotI  
409 restriction sites and ligated into the ePB vector. The identity of all plasmids was confirmed by  
410 Sanger (Genewiz/Azenta, South Plainfield, NJ) and whole-plasmid sequencing (Primordium,  
411 Monrovia, CA). See S1 File for a list of all primers used in this study.

#### 412 **Generation of ZAP inducible cell lines**

413 All ZAP inducible cell lines were made via the ePB transposon system in ZAP KO HEK293T cells.  
414 Specifically, ZAP KO HEK293T cells were transfected with equal amounts of the transposase  
415 plasmid and an ePB transposon vector containing WT or mutant ZAP using X-tremeGENE9 DNA  
416 Transfection Reagent (Roche Life Science, Basel, Switzerland) in Opti-MEM (Thermo Fisher  
417 Scientific) following manufacturer's instructions. 1µg/mL puromycin was added 48 hours post-  
418 transfection to select for ZAP KO 293T cells that have incorporated the ePB transposon. Our  
419 ZAPS WT and ZAPL WT cell lines were made by selecting single cell clones that follow two  
420 criteria: 1) robustly express ZAP following 24 hours of 1µg/mL doxycycline treatment, and 2)  
421 recapitulate differential alphaviral sensitivities (S3 Fig) similar to previously generated bulk cell  
422 lines with inducible ZAP expression (7,50). The mutant ZAP cell lines in this study were bulk cells  
423 that survived after puromycin selection. Comparable inducible ZAP expression in each cell line  
424 was validated by immunoblotting following treatment with 1µg/mL doxycycline.

#### 425 **Viruses and infections**

426 SINV (Toto1101) (51), SINV expressing luciferase (Toto1101/Luc and Toto1101/Luc:ts6) (35),  
427 SINV expressing enhanced green fluorescent protein (EGFP) (TE/5'2J/GFP) (52), RRV  
428 expressing EGFP (gift from Dr. Mark Heise, University of North Carolina) (53), ONNV expressing

429 EGFP (gift from Dr. Steve Higgs, Kansas State University) (54), CHIKV vaccine strain 181/clone  
430 25 (gift from Scott Weaver, The University of Texas Medical Branch at Galveston) (55) expressing  
431 EGFP, and VEEV vaccine strain TC-83 expressing EGFP (gift from Dr. Ilya Frolov, University of  
432 Alabama at Birmingham) have been previously described (8,50). All alphaviral stocks were  
433 generated and titered in BHK-21 cells (35).

434 ZAPS/L WT and mutant cell lines were induced for ZAP expression with 1 µg/mL of doxycycline 1  
435 day prior to virus infection. To quantify SINV replication, cells were infected with SINV with a  
436 luciferase reporter gene (Toto1101/Luc) and harvested 24 hours post-infection. To quantify SINV  
437 translation, cells were infected with a replication-deficient temperature-sensitive SINV  
438 (Toto1101/Luc:ts6) at 37°C for 1 hour to allow virus adsorption, followed by incubation at 40°C  
439 and harvested at the specified timepoints. Harvested lysates were measured for luciferase units  
440 following manufacturer's instructions of the Luciferase Assay System (Promega, Madison, WI).  
441 To determine fold inhibition, the average relative luciferase unit (RLU) of +dox was divided by that  
442 of -dox for each cell line.

443 To quantify infection by GFP-alphaviruses, infection was performed as described above and fixed  
444 in PBS with 1% FBS and 2% formaldehyde 24 hours post-infection. The fixed cells were analyzed  
445 on the Attune NxT Flow Cytometer (Thermo Fisher Scientific), courtesy of the UCLA Flow  
446 Cytometry Core. To determine fold change, the GFP percentage of +dox cells in each biological  
447 triplicate was divided by the average GFP percentage of -dox cells across biological triplicates for  
448 each independent experiment. Then, the fold changes were averaged across the independent  
449 experiments.

#### 450 **Quantification of SINV virion production via plaque assay**

451 To quantify SINV virion production in ZAPL WT or mutant cells, ZAP expression was induced by  
452 1 µg/mL doxycycline 1 day prior to infection and infected with SINV Toto1101. The viral

453 supernatant was collected at specific timepoints. To determine viral titers, BHK-21 cells were  
454 infected with the viral supernatant at 6 10-fold dilutions and incubated at 37°C for 1 hour with  
455 gentle rocking every 15 min. Avicel (RC-581 NF, pharm grade, DuPont Nutrition & Health) overlay  
456 consisting of 2X MEM and 4.5% Avicel was added to each well and the plate was incubated at  
457 37°C overnight. On the following day, cells were fixed with 7% formaldehyde for 15 minutes and  
458 stained with 1X crystal violet. The plates were washed and the plaques counted after drying.

#### 459 **Poly(I:C) stimulation, RNA extraction, and quantitative reverse transcription PCR (RT-** 460 **qPCR)**

461 To stimulate cells with a double-stranded RNA mimic, poly(I:C) diluted in Opti-MEM was  
462 incubated with Lipofectamine RNAiMax Transfection Reagent (Thermo Fisher Scientific) before  
463 being added to ZAPL WT or mutant cells. 1 day after poly(I:C) stimulation, total RNA was  
464 extracted from cells using the Quick-RNA kit (Zymo Research). The amount of RNA template was  
465 equalized for reverse transcription using the Protoscript II First Strand cDNA Synthesis Kit (New  
466 England Biolabs) and random hexamers. RT-qPCR was performed using 10-fold-diluted cDNA  
467 and the Luna Universal qPCR Master Mix (New England Biolabs) in the CFX Real-Time PCR  
468 system (Bio-Rad), courtesy of the UCLA Virology Core. qPCR conditions were as previously  
469 described (50). Target transcript levels were determined by normalizing the target transcript CT  
470 value to the RPS11 transcript CT value. Fold change was calculated using this normalized value  
471 relative to that of the corresponding cell line untreated with dox and unstimulated with poly(I:C)  
472 (CT method). For RT-qPCR primers, see S1 File.

#### 473 **Immunoblot analysis**

474 Proteins were visualized using SDS-PAGE with 4-20% Mini-PROTEAN TGX Precast Protein Gels  
475 (Bio-Rad) in NuPAGE MOPS SDS Running Buffer (Invitrogen) and transferred to a PVDF  
476 membrane (Bio-Rad). The proteins of interest were probed with the corresponding primary and

477 secondary antibodies, followed by visualization on a ChemiDoc imager (Bio-Rad, Hercules, CA)  
478 using the ProSignal Pico ECL Reagent detection reagent (Genesee Scientific, El Cajon, CA).

479 Primary antibody 1:20,000 anti-FLAG (Sigma-Aldrich), 1:20,000 anti-actin-HRP (Sigma-Aldrich),  
480 or 1:1000 anti-poly(ADP-ribose) (Abcam), and secondary antibody 1:20,000 goat anti-mouse  
481 HRP (Jackson ImmunoResearch, West Grove, PA), or 1:20,000 goat anti-rabbit HRP (Thermo  
482 Fisher Scientific) were used to probe the protein of interest. See Table S2 for more detail.

### 483 ***In vitro* biotinylation of SINV RNA and RNA pulldown assay**

484 The genomic SINV DNA template was digested by XhoI and *in vitro* transcribed using SP6 RNA  
485 polymerase (New England Biolabs) and 0.5mM biotin-16-UTP (Roche Life Science, Penzberg,  
486 Germany) as previously described (37). RNA biotinylation was confirmed by streptavidin-HRP dot  
487 blot as previously described (8).

488 *In vitro* RNA pulldown was performed as previously described (37). ZAP expression was induced  
489 in ePB ZAP cell lines and the protein lysates were harvested in CHAPS buffer (10mM Tris-HCl  
490 pH7.5, 1mM MgCl<sub>2</sub>, 1mM EDTA, 0.5% CHAPS, 10% glycerol, 5mM beta-mercaptoethanol, and  
491 protease inhibitor) 24 hours later. 0.4pmol of biotinylated SINV RNA was incubated with  
492 normalized amounts of protein lysates and RNA binding buffer containing RNaseOUT (Thermo  
493 Fisher), heparin (Sigma-Aldrich), and yeast tRNA (Thermo Fisher) to minimize non-specific  
494 binding. The lysate-RNA samples were incubated with Dynabeads M-280 Streptavidin  
495 (Invitrogen) on a shaker for 30 min at room temperature. Protein visualization on a ChemiDoc  
496 imager was as described above.

### 497 **Immunoprecipitation assays**

498 To test interaction with TRIM25, ZAP KO HEK293T cells were transfected with pcDNA3.1-  
499 3XFLAG-ZAPL and pcDNA3.1-myc-TRIM25. Cells were lysed in FLAG buffer (100mM Tris HCl

500 pH8.0, 150mM NaCl, 5mM EDTA, 5% glycerol, 0.1% NP-40, 1mM DTT, and protease inhibitor)  
501 and incubated on a rotator at 4°C for 30 min. After equilibration, FLAG beads were incubated with  
502 lysates on a rotator at 4°C for 45 min. Immunoprecipitated samples were washed 3 times with  
503 FLAG buffer and eluted in Laemmli buffer for immunoblotting.

504 PAR binding assay was based on (17) with modification. Briefly, ZAP inducible cells were lysed  
505 in lysis buffer containing 50mM Tris-HCl pH7.5, 150mM NaCl, 0.2% Triton X-100, protease  
506 inhibitor, and 1µM PARG inhibitor PDD 00017273 (Tocris Bioscience, Bristol, UK). After  
507 equilibration, FLAG beads were incubated with lysates on a rotator at 4°C for 1 hour and 30 min.  
508 Bound lysates were washed 3 times with IP buffer (50mM Tris-HCl pH7.5, 150mM NaCl, and  
509 0.2% Triton X-100) and eluted in Laemmli buffer for immunoblotting.

#### 510 **Sequence alignment, phylogenetic tree, and positive selection analysis**

511 The coding sequence (CDS) of human ZAPXL was used to search for orthologs in 260 other  
512 mammalian genome assemblies with a contig size of at least 30kb in the NCBI assembly database  
513 as of July 2020 to minimize truncated orthologous coding sequences. To extract the orthologous  
514 coding sequences of ZAP, we used best Blat reciprocal hits from the human CDS to every other  
515 mammalian genome, and back to the human genome (matching all possible reading frames,  
516 minimum identity of 30%, and the “fine” option activated).

517 The 261 orthologous ZAP were aligned to human ZAPXL with MACSE v2 with maximum accuracy  
518 settings (S2 File). The alignments generated by MACSE v2 were then cleaned by HMMcleaner  
519 using default parameters to remove errors from genome sequencing and “false exons” that might  
520 have been introduced during the Blat search. Visual inspection confirmed that the resulting  
521 alignment had a very low number of visibly ambiguous or erroneous segments.

522 The phylogenetic tree of the 261 mammals was built using IQ-Tree to generate the consensus,  
523 maximum likelihood tree with a GTR substitution model with six parameters (GTR-6) which  
524 provided the best fit (S2 File).

525 More complete details on the alignment and phylogenetic tree reconstruction are given in (56) as  
526 the same exact pipeline was used for this study.

527 The positive selection analyses FEL, MEME, and FUBAR were performed using HyPhy from the  
528 command line, with the aforementioned alignment and mammalian tree as inputs. Rodrigue *et*  
529 *al.*'s positive selection test based on a Mutation-Selection balance (MutSelomega) was used as  
530 described in (27). Briefly, Mutation-Selection balance tests attempt to provide higher statistical  
531 power to detect positive selection by better accounting for selective constraint in coding  
532 sequences, beyond the usual arbitrary use of the  $dN/dS > 1$  threshold by other selection tests.

### 533 **Statistical analysis**

534 Experiments were performed at least two independent times and statistical analyses were  
535 performed on biological replicates from triplicate wells using GraphPad Prism.

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698

## 699 **Figure captions**

### 700 **Figure 1. Identification of 7 positively selected sites across ZAP protein.**

701 (A) A schematic of the ZAPL isoform annotated with its domains. Triangles indicate positively selected  
702 sites identified from the overlap of four methods: Fixed Effects Likelihood; Mixed Effects Model of  
703 Evolution; Fast, Unconstrained Bayesian AppRoximation; and the Bayesian mutation-selection model  
704 by Rodrigue *et al.* (B) ZAP RNA binding domain bound to RNA. The structure (PDB: 6UEJ) (29) is  
705 visualized with UCSF Chimera. Positively selected Q28 and C38 residues shown in blue; RNA in  
706 orange; zinc fingers in salmon. (C) ZAP central domain bound to ADP-ribose. The structure (PDB:  
707 7TGQ) (17) is visualized with UCSF Chimera. Positively selected sites H551, S644, N658, and A672  
708 shown in green; ADP-ribose in dark orange.

### 709 **Figure 2. ZAP mutated at positively selected sites show differential antiviral activity against** 710 **SINV.**

711 (A, C) Western blot of (A) ZAPS or (C) ZAPL wild-type (WT) or positive selection mutants inducible  
712 ZAP KO 293T cell lysates. (B, D) (B) ZAPS or (D) ZAPL WT or mutant ZAP KO 293T cells were  
713 induced for ZAP 24 hours before infection with SINV Toto1101/Luc at a multiplicity of infection  
714 (MOI) of 0.01 plaque forming units (PFU)/cell and harvested at 24 hours post-infection (h.p.i.) for  
715 luciferase assay by measuring relative luciferase units (RLU). Data are combined from two  
716 independent experiments; error bars indicate standard deviation. 1µg/mL dox is used to induce  
717 ZAP expression. Asterisks indicate statistically significant differences as compared to the -dox  
718 condition for each cell line (Two-way ANOVA and Tukey's multiple comparisons test: \*, p<0.05;  
719 \*\*, p<0.01; \*\*\*, p<0.001; \*\*\*\*, p<0.0001).

### 720 **Figure 3. Mutating both positively selected sites in the second WWE domain of ZAP does** 721 **not further enhance antiviral activity.**

722 (A, C) Western blot of (A) ZAPS or (C) ZAPL WT, N658A, A672V, or N658A/A672V (NA) double  
723 mutant inducible ZAP KO 293T cell lysates. (B, D) (B) ZAPS or (D) ZAPL WT or mutant ZAP KO  
724 293T cells were induced for ZAP expression 24 hours before infection with SINV Toto1101/Luc  
725 at an MOI of 0.01 PFU/cell and harvested at 24 h.p.i for luciferase assay. Data are combined from  
726 three (B) and two (D) independent experiments; error bars indicate standard deviation. 1µg/mL  
727 dox is used to induce ZAP expression. Asterisks indicate statistically significant differences as  
728 compared to the -dox condition for each cell line (Two-way ANOVA and Tukey's multiple  
729 comparisons test: \*\*, p<0.01; \*\*\*\*, p<0.0001).

730 **Figure 4. The N658A mutant is better at inhibiting virion production and SINV RNA**  
731 **translation.**

732 ZAPL WT or N658A ZAP KO 293T cells were induced for ZAP expression with 1µg/mL dox 24  
733 hours prior to infection. Cells were infected with (A) SINV Toto1101 at an MOI of 0.01 PFU/cell,  
734 harvesting supernatant at 6, 12, 24, 36, and 40 h.p.i. for plaque assays. Viral titers of plaque  
735 assays are determined in BHK-21 cells. Data are combined from two independent experiments;  
736 error bars indicate standard deviation. Asterisks indicate statistically significant differences as  
737 compared to the -dox condition; or (B) SINV Toto1101/Luc:ts6 at an MOI of 0.01 PFU/cell, and  
738 harvested at 0, 3, and 6 h.p.i. for luciferase assay. Data are combined from two independent  
739 experiments; error bars indicate standard deviation. Asterisks indicate statistically significant  
740 differences as compared to the -dox condition for each cell line (Fisher's Least Significant  
741 Difference Test (A) or Two-way ANOVA and Tukey's multiple comparisons test (B): \*\*, p<0.01;  
742 \*\*\*, p<0.001; \*\*\*\*, p<0.0001).

743 **Figure 5. The ZAPL N658A mutant inhibits many other alphaviruses better than WT.**

744 After 24 hours of 1µg/mL dox treatment, ZAPL WT or N658A ZAP KO 293T cells were infected  
745 with (A) GFP-expressing Sindbis virus (SINV, MOI = 0.01), (B) Ross River virus (RRV, MOI = 1),



746 (C) o'nyong'nyong virus (ONNV, MOI = 1), (D) chikungunya virus (CHIKV, MOI = 0.1), or (E)  
747 Venezuelan equine encephalitis virus (VEEV, MOI = 0.1) PFU/cell for 24 hours before their  
748 percentage of infection was determined by flow cytometry. Data are combined from at least two  
749 independent experiments of biological replicates in triplicate wells; error bars indicate standard  
750 deviation.

751 **Figure 6. The improved antiviral activity of the N658A mutant is not due to changes in**  
752 **binding to SINV RNA or interaction with TRIM25, but changes in binding to poly(ADP-**  
753 **ribose) (PAR)**

754 (A) Western blot of ZAPL CY, WT, or N658A inducible ZAP KO 293T cell lysates bound to  
755 biotinylated SINV genomic RNA immunoprecipitated by streptavidin beads. Data are  
756 representative of three independent experiments. (B) Western blot of ZAP KO 293T transfected  
757 with pcDNA3.1-3XFLAG-ZAPL and pcDNA3.1-myc-TRIM25. Lysates were immunoprecipitated  
758 by FLAG beads. Data are representative of four independent experiments. (C) Western blot of  
759 ZAPL WT or N658A inducible ZAP KO 293T cell lysates immunoprecipitated by FLAG beads after  
760 treatment with 1 $\mu$ M PARG inhibitor. Data are representative of three independent experiments.  
761 1 $\mu$ g/mL dox is used to induce ZAP expression in ePB ZAP inducible cell lines.

762 **Figure 7. Asparagine is the predominant amino acid at site 658 yet confers weaker antiviral**  
763 **activity.**

764 (A, B) The distribution and (C) an abridged alignment of amino acids at sites 28, 657, 658, and  
765 659. (D) ZAPL WT or N658A ZAP KO 293T cells were induced for ZAP expression with 1 $\mu$ g/mL  
766 dox. Cells were infected with SINV Toto1101/Luc at an MOI of 0.01 PFU/cell and harvested at 24  
767 h.p.i for luciferase assay. Data are combined from two independent experiments; error bars  
768 indicate standard deviation. Asterisks indicate statistically significant differences as compared to

769 the -dox condition for each cell line (Two-way ANOVA and Tukey's multiple comparisons test: \*,  
770  $p < 0.05$ ; \*\*\*\*,  $p < 0.0001$ ).

## 771 **Supporting information**

### 772 **S1 Fig. Positive selection and domains of ZAP.**

773 (A) Positive selection analyses on ZAPXL of 261 mammalian species detected by the FEL,  
774 MEME, FUBAR, and Rodrigue methods. (B) ZAP isoforms annotated with their domains. The four  
775 ZAP splice variants are depicted here: ZAPS (short), ZAPM (medium), ZAPL (long), and ZAPXL  
776 (extra-long). All isoforms contain the zinc finger (Z1-Z5, pink) and WWE domains (green), but only  
777 ZAPXL and ZAPL have a catalytically inactive PARP-like domain (indigo). ZAPXL and ZAPM also  
778 share an extended exon 4 (teal). The amino acid numbering of domains is based on (6) and (7).

### 779 **S2 Fig. N658A mutant induces interferon (IFN) and interferon-stimulated gene (ISG) levels** 780 **similar to WT.**

781 ZAPL WT or N658A inducible ZAP KO 293T cells were untreated, treated with poly(I:C), or treated  
782 with both poly(I:C) and dox. RNA was harvested for RT-qPCR. mRNA levels of IFN or the ISG  
783 IFIT1 in each condition were normalized to that of the respective cell line without poly(I:C) and  
784 without dox. Data are combined from two independent experiments. Asterisks indicate statistically  
785 significant differences as compared to the -dox -poly(I:C) condition for each cell line (Two-way  
786 ANOVA and Tukey's multiple comparisons test: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*\*,  $p < 0.0001$ ).

### 787 **S3 Fig. Characterization of WT ZAP inducible single clone cell lines.**

788 (A) Western blot of ZAPS and ZAPL WT inducible ZAP KO 293T cell lysates. Each single clone  
789 cell line was treated with dilutions of doxycycline 24 hours after seeding. Cell lysates were  
790 harvested 24 hours after dox treatment. (B) ZAPS and ZAPL WT inducible ZAP KO 293T cells

791 were induced for ZAP expression 24 hours before infection by GFP-expressing alphaviruses and  
792 harvested at the time listed for flow cytometry (SINV, MOI = 10, harvest 8 h.p.i.; RRV, MOI = 10,  
793 harvest 24 h.p.i.; ONNV, MOI = 0.1, harvest 18 h.p.i.).

794 **S1 File. Primers used in this study.**

795 **S2 File. Alignment and phylogenetic tree from the 261 mammalian ZAP sequences.**

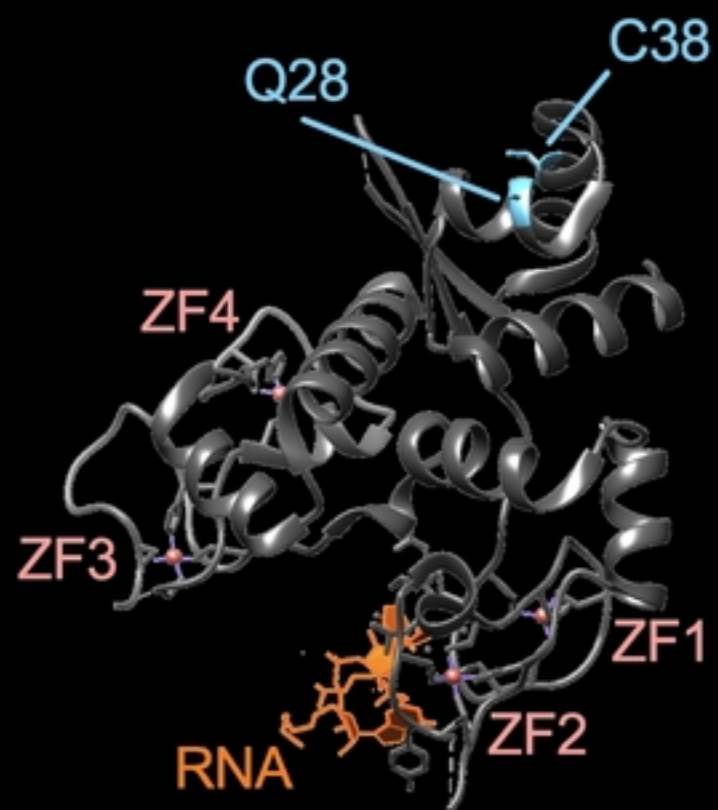


Fig1

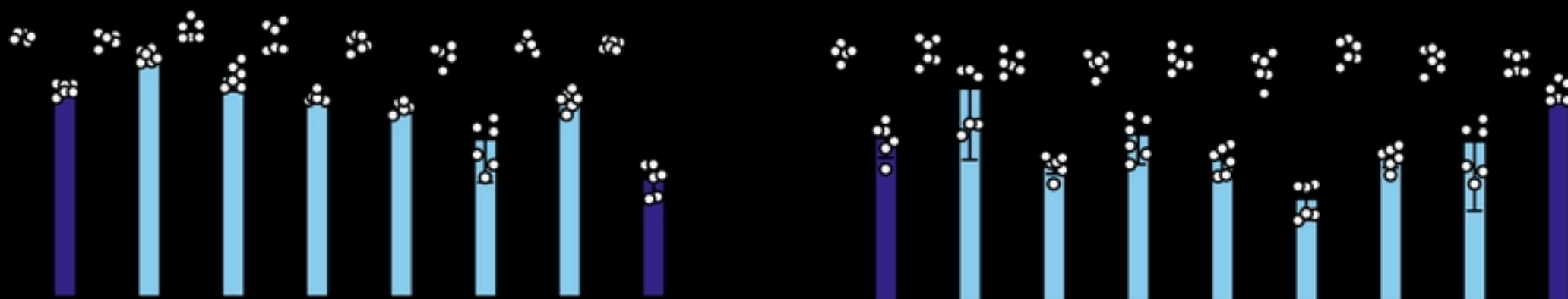
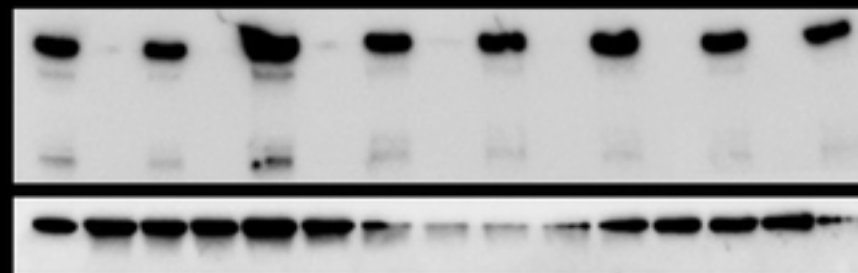
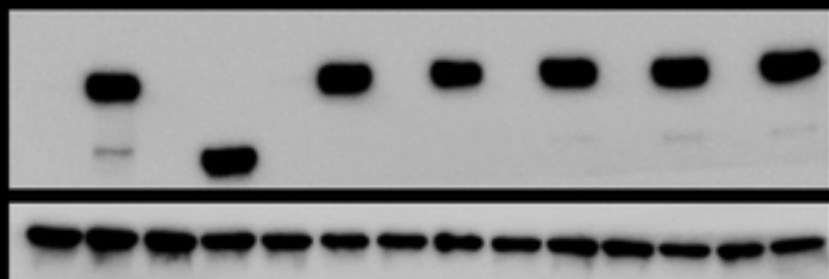


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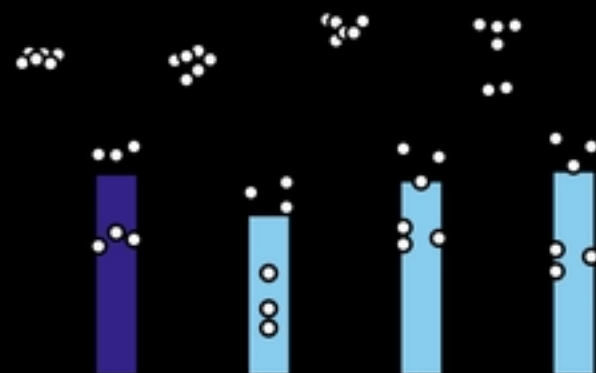
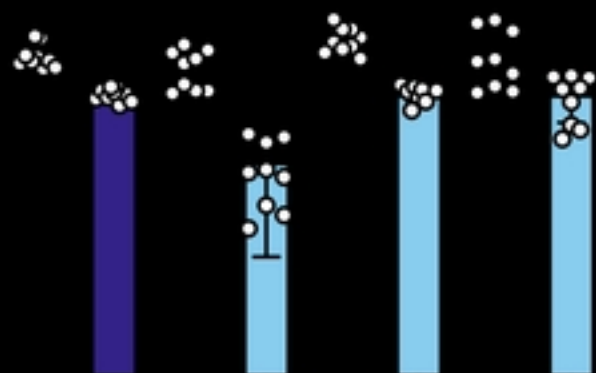
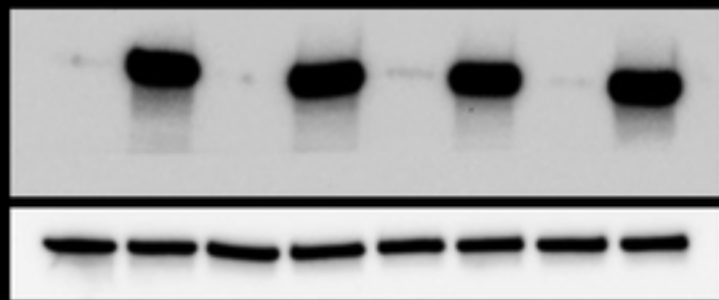
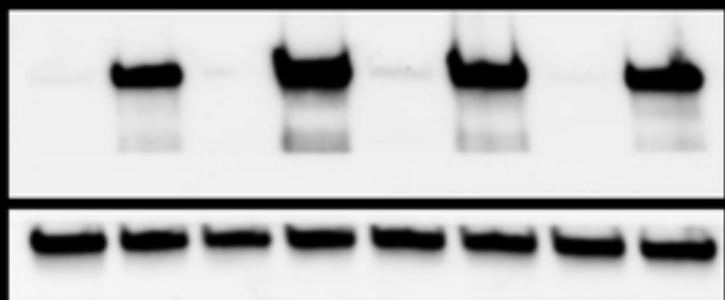


Fig3

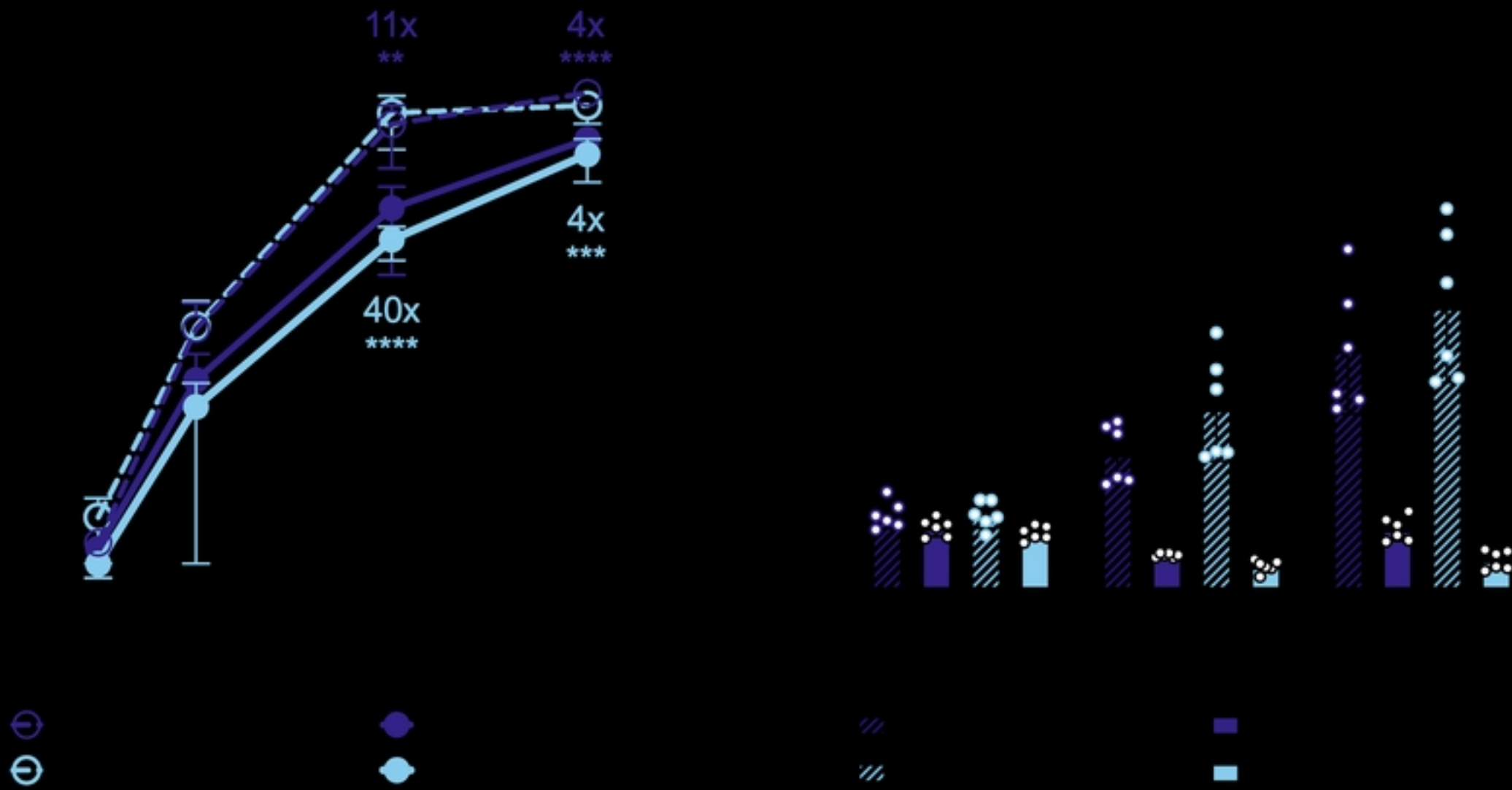


Fig4



Fig5



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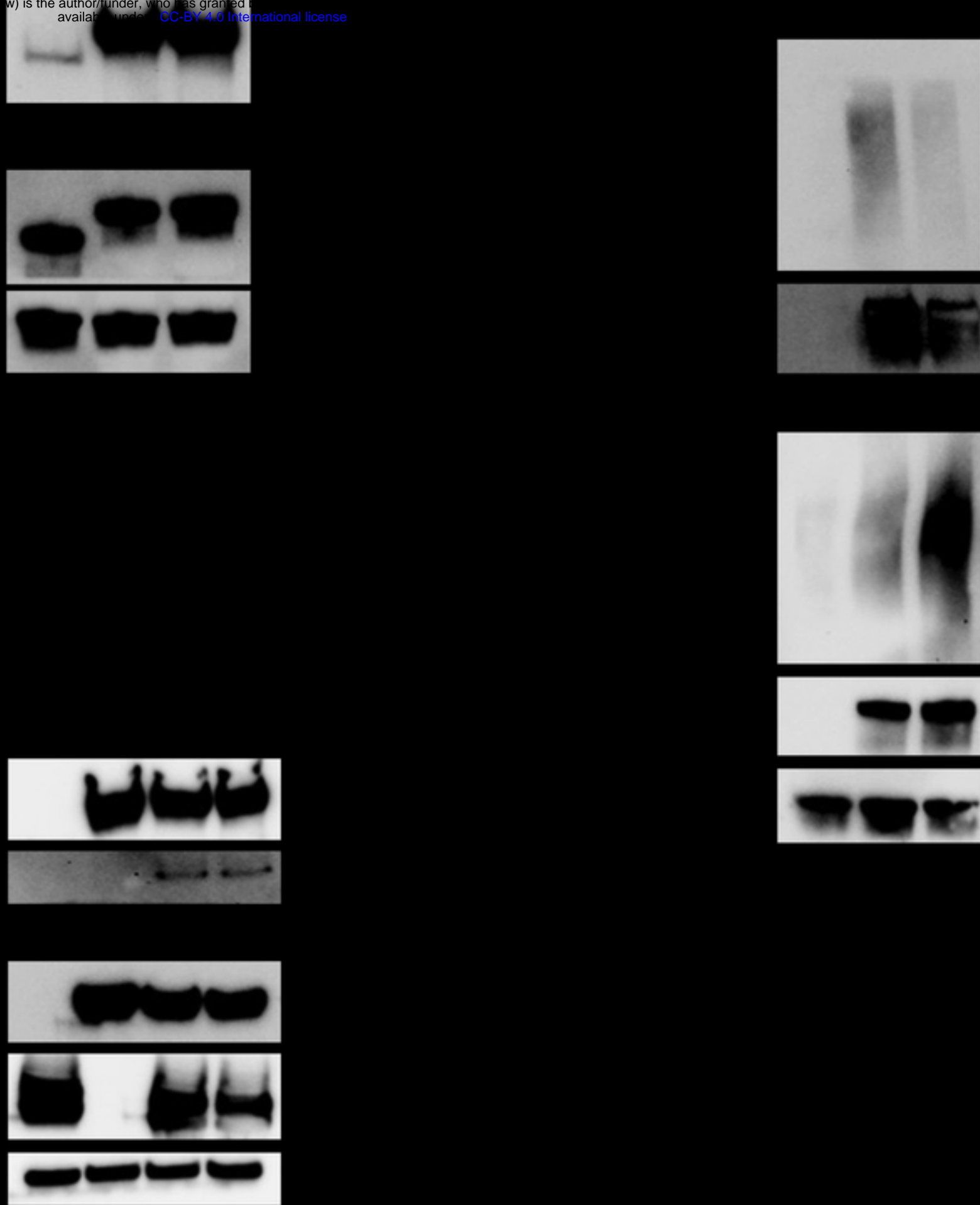
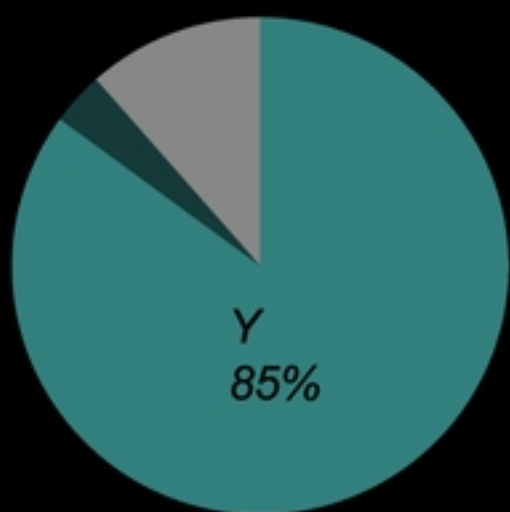
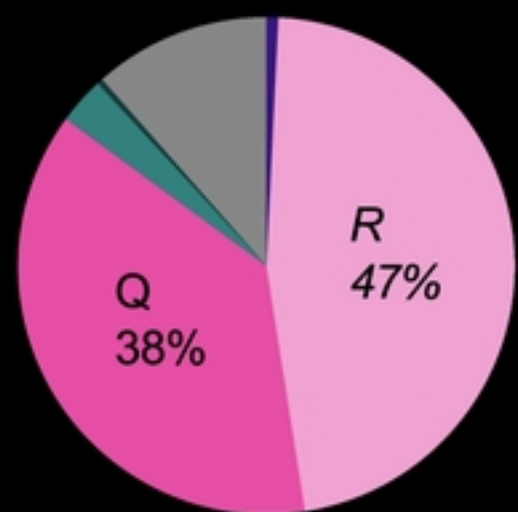
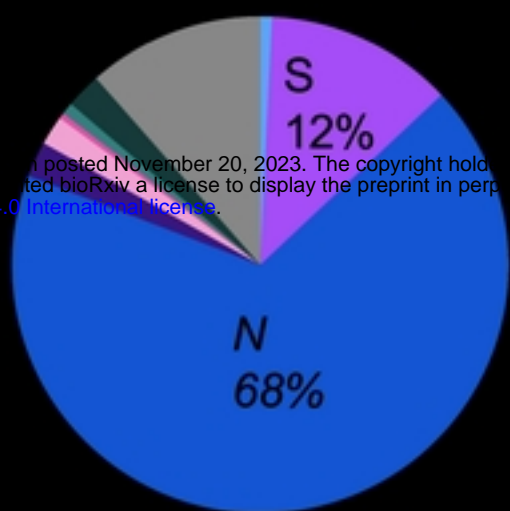
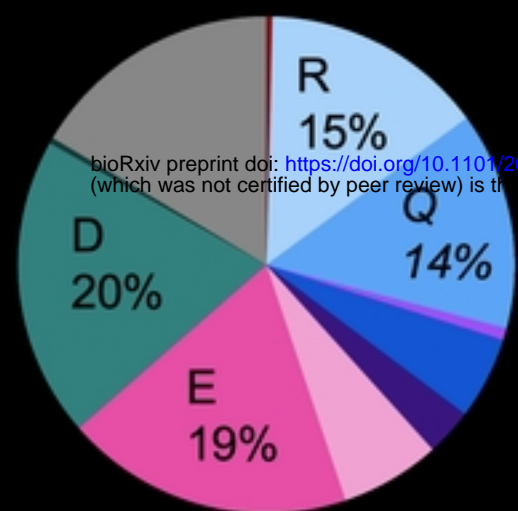


Fig6

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	K	R	N	Y
	G	Q	N	Y
	H			
	G	Q	S	Y
	D	R	N	Y

nonpolar (aliphatic)  
nonpolar (aromatic)  
polar (uncharged)  
polar (positive)  
polar (negative)

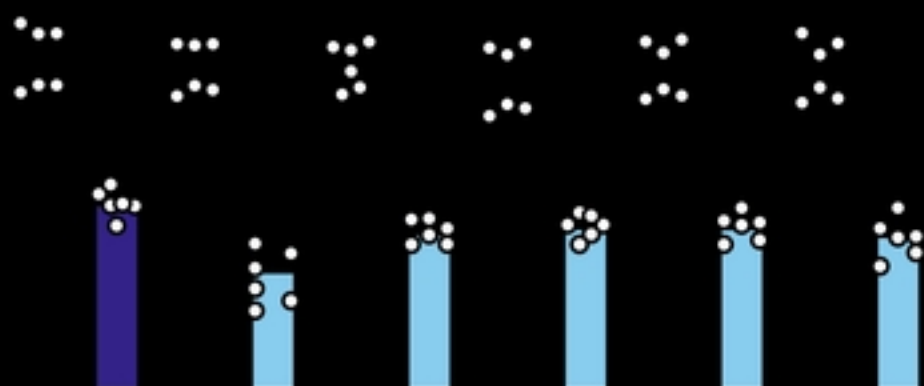


Fig7