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A library-on-library screen reveals the breadth expansion landscape of a broadly neutralizing betacoronavirus antibody

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19 **ABSTRACT**

20 Broadly neutralizing antibodies (bnAbs) typically evolve cross-reactivity breadth through acquiring
21 somatic hypermutations. While evolution of breadth requires improvement of binding to multiple
22 antigenic variants, most experimental evolution platforms select against only one antigenic variant
23 at a time. In this study, a yeast display library-on-library approach was applied to delineate the
24 affinity maturation of a betacoronavirus bnAb, S2P6, against 27 spike stem helix peptides in a
25 single experiment. Our results revealed that the binding affinity landscape of S2P6 varies among
26 different stem helix peptides. However, somatic hypermutations that confer general improvement
27 in binding affinity across different stem helix peptides could also be identified. We further showed
28 that a key somatic hypermutation for breadth expansion involves long-range interaction. Overall,
29 our work not only provides a proof-of-concept for using a library-on-library approach to analyze
30 the evolution of antibody breadth, but also has important implications for the development of
31 broadly protective vaccines.

32 INTRODUCTION

33 Many RNA viruses have high genetic diversity and undergo rapid antigenic drift due to the
34 selection pressure from host humoral immune responses. As a result, it is often challenging to
35 develop effective vaccines with a high breadth of protection against RNA viruses. Nevertheless,
36 in the past two decades, a number of broadly neutralizing antibodies (bnAbs) against different
37 RNA viruses, including influenza virus¹⁻⁴, human immunodeficiency virus⁵⁻⁷, coronavirus⁸⁻¹¹, and
38 flavivirus^{12,13}, have been isolated. These bnAbs protect against antigenically distinct strains of a
39 given virus species, or even genus and family. The discovery of bnAbs has provided crucial
40 insights into the development of broadly protective vaccines^{9,14-16}.

41
42 Most bnAbs evolve from an unmutated common ancestor (UCA) with narrow binding
43 specificities¹⁷⁻²⁰. Their cross-reactivity breadth is subsequently acquired through the accumulation
44 of somatic hypermutations (SHMs) during affinity maturation²¹⁻²³. Yeast display is a common
45 approach for studying the evolutionary landscapes of antibody affinity maturation²⁴⁻³². This
46 process typically involves the construction of an antibody mutant library, which is then displayed
47 on yeast surface and selected for antigen binding^{24,26-28,30-32}. However, conventional yeast display
48 selection focuses on only one antigen at a time, which imposes a challenge in studying the
49 evolution of antibody breadth. Improving binding affinity of an antibody against one antigenic
50 variant sometimes diminishes its binding affinity against another variant²⁷. Yet, evolution of
51 breadth requires improvement of binding affinity across multiple antigenic variants simultaneously.
52 As a result, characterization of the evolutionary pathways for antibody breadth expansion requires
53 selection against multiple antigenic variants in parallel.

54
55 In the past few years, studies of human antibody responses to SARS-CoV-2 have led to the
56 discovery of bnAbs that target the highly conserved stem helix peptide in the S2 domain of the
57 coronavirus spike glycoprotein^{8,8,33,34}. S2P6, which was isolated from a COVID-19 convalescent

58 individual, is a representative bnAb to stem helix peptide¹⁰. While S2P6 neutralizes antigenically
59 distinct betacoronavirus (β -CoV) strains including SARS-CoV-2, antibody binding data suggest
60 that it arose in response to HCoV-OC43 infection and gained cross-reactivity to other β -CoV
61 strains via SHMs¹⁰. Given that stem helix peptide is a target for the development of broadly
62 protective coronavirus vaccines³⁵, it is important to understand the evolutionary trajectories that
63 lead to breadth expansion of bnAbs to the stem helix peptide.

64

65 Recently, a yeast display platform for coevolving protein-protein interfaces was developed³⁶.
66 Here, we adopted this platform to screen a library of 27 unique β -CoV stem helix peptides against
67 a mutant library of S2P6 encoding all combinations of SHMs that lie on or near the paratope. This
68 approach enabled us to map the binding affinity landscapes of S2P6 against stem helix peptides
69 from all β -CoV subgenera. We observed weak correlations of binding affinity landscapes of S2P6
70 across different β -CoV stem helix peptides, indicating that the effect of a given SHM on S2P6
71 binding could vary depending on the sequence of the target stem helix peptide. At the same time,
72 several key SHMs for breadth expansion could be identified and experimentally validated. Our
73 results further highlight the importance of long-range interaction in the affinity maturation of S2P6.
74 Throughout this study, the Kabat numbering scheme is used for antibody residues unless
75 otherwise stated.

76

77 **RESULTS**

78 **Detecting interaction between S2P6 and SARS-CoV-2 stem helix peptide by yeast display**

79 To determine if we could adopt a previously developed protein-protein coevolution platform³⁶ to
80 screen an antibody mutant library against an antigen library, a pilot experiment was performed
81 using the SARS-CoV-2 stem helix peptide and S2P6¹⁰. We cloned a yeast display construct that
82 consisted of (from N-terminal to C-terminal): Aga2p, stem helix peptide (SP), 3C protease
83 cleavage site and linker (3C), S2P6 in single-chain variable fragment (scFv) format, and HA tag

84 **(Fig. 1A and S1)**. After the construct was displayed on the yeast surface, the yeast cells were
85 treated with 3C protease. If S2P6 bound to the stem helix peptide, the HA tag would be retained
86 at the yeast cell surface. If S2P6 did not bind to the stem helix peptide, the HA tag would be lost
87 **(Fig. 1A)**. For the yeast displaying the SARS-CoV-2 stem helix peptide and S2P6, the HA tag was
88 readily detected both before and after protease treatment **(Fig. 1D)**. This result indicated that
89 S2P6 bound strongly to the SARS-CoV-2 stem helix peptide on yeast surface as expected. By
90 contrast, for the yeast displaying the SARS-CoV-2 stem helix peptide and S2P6 UCA, which was
91 the fully germline-reverted S2P6¹⁰, we observed loss of the HA tag after protease treatment **(Fig.**
92 **1D)**. This observation indicated that the S2P6 UCA did not bind to the SARS-CoV-2 stem helix
93 peptide, consistent with the previous study¹⁰. Overall, our pilot experiment demonstrated that the
94 interaction between S2P6 and stem helix peptide could be captured by the yeast display platform
95 that was previously developed for studying protein-protein coevolution³⁶.

96

97 **Screening an S2P6 mutant library against a stem helix peptide library**

98 We sought to analyze the affinity maturation pathway of S2P6 by screening an S2P6 mutant
99 library encoding all possible combinations of SHMs against a library of 27 unique β -CoV stem
100 helix peptides **(Fig. 1B and Table S1)**. Among the 12 SHMs in S2P6 **(Fig. 1C and S2A-B)**, V_H
101 Q1E and V_L D105E were distal from the binding interface. Reverting these positions in S2P6 to
102 the germline sequence (V_H Q1 and V_L D105) did not result in loss of HA tag in our yeast display
103 system after protease cleavage **(Fig. 1D)**. This observation indicated that V_H Q1E and V_L D105E
104 played a minimal, if any, role in the affinity maturation of S2P6. As a result, we moved forward
105 with constructing the S2P6 mutant library without V_L D105E and V_H Q1E mutations **(Fig. S2C)**. In
106 other words, our S2P6 mutant library contained 1,024 variants ($2^{10} = 1,024$). Together with the 27
107 unique β -CoV stem helix peptides and one negative control peptide, there were a total of 28
108 peptide variants \times 1,024 S2P6 variants = 28,672 combination variants in our library-on-library
109 screen.

110

111 To track the frequency of different combination variants in our library, next-generation sequencing
112 was required. While the region of interest in the previous study that developed the protein-protein
113 coevolution platform was <600 bp³⁶, which is compatible with Illumina short-read sequencing, the
114 amplicon spanning both S2P6 scFv and stem helix peptide was too long (>800 bp). To circumvent
115 this issue, we adopted a barcoding strategy from a previous study³⁷. Briefly, we included a 16-
116 nucleotide “barcode” downstream of the coding region, such that different combination variants
117 had their own barcodes (**Fig. S1 and S3**). The linkages between individual combination variants
118 and their corresponding barcodes were then mapped by PacBio sequencing, which had
119 sufficiently long read lengths to cover both S2P6 scFv and stem helix peptide but insufficient read
120 depths to track the frequency of each combination variant in the library. Subsequently, the
121 frequency of different variants could be tracked by sequencing the barcode region using Illumina
122 short-read sequencing.

123

124 Flow cytometry analysis showed that combination variants in our library had a wide range of
125 binding affinity (**Fig. 1D**). The library was subjected to fluorescence-activated cell sorting both
126 before and after protease cleavage (**Fig. S4**). Four bins were collected from each sort according
127 to the amount of HA tag detected on the yeast surface. The frequency of each combination variant
128 in each bin was quantified by next-generation sequencing as described above. An expression
129 score and a binding score were then computed for each combination variant based on its
130 frequency distribution in different bins, hence a proxy for HA tag signal, before and after protease
131 cleavage, respectively (**Table S2, see Materials and Methods**). As shown previously, binding
132 affinity strongly correlates with HA tag signal after protease cleavage³⁶. Combination variants in
133 our library had a broad distribution of binding scores (**Fig. S5A**), consistent with the flow cytometry
134 analysis of the library (**Fig. 1D**). At the same time, they had a narrow distribution of expression
135 scores (**Fig. S5B**), indicating that most combination variants expressed well. We also observed a

136 Pearson correlation of 0.70 between the binding scores of two independent experimental
137 replicates (**Fig. S5C**), demonstrating reproducibility of our library-on-library screen. By contrast,
138 the Pearson correlation between binding score and expression score was 0.29, showing that the
139 expression levels of combination variants had a relatively small influence on their binding scores
140 (**Fig. S5D**).

141

142 **Breadth expansion of S2P6 is restricted by sequence variations of stem helix peptides**

143 To analyze the breadth expansion of S2P6, we computed the average binding scores of the S2P6
144 mutant library for each β -CoV stem helix peptide (**Fig. 2A**). High average binding scores were
145 observed for stem helix peptides from all sarbecovirus strains as well as embecovirus strains,
146 except PHEV and HCoV-HKU1. Nevertheless, the average binding scores for stem helix peptides
147 from PHEV and HCoV-HKU1 were still higher than the negative control peptide. For stem helix
148 peptides from the embecovirus, amino acid variants at position 1153 appeared to play a critical
149 role in binding to S2P6. At position 1153, YakCoV, HCoV-HKU1, and PHEV have N1153, S1153,
150 and Y1153, respectively, whereas all other embecovirus strains have D1153 (**Fig. 1B**). As
151 previously shown by a mutational analysis of SARS-CoV-2 stem helix peptide, D1153 has the
152 highest binding activity to S2P6, followed by N1153, S1153, and then Y1153¹⁰. Consistently, stem
153 helix peptides from those embecovirus strains with D1153 had the highest average binding scores
154 in our data, followed by YakCoV (N1153), HCoV-HKU1 (S1153), then PHEV (Y1153).

155

156 Low average binding scores were observed for stem helix peptides from all merbecovirus strains,
157 except MERS-CoV and Bat-NeoCoV. Notably, both MERS-CoV and Bat-NeoCoV have D1153
158 whereas all other merbecovirus strains have E1153 (**Fig. 1B**), again substantiating the importance
159 of Asp at this position for S2P6 binding¹⁰. All hibecovirus strains also have E1153, which explained
160 the low average binding scores to their stem helix peptides. Furthermore, stem helix peptides
161 from all nobecovirus strains had low average binding scores. While the stem helix peptides from

162 other β -CoV strains have L1152, all nobecovirus strains have F1152 (**Fig. 1B**), which has
163 previously been shown to abolish binding to S2P6¹⁰.

164

165 **S2P6 binding landscape varies across stem helix peptides**

166 It is known that S2P6 UCA binds strongly to the stem helix peptides of HCoV-OC43, but not those
167 from the other two β -CoV strains that circulate in human population, namely HCoV-HKU1 and
168 SARS-CoV-2¹⁰. As a result, S2P6 is hypothesized to have been initially elicited in response to
169 HCoV-OC43 infection with specificity broadened through subsequent recall response to either or
170 both HCoV-HKU1 and SARS-CoV-2 infections¹⁰. Consistently, even S2P6 variants with a low
171 number of SHMs had high binding scores for HCoV-OC43 stem helix peptide (**Fig. 2B**). By
172 contrast, the binding scores for stem helix peptides from all sarbecovirus strains, namely SARS-
173 CoV-2, BatCoV-BtkY72, BtRf-BetaCoV, and BatCoV-GX2013, increased as S2P6 accumulated
174 more SHMs (**Fig. 2B and S6**).

175

176 We further aimed to compare the binding landscapes of S2P6 across different β -CoV stem helix
177 peptides (**Fig. 3 and S7**). Weak correlations of binding scores were observed among stem helix
178 peptides from sarbecovirus strains (Pearson correlation = 0.14 to 0.21, **Fig. 3**). There was also a
179 weak correlation of binding scores between BCoV-ENT (embecovirus) and sarbecovirus stem
180 helix peptides (Pearson correlation = 0.12 to 0.26). In comparison, the correlations of binding
181 scores between experimental replicates for each of these stem helix peptides were higher
182 (Pearson correlation = 0.35 to 0.41, **Fig. 3**). Similarly, there was a lack of correlation of binding
183 scores among embecovirus and merbecovirus stem helix peptides (Pearson correlation = -0.13
184 to 0.08), whereas moderate correlations of binding scores were observed between their
185 experimental replicates (Pearson correlation = 0.34 to 0.56). These results demonstrated that the
186 S2P6 binding landscape differed among stem helix peptides from different β -CoV strains. In other

187 words, depending on the sequence of the target stem helix peptide, a given SHM of S2P6 could
188 have different effects on binding affinity.

189

190 **Affinity maturation of S2P6 involves long range interaction**

191 Next, we aimed to analyze the impacts of individual SHMs on S2P6 binding landscape. Briefly,
192 the S2P6 binding landscape was decomposed into additive effects of individual SHMs and
193 pairwise epistasis effects between SHMs (**Fig. 4A and S8, see Materials and Methods**)³⁸. Three
194 SHMs on heavy chain, namely V_H Y32Q, V_H M48I, and V_H S56H, stood out as having positive
195 additive effects on binding to stem helix peptides from most β -CoV strains (**Fig. 4A**). To
196 experimentally validate this finding, these mutations were introduced individually and in
197 combinations (single, double, and triple mutants) to S2P6 UCA and expressed as fragment
198 antigen-bindings (Fabs). Biolayer interferometry experiments showed that each single mutation
199 improved the binding response to the stem helix peptides from BatCoV-BtkY72, SARS-CoV-2,
200 BtRf-BetaCoV, and MHV in comparison to S2P6 UCA (**Fig. 4B and S9**). Consistently, reverting
201 V_H Q32 to V_H Y32 has been shown to decrease the binding affinity of S2P6 to multiple β -CoV
202 stem helix peptides¹⁰. Nevertheless, among the three SHMs tested, V_H M48I conferred the largest
203 improvement in binding response to all four tested stem helix peptides. V_H M48I alone led to a
204 higher binding response than V_H Y32Q/S56H and V_H Y32Q/M48I double mutants for BatCoV-
205 BtkY72, SARS-CoV-2, and BtRf-BetaCoV stem helix peptides. Similarly, for the stem helix
206 peptides from BatCoV-BtkY72, SARS-CoV-2, and BtRf-BetaCoV, V_H M48I/S56H double mutant
207 had higher binding response than the V_H Y32Q/M48I/S56H triple mutant. Since V_H Y32Q did not
208 improve the binding response in the presence of V_H M48I, our data also suggested that negative
209 epistasis existed between V_H Y32Q and V_H M48I, which was also observed in our decomposition
210 analysis, albeit mildly (**Fig. S8**).

211

212 Since V_H Y32Q and V_H S56H are in the paratope of S2P6¹⁰ (**Fig. 4C**), it may not be surprising that
213 they could improve the binding of S2P6 to β -CoV stem helix peptides. By contrast, V_H M48I is not
214 in the paratope and instead resides in the hydrophobic core of the heavy chain variable domain
215 (**Fig. 4C**). This observation indicated that V_H M48I improved the interaction between S2P6 and β -
216 CoV stem helix peptides via long-range interaction. In fact, it is not uncommon for non-paratope
217 SHMs to improve antibody binding affinity by modulating the conformation of complementarity
218 determining region (CDR) loops that are part of the paratope^{17,39}. It is possible that a similar
219 mechanism applies to V_H M48I in S2P6, although additional structural analysis is required to verify
220 this speculation.

221

222 **DISCUSSION**

223 Conventional yeast display experiments enable high-throughput analysis of antibody affinity
224 maturation pathways^{28,30,31,40}. However, they are often limited to analyzing antibody binding to one
225 target antigen at a time, which restricts our understanding in the evolution of cross-reactivity
226 breadth. A platform that allows analysis of affinity maturation pathways to multiple antigenic
227 variants in parallel is therefore needed. In this study, we addressed this gap by determining the
228 binding affinity landscapes of S2P6¹⁰ against 27 unique stem helix peptides across all β -CoV
229 subgenera in a single yeast display experiment. Our work provides not only a proof-of-concept
230 for using a yeast display library-on-library approach to analyze the evolution of antibody cross-
231 reactivity breadth, but also molecular insights into the development of broadly protective
232 coronavirus vaccines.

233

234 A notable finding in this study is the weak correlations of S2P6 binding landscapes among different
235 β -CoV stem helix peptides. This indicates the varying effects of a given SHM on binding affinity
236 to different β -CoV stem helix peptides. Consistently, our decomposition analysis showed that most
237 SHMs had varying additive effects. Similar observations have also been made with an influenza

238 bnAb in a previous study³⁰. Nevertheless, we also identified SHMs that improve the binding of
239 S2P6 to most β -CoV stem helix peptides. An effective broadly protective vaccine should facilitate
240 the acquisition of SHMs that improve binding affinity to many antigenic variants. On the other
241 hand, SHMs that confer trade-offs for binding different antigenic variants should be avoided.
242 Consequently, future studies need to focus on the structural mechanisms underlying the
243 differential impacts of SHMs on breadth expansion, which in turn will be informative for the
244 development of vaccines that optimally elicit bnAbs.

245

246 Using library-on-library approaches to screen antibody-antigen interactions has been described
247 since at least 15 years ago, based on co-selection of a phage-displayed antibody library and a
248 yeast-displayed antigen library⁴¹. Since this strategy requires cell sorting into 96-well plates
249 followed by sequencing the antibody and antigen in each well individually⁴¹, its throughput is low.
250 More recently, library-on-library approaches that utilize lentivirus-displayed antigen library and
251 human B cell library have been described^{42,43}. However, the throughput of these approaches
252 remains moderate due to their reliance on single-cell sequencing, which analyzes at most $\sim 10^4$
253 cells per sample. By contrast, the yeast display library-on-library approach in our present study,
254 which was adopted from Yang et al.³⁶, has a much higher throughput since it is compatible with
255 amplicon-based next-generation sequencing. If nucleotide barcode is needed as described in this
256 study, the maximum throughput would be $\sim 10^6$, as determined by the throughput of a PacBio
257 sequencing run. In fact, another yeast display library-on-library approach has been described
258 previously that relies on yeast mating and is also compatible with amplicon-based next-generation
259 sequencing⁴⁴. Nevertheless, this approach requires an engineered yeast strain that is not
260 commercially available⁴⁴. In comparison, our approach is more accessible since it largely follows
261 conventional yeast display protocol without the need of any special reagents.

262

263 We acknowledge that our proof-of-concept in this study is based on a small peptide antigen.
264 However, given that larger antigens, such as influenza hemagglutinin, HIV gp120, and SARS-
265 CoV-2 receptor-binding domain, have been successfully displayed on the yeast surface⁴⁵⁻⁴⁷, we
266 anticipate that our library-on-library approach can be applied to a wide range of antibody-antigen
267 pairs. In addition, since our approach does not rely on yeast biology, unlike the yeast mating-
268 based approach mentioned above⁴⁴, it can potentially be adopted to a mammalian cell display
269 system, which is essential for even larger antigens or if glycosylation matters. Future studies
270 should continue to improve existing library-on-library approaches for screening antibody-antigen
271 interactions as well as develop new ones.

272

273 **MATERIALS AND METHODS**

274 **Yeast display plasmid**

275 A DNA fragment encoding (from N-terminal to C-terminal) a cMyc tag, SARS-CoV-2 spike stem
276 helix peptide sequence (residues 1146-1159), 3C protease cleavage site and linker, S2P6 heavy
277 chain variable domain (V_H) and light chain variable domain (V_L) connected with a GS flexible
278 linker, and an HA tag was synthesized as an eBlock (Integrated DNA Technologies).
279 Subsequently, this fragment was cloned into the pCTcon2 vector (Addgene, Cat. No. 41843)⁴⁸ in
280 frame with the Aga2 coding region at the N-terminal end using Gibson assembly (**Fig. S1**).

281

282 **Construction of the combination variant library**

283 The cMyc-SP-3C-S2P6_VH-GS-S2P6_VL-HA library insert was divided into six fragments and
284 assembled using overlap PCR (**Fig. S3**). All fragments were gel-purified using a Monarch DNA
285 Gel Extraction Kit (NEB). To generate the full-length library insert, all fragments were pooled
286 together and underwent 10 cycles of PCR with the following conditions: 98 °C for 30 s, 10 cycles
287 of (98 °C for 10 s, 50 °C for 15 s, 72 °C for 15 s), 72 °C for 2 min, 4 °C indefinitely. Extension
288 primers Fragment 1_F and Fragment 6_R_3 were added into the reaction, which were then ran

289 for another 25 cycles using conditions: 98 °C for 30 s, 25 cycles of (98 °C for 10 s, 55 °C for 5 s,
290 72 °C for 15 s), 72 °C for 2 min, 4 °C indefinitely. The full-length library insert was gel-purified. To
291 generate the linearized vector for the library, the plasmid encoding the SARS-CoV-2 stem helix
292 peptide and S2P6 WT, which was described above, was used as a template for PCR using primers
293 S2P6_vector_F and S2P6_vector_R. The linearized vector was digested with DpnI (NEB) for 2 h
294 at 37 °C and gel purified. All PCRs were performed using PrimeSTAR Max polymerase (Takara
295 Bio). The sequences of all primers in this study can be found in **Table S3**. All primers in this study
296 were synthesized by Integrated DNA Technologies.

297

298 **Yeast transformation**

299 *Saccharomyces cerevisiae* EBY100 cells (American Type Culture Collection, Cat. No. MYA-4941)
300 were grown overnight in YPD medium (1% w/v yeast nitrogen base, 2% w/v peptone, 2% w/v
301 D(+)-glucose) at 30 °C with shaking at 225 rpm until OD₆₀₀ reached 3. An aliquot of the overnight
302 culture was used to inoculate 100 mL fresh YPD media with an initial OD₆₀₀ of 0.3. This culture
303 was incubated at 30 °C with shaking at 225 rpm until OD₆₀₀ reached 1.6. The yeast cells were
304 collected by centrifugation at 4600 × g for 3 min at room temperature. YPD media was removed,
305 and the cell pellet was washed twice with 50 mL ice-cold water, and then once with 50 mL of ice-
306 cold electroporation buffer (1 M sorbitol and 1 mM calcium chloride). The cells were then
307 resuspended in 20 mL conditioning media (0.1 M lithium acetate and 10 mM dithiothreitol) and
308 incubated at 30 °C with shaking at 225 rpm. The conditioned yeast cells were then collected via
309 centrifugation at 4600 × g for 3 min at room temperature, washed once with 50 mL ice-cold
310 electroporation buffer and resuspended in electroporation buffer to reach a final volume of 1 mL.
311 The cells were kept on ice until used.

312

313 5 µg of the purified linearized vector and 2.7 µg of the purified library insert were added to 400 µL
314 of conditioned yeast. The mixture was transferred to a pre-chilled BioRad GenePulser cuvette

315 with 2 mm electrode gap and kept on ice for 5 min prior to electroporation. Cells were
316 electroporated at 2.5 kV and 25 μ F, achieving a time constant between 3.7 and 4.1 ms.
317 Electroporated cells were recovered into 4 mL of YPD media supplemented with 4 mL of 1 M
318 sorbitol and incubated at 30 °C with shaking at 225 rpm for 1 h. Recovered cells were collected
319 via centrifugation at 1700 \times g for 3 min at room temperature, resuspended in 0.6 mL SD-CAA
320 medium (2% w/v D-glucose, 0.67% w/v yeast nitrogen base with ammonium sulfate, 0.5% w/v
321 casamino acids, 0.54% w/v Na₂HPO₄, and 0.86% w/v NaH₂PO₄·H₂O, all dissolved in deionized
322 water), plated across 3 \times 150 mm SD-CAA plates (2% w/v D-glucose, 0.67% w/v yeast nitrogen
323 base with ammonium sulfate, 0.5% w/v casamino acids, 0.54% w/v Na₂HPO₄, 0.86% w/v
324 NaH₂PO₄·H₂O, 18.2% w/v sorbitol, and 1.5% w/v agar, all dissolved in deionized water) and
325 incubated at 30 °C for 48 h. After 48 h, approximately 200,000 colonies were collected in SD-CAA
326 medium, centrifuged at 4600 \times g for 5 min at room temperature, and resuspended in SD-CAA
327 medium with 15% v/v glycerol such that OD₆₀₀ was 50. Glycerol stocks were stored at -80 °C for
328 later use. This protocol was modified from a previously described protocol⁴⁹.

329

330 **PacBio sequencing of the combination variant library**

331 Plasmids from the transformed yeast cells were extracted using a Zymoprep Yeast Plasmid
332 Miniprep II Kit (Zymo Research) following the manufacturer's protocol. The library insert and
333 barcode were amplified using primers PacBio Recovery_F and PacBio Recovery_R (**Table S3**).
334 This PCR was performed using Q5 high-fidelity DNA polymerase (NEB) with the following
335 settings: 98 °C for 30 s, 25 cycles of (98 °C for 10 s, 62 °C for 15 s, 72 °C for 30 s), 72 °C for 2 min,
336 4 °C indefinitely. PCR products were gel purified and sequenced on one SMRT Cell 8M on a
337 PacBio Sequel IIe using the CCS sequencing mode and a 15-hour movie time.

338

339 **Analysis of PacBio sequencing data**

340 Circular consensus sequences (CCSs) were generated from the raw subreads using the ccs
341 program (<https://github.com/PacificBiosciences/ccs>, version 6.4.0), setting the parameters to
342 require 99.9% accuracy and a minimum of 3 passes. CSSs in FASTQ format were parsed using
343 SeqIO module in BioPython⁵⁰. For each read, the positions of the stem helix peptide, S2P6 heavy
344 and light chain variable domains, as well as the 16-nucleotide barcode were identified by aligning
345 their flanking sequences to the read. If the lengths of these regions deviated from the expected
346 lengths, the read would be discarded. The nucleotide sequences of the stem helix peptide, as
347 well as S2P6 heavy and light chain variable domains were then translated. If the amino acid
348 sequence of the stem helix peptide did not match any of the 27 stem helix peptides, the read
349 would be discarded. Similarly, if the amino acid sequences at the mutated positions of S2P6 did
350 not match our library design, the read would be discarded. Subsequently, the combination variant
351 and the 16-nucleotide barcode in each read were identified.

352

353 Since reads that shared the same 16-nucleotide barcode should have the same combination
354 variant, we compared the amino acid sequences at the positions of interest among different reads
355 that shared the same 16-nucleotide barcode. For each position of interest, an amino acid variant
356 present in at least 80% of the reads that shared the same 16-nucleotide barcode was regarded
357 as the true variant. If none of the amino acid variant was present in at least 80% of the reads that
358 shared the same 16-nucleotide barcode, all reads that shared the given 16-nucleotide barcode
359 would be discarded. Besides, those 16-nucleotide barcodes that appeared in only one read were
360 discarded.

361

362 **Fluorescence-activated cell sorting (FACS) of yeast display library**

363 A 150 μ L glycerol stock of the transformed yeast display library was recovered in 50 mL SD-CAA
364 medium by incubating at 27 °C with shaking at 250 rpm until OD₆₀₀ reached between 1.5-2.0.
365 Then 15 mL of the yeast culture was harvested via centrifugation at 4600 \times g at 4 °C for 5 min.

366 The supernatant was discarded, and SGR-CAA induction media (2% w/v galactose, 2% w/v
367 raffinose, 0.1% w/v D-glucose, 0.67% w/v yeast nitrogen base with ammonium sulfate, 0.5% w/v
368 casamino acids, 0.54% w/v Na₂HPO₄, and 0.86% w/v NaH₂PO₄·H₂O, all dissolved in deionized
369 water) was added to a final volume of 50 mL with an initial OD₆₀₀ of 0.5. The SGR-CAA yeast
370 culture was transferred to a baffled flask and incubated at 18 °C with shaking at 250 rpm for about
371 24 h until the OD₆₀₀ reached between 1.3-1.6.

372

373 For each staining replicate, 1 mL of yeast culture was harvested via centrifugation at 4600 × g at
374 4 °C for 5 min. The pellet was washed twice with 1 mL of 1× PBS and resuspended in 1 mL of 1×
375 PBS. For the library binding sort, anti-HA-tag mouse antibody with Alexa Fluor 647 conjugate
376 (6E2, Cell Signaling Technology, Cat. No. 3444) was added to washed cells at a 1:100 dilution. A
377 no-stain negative control was included in which nothing was added to the PBS-resuspended cells.
378 Samples were incubated overnight at 4 °C with rotation. For protease treatment, the stained cells
379 were spun down and the supernatant was removed. 10 µL HRV 3C protease (ThermoFischer,
380 Cat. No. 88946), 10 µL protease reaction buffer, and 80 µL water was added to the stained cells.
381 The cells incubated with protease at 4 °C for 1 h.

382

383 The yeast cells were pelleted and washed twice in 1× PBS and resuspended in FACS tubes
384 containing 2 mL 1× PBS. Using a BD FACS Aria II cell sorter (BD Biosciences) and FACS Diva
385 software v8.0.1 (BD Biosciences), cells in the selected gates were collected in 1 mL of SD-CAA
386 containing 1× penicillin/streptomycin. Single yeast cells were gated by forward scatter (FSC) and
387 side scatter (SSC). For the binding sort, single cells were gated into 4 bins along the Alexa Fluor
388 647 axis. Cells expressing the highest Alexa Fluor 647 fluorescence were sorted into “bin 4”, then
389 the next highest into “bin 3”, followed by “bin 2” and then “bin 1”. Gating strategy used is shown
390 in **Fig. S4A**. Number of cells collected per bin per replicate is shown in **Fig. S4B**. Cells were then
391 collected and grown overnight in SD-CAA at 30 °C with shaking at 225 rpm. FlowJo v10.8 software

392 (BD Life Sciences) was used to analyze FACS data. Replicates were performed on the same day,
393 starting from separate glycerol stocks of the transformed library.

394

395 **Illumina sequencing of the post-sorted yeast display library**

396 Plasmids from the sorted yeast cells were extracted using a Zymoprep Yeast Plasmid Miniprep II
397 Kit (Zymo Research) following the manufacturer's protocol. The 16-nucleotide barcode was
398 amplified using primers Barcode_Recovery_F and Barcode_Recovery_R (**Table S3**), which
399 contained part of the adapter sequence required for Illumina sequencing. A maximum of 100 ng
400 of genomic DNA per 50 μ L PCR reaction was used as template. This PCR was performed using
401 Q5 high-fidelity DNA polymerase (NEB) with the following settings: 98 °C for 30 s, 25 cycles of
402 (98 °C for 10 s, 62 °C for 15 s, 72 °C for 15 s), 72 °C for 2 min, 4 °C indefinitely. The PCR products
403 were gel purified. For each sample, 20 ng of the purified PCR product was appended with the rest
404 of adapter sequence and index via PCR using primers: 5'-AAT GAT ACG GCG ACC ACC GAG
405 ATC TAC ACX XXX XXX XAC ACT CTT TCC CTA CAC GAC GCT-3', and 5'-CAA GCA GAA GAC
406 GGC ATA CGA GAT XXX XXX XXG TGA CTG GAG TTC AGA CGT GTG CT-3'. Positions
407 annotated by an "X" represented the nucleotides for the index sequence. This PCR was performed
408 by PrimeSTAR Max polymerase (Takara Bio) with the following settings: 98 °C for 30 s, 10 cycles
409 of (98 °C for 10 s, 55 °C for 15 s, 72 °C for 15 s), 72 °C for 2 min, 4 °C indefinitely. Indexed products
410 were gel purified, mixed at equimolar ratios, and submitted for next generation sequencing using
411 Illumina NovaSeq X PE150.

412

413 **Analysis of Illumina sequencing data**

414 The Illumina NovaSeq sequencing data were obtained in FASTQ format. Forward and reverse
415 reads of each paired-end read were merged by PEAR⁵¹. The merged reads were then parsed by
416 SeqIO module in BioPython⁵⁰, followed by primer trimming to remove primer sequences. Reads
417 with incorrect primer sequences or lengths were discarded. Reads that did not match any

418 barcodes from our PacBio data were also discarded. The combination variant, which consisted of
419 an S2P6 variant i and a stem helix peptide variant s , in each read was then identified using the
420 barcode. The frequency (F) of a combination variant within bin n at timepoint t of replicate r was
421 computed as follows:

$$422 \quad F_{s,i,n,t,r} = \frac{\text{readcount}_{s,i,n,t,r} + 1}{\sum_i \sum_s (\text{readcount}_{s,i,n,t,r} + 1)} \quad (1)$$

423 A pseudocount of 1 was added to the read counts of each mutant to avoid division by zero in
424 subsequent steps. The frequency (F) of each combination variant among bins at $t = 0$ h (i.e. pre-
425 protease cleavage) was used to calculate the expression score (E)^{37,52}:

$$426 \quad E_{s,i,r} = \frac{F_{s,i,bin1,t_0,r} \times 0.25 + F_{s,i,bin2,t_0,r} \times 0.5 + F_{s,i,bin3,t_0,r} \times 0.75 + F_{s,i,bin4,t_0,r} \times 1}{\sum_{n=1}^4 F_{s,i,n,t_0,r}} \quad (2)$$

427 The frequency (F) of each combination variant among bins at $t = 1$ h (i.e. post-protease cleavage)
428 was then used to calculate the binding score (B):

$$429 \quad B_{s,i,r} = \frac{F_{s,i,bin1,t_1,r} \times 0.25 + F_{s,i,bin2,t_1,r} \times 0.5 + F_{s,i,bin3,t_1,r} \times 0.75 + F_{s,i,bin4,t_1,r} \times 1}{\sum_{n=1}^4 F_{s,i,n,t_1,r}} \quad (3)$$

430 The average binding score for combination variants that contained the negative control peptide
431 (DSAKEALDKYFKNH) were calculated ($B_{negctrl}$). The binding score of the combination variant that
432 contained SARS-CoV-2 stem helix peptide (DSFKEELDKYFKNH) and S2P6 WT was also
433 extracted ($B_{SARS2-S2P6WT}$). The normalized binding score ($NormB$) of each combination variant i
434 was then calculated as followed:

$$435 \quad NormB_{s,i,r} = \frac{B_{s,i,r} - \overline{B_{negctrl,r}}}{B_{SARS2-S2P6WT,r} - \overline{B_{negctrl,r}}} \quad (4)$$

436 The reported $NormB$ was the average between replicates.

437

438 **Expression and purification of Fabs**

439 Codon-optimized oligonucleotides encoding S2P6 V_H variants were synthesized as eBlocks
440 (Integrated DNA Technologies), and then cloned into pHCMV3 vector in Fab format using Gibson
441 assembly. The plasmids were co-transfected into Expi293F cells at a 2:1 (HC:LC) mass ratio
442 using ExpiFectamine 293 Reagent (Thermo Fisher Scientific) following the manufacturer's
443 protocol for a 25 mL culture. 6 days post-transfection, the supernatant of each Expi293F culture
444 was collected and clarified by centrifugation at 4600 × g for 45 min at 4 °C to remove cells and
445 debris. After clarification, the supernatant was filtered through a 0.22 μm polyethersulfone
446 membrane filter (Millipore).

447

448 Fabs were purified using IgG-CH1-XL beads (Thermo Fisher Scientific). The beads were washed
449 with MilliQ H₂O three times and resuspended in 1× PBS. The clarified and filtered supernatant
450 was incubated with washed beads overnight at 4 °C with gentle rocking. The supernatant with
451 beads was then loaded into a column. The flowthrough was discarded, and the beads were
452 washed once with 1× PBS. To elute the antibody, the beads were incubated in 60 mM sodium
453 acetate, pH 3.7 for 10 min at 4 °C and the flow-through was collected. The eluate containing Fab
454 was buffer exchanged into 1× PBS using a centrifugal filter unit with a 10 kDa molecular weight
455 cut-off (Millipore). Fabs were stored at 4 °C.

456

457 **Biolayer interferometry binding assays**

458 Biolayer interferometry (BLI) experiments were performed at room temperature using Octet
459 Red96e instrument (Sartorius). Biotin-labeled stem peptides (GenScript) at 4 μg/mL in 1× kinetics
460 buffer (1× PBS, pH 7.4, 0.01% w/v BSA, and 0.002% v/v Tween 20) were loaded onto streptavidin
461 (SA) biosensors and incubated with Fabs at 500 nM, 750 nM, and 1000 nM. The assay consisted
462 of five steps: (1) baseline: 60 s with 1× kinetics buffer; (2) loading: 120 s with biotin-labeled stem
463 peptides; (3) baseline: 60 s with 1× kinetics buffer; (4) association: 120 s with Fab samples; and
464 (5) dissociation: 120 s with 1× kinetics buffer. For estimating the exact K_D, a 1:1 binding model

465 was used. When a 1:1 binding model did not fit well due to non-specific binding, a 2:1
466 heterogeneous ligand model was used to improve fitting.

467

468 **DATA AVAILABILITY**

469 Raw deep sequencing data generated in this study have been submitted to the NIH Sequence
470 Read Archive under accession number: PRJNA1064076 and PRJNA1113356.

471

472 **CODE AVAILABILITY**

473 Custom codes for data analysis have been deposited to:

474 https://github.com/nicwulab/S2P6_lib-on-lib_screen.

475

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483

484 **AUTHOR CONTRIBUTIONS**

485 M.Y.O. and N.C.W. designed research. M.Y.O. performed research. M.Y.O., W.O.O., and N.C.W.
486 analyzed data. M.Y.O. and N.C.W. wrote the paper.

487

488 **COMPETING INTERESTS**

489 N.C.W. serves as a consultant for HeliXon. All authors declare no other competing interests.

490

491 **REFERENCES**

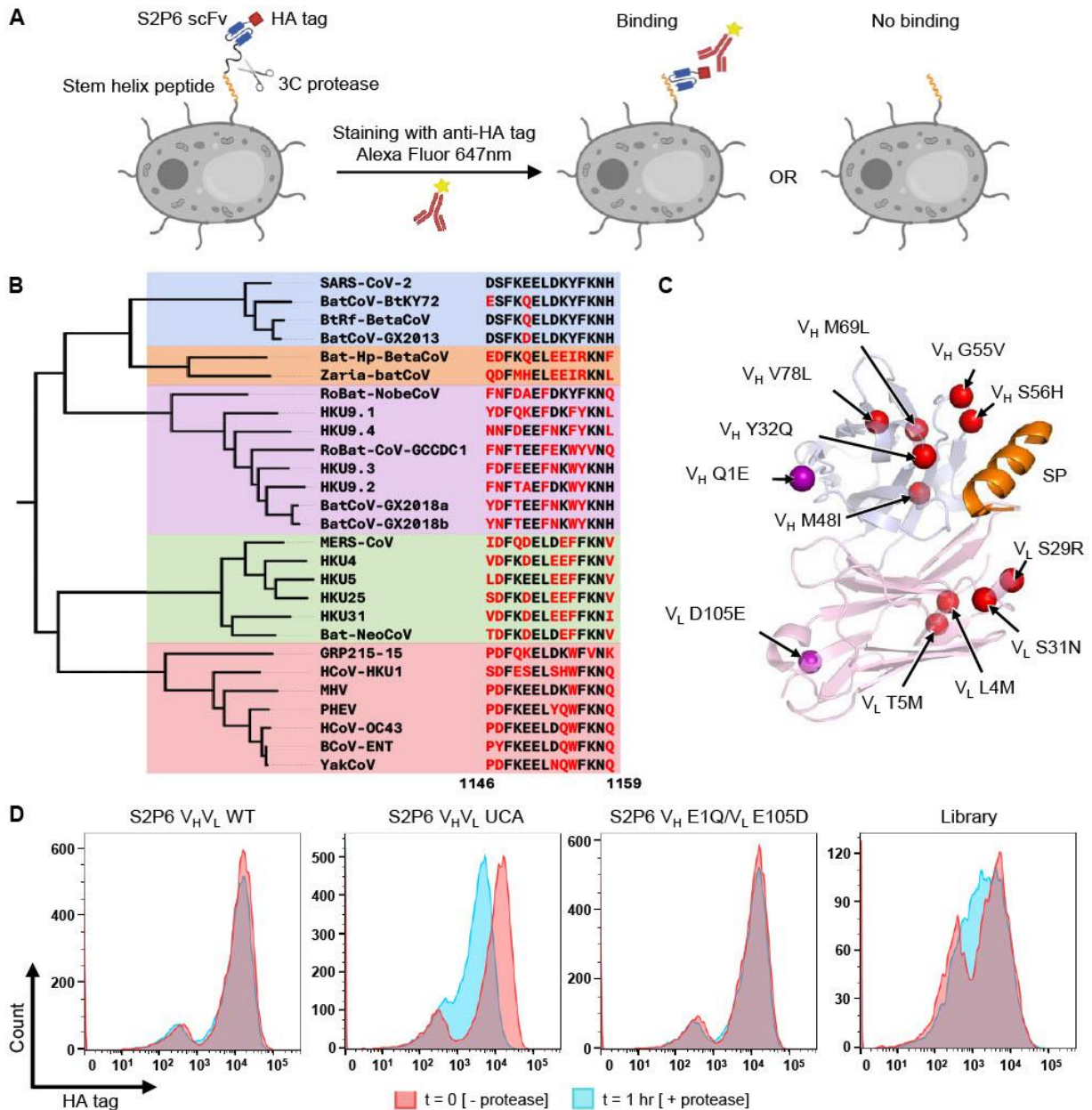
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602

603 **Figure 1. Overview of library-on-library experimental design. (A)** Each yeast cell displayed a

604 S2P6 variant in single-chain variable fragment (scFv) format and a stem helix peptide variant with

605 a 3C protease cleavage site in between. After treatment with 3C protease and staining with anti-

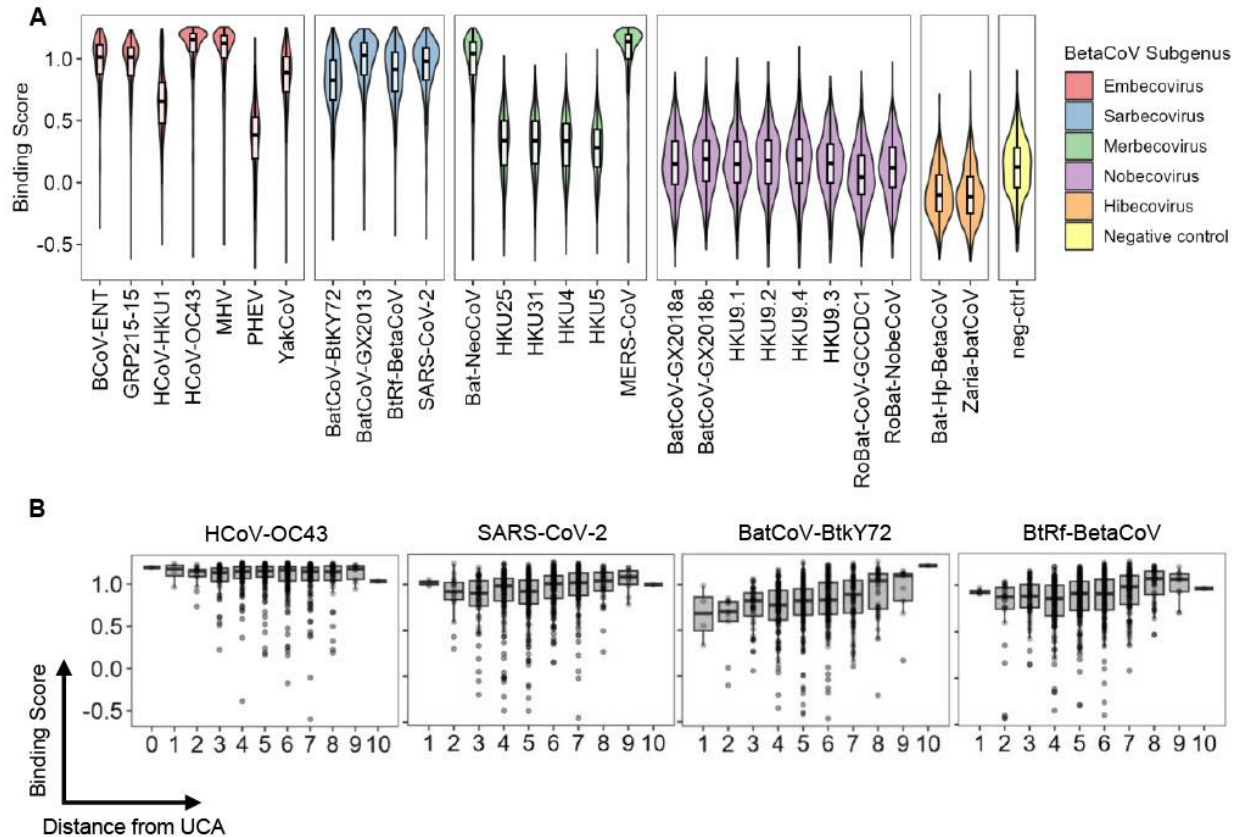
606 HA tag Alexa Fluor 647 nm, S2P6-SP binding could be assessed using flow cytometry. **(B)** A

607 phylogenetic tree was constructed using the spike sequences of the β -CoV strains included in

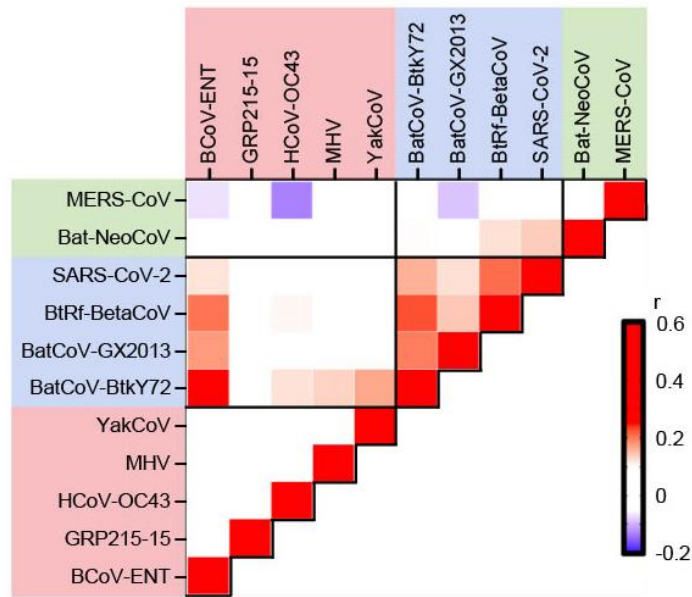
608 this study. Sequences of their spike stem helix peptides (residues 1146-1159, SARS-CoV-2

609 numbering) are shown on the right. Box color denotes β -CoV subgenus: sarbecovirus (blue),

610 hibecovirus (orange), nobecovirus (purple), merbecovirus (green), and embecovirus (red). **(C)**
611 The structure of S2P6 Fab in complex with SARS-CoV-2 stem helix peptide (orange) is shown
612 (PDB 7RNJ)¹⁰, with heavy chain variable domain (V_H) in light blue and light chain variable domain
613 (V_L) in light pink. Somatic hypermutations included in our library design are shown in red and
614 those excluded are in purple. **(D)** Flow cytometry was performed to analyze the HA tag signal
615 (Alexa Flour 647 nm) of yeast cells displaying S2P6 WT, S2P6 UCA, and S2P6 V_H E1Q/V_L E105D
616 with SARS-CoV-2 stem helix peptide, as well as the combination variant library. “- protease” and
617 “+ protease” indicate before and after protease treatment, respectively.



618 **Figure 2. Analysis of S2P6 binding landscapes across stem helix peptides from all β -CoV**
 619 **subgenus. (A)** Average binding scores of S2P6 variants to the indicated stem helix peptide
 620 variants are shown as a violin plot and a boxplot. SARS-CoV-2 stem helix peptide with two alanine
 621 mutations introduced (DSAKEALDKYFKNH), which has previously been shown to abolish binding
 622 to S2P6¹⁰, was used as a negative control. **(B)** Binding scores of S2P6 variants with different
 623 numbers of somatic hypermutations (i.e. distance from UCA) to HCoV-OC43, SARS-CoV-2,
 624 BatCoV-BtkY72, and BtRf-BetaCoV stem helix peptides are shown. Each data point represents
 625 the one S2P6 variant. For the boxplot, the middle horizontal line represents the median. The lower
 626 and upper hinges represent the first and third quartiles, respectively. The upper whisker extends
 627 to the highest data point within a 1.5 \times inter-quartile range (IQR) of the third quartile, whereas the
 628 lower whisker extends to the lowest data point within a 1.5 \times IQR of the first quartile.



629

630 **Figure 3. Correlation of S2P6 binding landscapes between stem helix peptides.** Pairwise

631 Pearson correlations of binding scores between stem helix peptides are shown. Only those stem

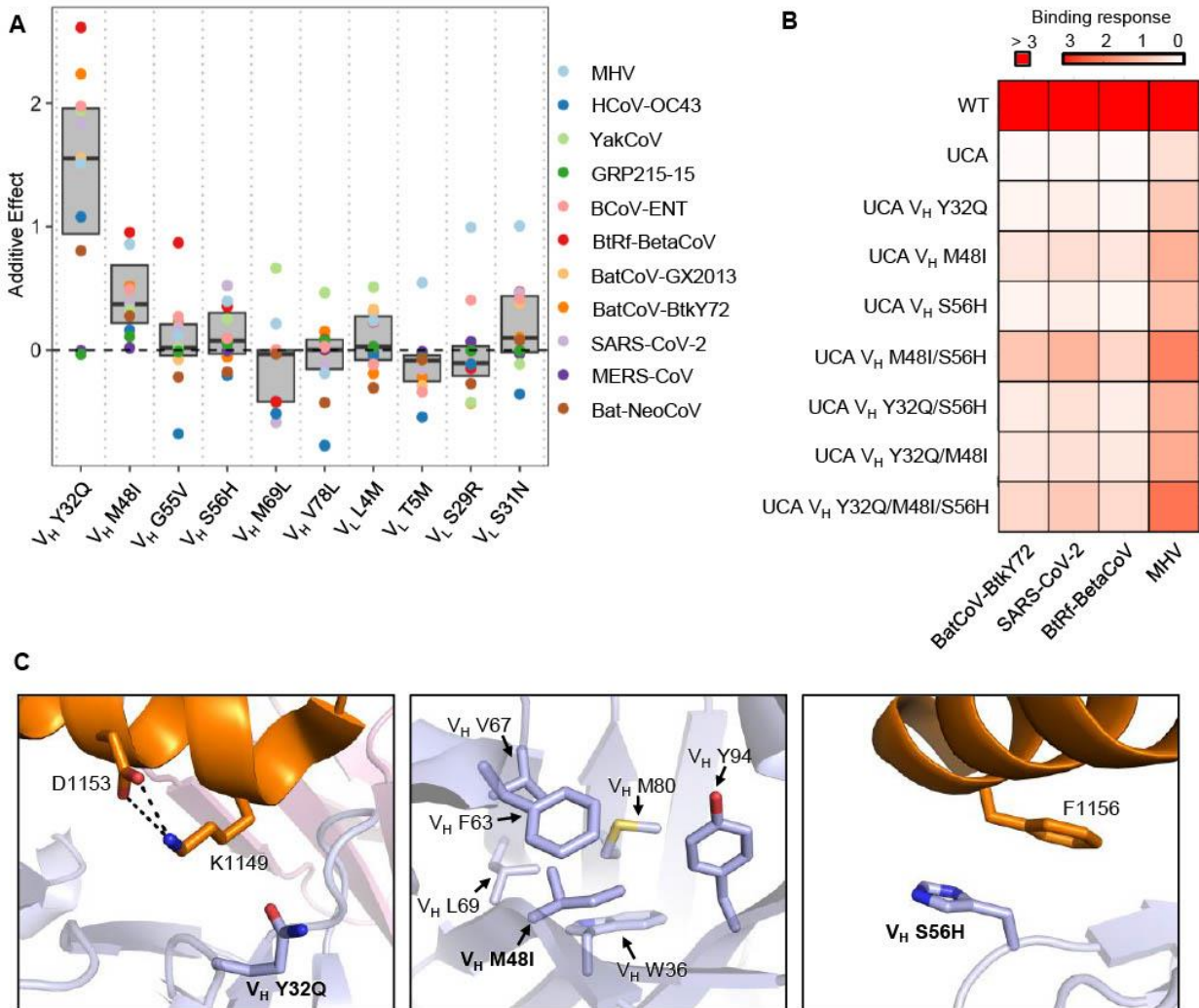
632 helix peptides with an average binding score >0.75 are analyzed here. Red indicates positive

633 correlation coefficient, whereas blue indicates negative correlation coefficient. Diagonal

634 represents the Pearson correlation coefficients between experimental replicates of the indicated

635 stem helix peptides. Shading of axis labels indicates beta-CoV subgenus: merbecovirus (green),

636 sarbecovirus (blue), and embecovirus (red).



637 **Figure 4. Key somatic hypermutations for breadth expansion.** (A) The additive effects of the
 638 indicated somatic hypermutations on binding to different stem helix peptides are shown. Only
 639 those stem helix peptides with an average binding score >0.75 are included here. (B) Response
 640 levels (nm) of S2P6 WT, UCA, and UCA mutant Fabs binding to BatCoV-BtkY72, SARS-CoV-2,
 641 BtRf-BetaCoV, and MHV stem helix peptides were measured using biolayer interferometry. (C)
 642 Structural analysis of somatic hypermutations V_H Y32Q, V_H M48I, and V_H S56H is shown. SARS-
 643 CoV-2 stem helix peptide and S2P6 V_H are shown in orange and blue, respectively (PDB: 7RNJ)¹⁰.