

1 **Title:** Inverse folding of protein complexes with a structure-informed language model enables  
2 unsupervised antibody evolution

3

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16

17 **Abstract**

18 Large language models trained on sequence information alone are capable of learning high level  
19 principles of protein design. However, beyond sequence, the three-dimensional structures of  
20 proteins determine their specific function, activity, and evolvability. Here we show that a general  
21 protein language model augmented with protein structure backbone coordinates and trained on  
22 the inverse folding problem can guide evolution for diverse proteins without needing to  
23 explicitly model individual functional tasks. We demonstrate inverse folding to be an effective

24 unsupervised, structure-based sequence optimization strategy that also generalizes to multimeric  
25 complexes by implicitly learning features of binding and amino acid epistasis. Using this  
26 approach, we screened ~30 variants of two therapeutic clinical antibodies used to treat SARS-  
27 CoV-2 infection and achieved up to 26-fold improvement in neutralization and 37-fold  
28 improvement in affinity against antibody-escaped viral variants-of-concern BQ.1.1 and XBB.1.5,  
29 respectively. In addition to substantial overall improvements in protein function, we find inverse  
30 folding performs with leading experimental success rates among other reported machine  
31 learning-guided directed evolution methods, without requiring any task-specific training data.

## 37 **Introduction**

38 Evolution generates diverse proteins at the level of biological sequences by exploring a  
39 vast search space of potential mutations and acquiring those that improve fitness. However, it is  
40 the three-dimensional structure encoded by these sequences that ultimately determines the  
41 function and activity of a protein. Consequently, as proteins accumulate mutations, they undergo  
42 corresponding structural changes, which in turn facilitate functional adaptations<sup>1</sup>.

43 In the laboratory, this tendency for greater sequence change to cause structural  
44 divergence poses a major challenge to engineering better proteins via a stepwise evolutionary  
45 process. Mutations added in sequential rounds of artificial evolution are increasingly likely to  
46 destabilize the structure and therefore diminish the protein's evolvability<sup>2</sup>. Identifying beneficial  
47 mutations is further challenged by the fact that almost all mutations to a prototypical protein are  
48 deleterious, or at best neutral, and only a rare subset are beneficial on its fitness landscape<sup>3-8</sup>. In  
49 total, these phenomena can often reduce the evolutionarily accessible paths and make evolution  
50 more susceptible to local fitness optima<sup>9,10</sup>, further complicating attempts to increase fitness.

51 To address both the structural constraints of protein design and the high dimensionality of  
52 the mutational search space, we utilized a general protein language model augmented with  
53 structural information and trained across millions of non-redundant single sequence-structure  
54 pairs on the inverse folding objective<sup>11</sup>. Most simply, the inverse folding problem considers the  
55 task opposite of that performed by many of the recent powerful structure-prediction tools,  
56 including AlphaFold and ESMFold<sup>12,13</sup>: recovery of a protein's native sequence, given its three-  
57 dimensional backbone coordinates (**Figure 1a**). This is accomplished by predicting the identity  
58 of an amino acid given both the preceding amino acid sequence (referred to as autoregressive  
59 modeling) and the entire structure's backbone coordinates (**Methods**). Thus, sequences assigned

60 high likelihood scores by the inverse folding language model are expected to fold into the  
61 backbone of the input structure with high confidence (**Figure 1b**).

62 Our inverse folding framework for protein design does not model an explicit protein  
63 function or definition of protein fitness. Rather, using a structure-guided paradigm, we indirectly  
64 explore the underlying fitness landscape by focusing exploration to regions where the backbone  
65 fold of the protein is preserved. We hypothesize constraining evolution to regimes of high  
66 inverse folding likelihood can serve as an effective prior for high-fitness variants, and thereby  
67 improve the efficiency of evolution (**Figure 1c**).

68 We reasoned that this approach may be particularly valuable for the evolution of human  
69 antibodies, which are used clinically to treat a broad range of diseases<sup>14</sup>. Antibodies are used  
70 therapeutically to bind to a target antigen mediating pathogenesis, and modify or disrupt its  
71 function<sup>15</sup>. A central concept of this study is to use the complete structure of the antibody-  
72 antigen complex to guide evolution. By conditioning the inverse folding model on the entire  
73 antibody-antigen complex, we sought to enable the discovery of mutations that preserve or  
74 enhance the stability of the entire complex, and thus that improve antibody function.

75 Indeed, we show that as an unsupervised machine learning-guided evolution strategy,  
76 inverse folding is capable of identifying high fitness mutations across several protein families  
77 and tasks, performing better than sequence-only methods. We found that inverse folding  
78 generalizes to protein complexes with improved antibody variant prediction when antigen  
79 structural information is also included as input. To demonstrate the practical utility of this  
80 method, we improved the potency of mature, clinical SARS-CoV-2 monoclonal antibody  
81 therapies, in a low-throughput setting, against both their original viral target as well as viral  
82 escape variants that reduced their efficacy, namely variants-of-concern (VOC) BQ.1.1 and

83 XBB.1.5. We achieved up to 26-fold improvement in the neutralization potency of Ly-1404  
84 (Bebtelovimab) against BQ.1.1, and 11-fold for SA58, testing only a total of 31 and 25 antibody  
85 variants, respectively. We also achieved 27-fold improvement in affinity against BQ.1.1 and 37-  
86 fold improvement in affinity against XBB.1.5. Notably, all experimentally tested combinations  
87 of inverse folding-recommended mutations showed improved activity, with many designs  
88 comprising multiple synergistic mutations. With our approach, we report experimental success  
89 rates that surpass all previous machine learning-guided protein evolution methods<sup>8,16-28</sup>, including  
90 those based on supervision with task-specific training data. These findings highlight the  
91 advantage of an unsupervised, structure-based paradigm to identify efficient evolutionary  
92 trajectories.

93

## 94 **Results**

95 *Inverse folding enriches sequence exploration for high function protein variants across diverse*  
96 *tasks*

97 We evaluated whether inverse folding can be used to guide protein evolution, without  
98 needing to explicitly model specific functional tasks, by assessing its ability to identify mutations  
99 resulting in high levels of protein activity for a desired functional property, or fitness measure.  
100 Accordingly, for 10 proteins from diverse families among four organisms, and with functions  
101 ranging from enzyme catalysis (TPMT) to oncogenesis (HRAS) to transcriptional regulation  
102 (GAL4), we used inverse folding likelihoods to score variants profiled in large datasets from  
103 deep mutational scanning experiments<sup>29-38</sup> against a target backbone of the wild-type protein<sup>39-48</sup>  
104 **(Methods, Supplementary Table 1).**

105 From the thousands of tested variants for each of the 10 proteins, we identified numerous  
106 with experimentally determined protein activities ranking in the top percentiles of the entire  
107 screen within just the set of top ten inverse-folding predictions (**Figure 1d**). Our analysis also  
108 demonstrates that conditioning on structural information serves to improve predictive capabilities  
109 of protein language models as we successfully identified mutations in the top fifth percentile for  
110 9 out of the 10 proteins using inverse folding compared to just 2 proteins using a state-of-the-art  
111 general protein language model trained only on sequence information and specifically for variant  
112 prediction (ESM-1v)<sup>49</sup> (**Figure 1d**). This improvement in prediction also holds with increasingly  
113 relaxed thresholds for classification as high-fitness variants.

114 These results suggest that inverse folding offers a promising alternative to brute force  
115 experimental searches for beneficial mutations. Notably, some of the top mutations predicted by  
116 inverse folding are also the same ones recovered from exhaustive experimental exploration. For  
117 example, for restriction enzyme haeIIIM, variant Q18E is recommended within the top five  
118 inverse folding predictions and experimentally ranks as the second-best substitution (and > 5  
119 standard deviations above the mean) out of the nearly 2000 substitutions screened to the  
120 endonuclease<sup>38</sup>. Another key advantage of our task-independent framework, in addition to being  
121 broadly applicable across diverse proteins, is the ability to improve a single protein for multiple  
122 desired properties without needing to develop specialized high-throughput assays to screen each  
123 independently. From just the top 10 inverse folding predictions for MAPK1, we identify  
124 substitutions Q105M and Y64D, which are experimentally shown to confer resistance to two  
125 different oncogenic-targeting MAPK1 kinase inhibitors<sup>32</sup>.

126

127 *Inverse folding is a state-of-the-art zero-shot mutational effect predictor for antibodies*

128           To analyze the effectiveness of augmenting a general protein language model with  
129 structural information, specifically for antibody variant prediction, we compared the inverse  
130 folding likelihoods of sequences across entire mutational landscapes against the corresponding  
131 experimental fitness values from three existing mutagenesis datasets. The first two of the datasets  
132 profile the scFv equilibrium dissociation constants ( $K_D$ ) of all possible evolutionary intermediates  
133 between the inferred germline and somatic sequence of naturally affinity-matured influenza  
134 broadly neutralizing antibodies (bnAbs) CR9114 and CR6261, which bind the conserved stem  
135 epitope of influenza surface protein hemagglutinin (HA)<sup>50</sup>. For both bnAbs, only mutations in the  
136 heavy chain, which is responsible for antigen binding, were characterized. The profiled  
137 mutational landscape of CR9114 includes all possible combinations of 16 substitutions while that  
138 of CR6261 includes all possible combinations of 11 substitutions, totaling  $2^{16} = 65,536$  and  $2^{11} =$   
139 2,048 variant antibody sequences respectively. Each of these libraries were screened for binding  
140 against two distinct influenza HA subtypes (H1 and H3 for CR9114 and H1 and H9 for  
141 CR6261). The third dataset assesses the effects of all possible single amino acid substitutions  
142 with a deep mutational scan profiling 4,275 mutations in the variable regions for both heavy  
143 chain (VH) and light chain (VL) of antibody G6.31 to binding with its ligand, vascular  
144 endothelial growth factor A (VEGF-A)<sup>51</sup>.

145           For each dataset, we computed the Spearman correlation between the log likelihood  
146 estimated by the inverse folding model and the experimentally determined binding measure for a  
147 given antigen, across all sequences in the mutational library. We scored the inverse folding  
148 likelihood of each candidate sequence in the library using the backbone coordinates of a structure  
149 with the mature antibody bound to its target antigen<sup>52-54</sup>.

150           Across all five experimental binding datasets, we found that inverse folding performs  
151 better than both a sequence-only language model, ESM-1v<sup>49</sup>, and a site-independent model of  
152 mutational frequency curated with extensive antibody sequence alignments, abYsis<sup>55</sup>. In nearly  
153 all experimental scenarios, supplementing sequence information with the backbone coordinates  
154 of the antibody alone, without providing antigen information, as input to inverse folding is  
155 sufficient to outperform other sequence-only methods. A notable feature of the autoregressive  
156 architecture is that it computes the joint likelihood over all positions in a sequence, making it  
157 well-suited to score combinatorial sequence changes. We find that inverse folding can capture  
158 complex epistatic interactions, or potential interdependence among individual amino acids, as it  
159 performs well on the CR9114 and CR6261 libraries composed of sequences with multiple  
160 mutations (**Figure 2a,b**).

161           We achieved the greatest improvement in performance on all five experimental screens  
162 by incorporating the structure of both the antibody and antigen (**Figure 2a**), indicating that the  
163 inverse folding model can implicitly learn features of binding (**Figure 2c**). This result is  
164 particularly significant, given that the inverse folding model is only trained on single-chain  
165 protein structures, while the antibody-antigen complexes we use as inputs are composed of either  
166 three (G6.31) or four (CR9114, CR6261) protein chains. The most substantial contribution of  
167 antigen information is observed in the case of CR9114-H1, for which the correlation increases  
168 from 0.17 with only antibody information to 0.65 with sequence and backbone coordinates of the  
169 entire complex.

170           Remarkably, we could still predict effects of mutations on binding for a cross-reactive  
171 antibody while using a different antigen as input to the model. (**Figure 2a,b**). Despite using a  
172 complex with HA from H5N1 influenza as input to score CR9114 variants, we obtain



173 correlations of 0.65 and 0.50 with experimental binding data for H1 and H3, respectively. This is  
174 particularly striking since, for example, H5 and H1 only share 63% sequence identity across both  
175 HA subunits (**Supplementary Figure 3**). This same cross-reactive predictive capability is  
176 observed for CR6261, which is tested experimentally against H1 and H9 while we use an input  
177 structure with HA from 1918 H1N1 influenza (**Figure 2a**). Although inverse folding cannot learn  
178 explicit chemical rules of binding (e.g., hydrogen bonding or disulfide bridge formation) since it  
179 does not have access to amino acid side chain atomic coordinates, these results suggest that  
180 structural principles like interface packing or potential steric interference are not only implicitly  
181 accessible from residue identities, but are also informative for binding prediction.

182 Our model's top recommended mutations are made independently of a specific definition  
183 of fitness; they simply represent a set of variants with a high likelihood of folding into the input  
184 backbone structure. Therefore, our model's recommendations may also help identify mutations  
185 that improve other useful biochemical properties beyond affinity. Impressively, for example, the  
186 top inverse folding-recommended mutation to the VL of G6.31 is F83A, which was identified in  
187 the original screening study<sup>51</sup> to be particularly interesting as it confers a three-fold increase in  
188 VEGF-A binding affinity and a 5°C improvement in melting temperature, despite being 25Å  
189 from the antigen and in the antibody framework region. It was determined that the VL F83A  
190 substitution induces more compact packing and the site serves as a conformational switch that  
191 affects biological activity at the antibody-antigen interface by modulating both interdomain and  
192 elbow angle dynamics<sup>51</sup>.

193

194 *Engineering therapeutic antibodies for increased potency and resilience*

195 Finally, we aimed to assess if the structure-augmented language model's predictive  
196 capabilities could not only resolve trends on large sets of experimental data, but also enable  
197 efficient and successful directed evolution campaigns while testing only a small number (on the  
198 order of tens) of variants. To do so, we considered the task of improving the potency and  
199 resilience (effectiveness against a virus as it mutates over time) of two mature, clinical  
200 monoclonal antibody therapies.

201

- 202 • Ly-1404 (Bebtelovimab) was isolated from a COVID-19 convalescent donor and binds to  
203 the receptor binding domain (RBD) of the SARS-CoV-2 Spike protein<sup>56</sup>. It was approved  
204 by the U.S. F.D.A. on February 11, 2022 given its activity against both the original  
205 Wuhan and Omicron SARS-CoV-2 variants and was the last remaining approved  
206 monoclonal antibody therapy withstanding against viral evolution<sup>57</sup> until its  
207 discontinuation on November 30, 2023 due to antibody evasion by VOC BQ.1.1.<sup>58</sup>
- 208 • SA58 (BD55-5840) was isolated from a vaccinated individual and is one of two RBD-  
209 targeting neutralizing antibodies (NAb) in a rationally developed antibody cocktail. SA58  
210 alone retained efficacy against all Omicron subvariants, including *in vivo* protection  
211 against BA.5<sup>59,60</sup> and was shown to be effective as a post-exposure prophylaxis in a  
212 clinical study<sup>61</sup>.

213

214 For both antibody engineering campaigns, we used the inverse folding language model to  
215 compute likelihoods of all ~4,300 possible single-residue substitutions in the VH or VL regions  
216 of the antibody. In the first round of evolution, we selected only the top ten predictions at unique  
217 residues in each chain for experimental validation. An important practical benefit of our method

218 is the ability to optimize against measures of fitness most relevant to the protein's downstream  
219 function, rather than being limited to indirect and less accurate surrogate measures that are more  
220 amenable to high-throughput screening<sup>4,16</sup>. We leverage this advantage to directly evolve these  
221 antibodies for their ability to more potently neutralize SARS-CoV-2 pseudotyped lentivirus.

222 Variants recommended by the inverse folding language model were assessed by  
223 comparing the half-maximal inhibitory concentration (IC<sub>50</sub>) relative to the wild-type antibody.  
224 Remarkably, although we chose to only test 20 single-site substitutions for each of the two  
225 clinical monoclonal antibody therapies, approximately one-third of them improved neutralizing  
226 potency. Notably, several of these variants improve neutralization IC<sub>50</sub> by approximately 2-fold  
227 with just a single amino acid change (**Figure 3a, Supplementary Data 1**).

228 Prompted by recent evidence showing that conservation of the overall RBD structure is  
229 robust to SARS-CoV-2 evolution<sup>62</sup>, we next sought to determine whether we could also evolve  
230 the previously mature antibodies against SARS-CoV-2 BQ.1.1, the variant responsible for  
231 diminished therapeutic efficacy. Although the antibodies were previously effective, a change in  
232 antigen conceptually represents a fundamental shift in the underlying fitness landscape (**Figure**  
233 **3b**). From the same set of 20 single amino acid substitutions to Ly-1404, we found that nearly  
234 half improve neutralization of variant BQ.1.1. In addition to a high success rate, we also found  
235 multiple of these mutations provided a large magnitude of improvement. Several single amino  
236 acid substitutions to Ly-1404 individually result in over a 3-fold improvement while the most  
237 beneficial mutation to SA58 results in a nearly 7-fold improvement (**Figure 3c**).

238 Taken together, approximately two-third and one-third of tested single amino acid  
239 substitutions to Ly-1404 and SA58, respectively, were beneficial for neutralization of either the  
240 original strain or BQ.1.1. These results reinforce that, despite all being predicted to have the

241 same backbone fold, inverse folding variants feature functional diversity and can be used for  
242 distinct notions of protein fitness. Interestingly, for both antibodies, the most beneficial mutation,  
243 is not shared by the each of the strains tested (**Supplementary Figure 4**).

244 A common challenge in directed evolution is contending with the combinatorial  
245 explosion of possible sequences which emerges from trying to combine a set of individually  
246 beneficial mutations. In the second round of evolution, we simply use the inverse folding model  
247 again to acquire up to five top-scoring unique combinations of mutations to each antibody chain  
248 (**Methods**). Notably, across both evolutionary trajectories, all 15 antibody designs with multiple  
249 mutations have  $IC_{50}$  values better than wild-type, with many designs showing synergistic effects  
250 upon combination. For example, just a single amino acid mutation in each of the two chains of  
251 SA58 leads to over an 11-fold improvement (**Figure 3c,d**). Similarly, the most potent evolved  
252 design of Ly-1404 is a combination of seven of the eight beneficial single amino acid  
253 substitution to the VH and improves neutralization 26-fold (**Figure 3d**). Critically, these  
254 improvements to neutralizing potency against BQ.1.1 do not sacrifice potency against the  
255 original strains. We found that the top SA58 design against BQ.1.1 after the second round of  
256 evolution also improves BA.1 neutralization nearly 3-fold (**Supplementary Data 1**).

257

#### 258 *Additional characterization of evolved antibodies*

259 To further characterize the basis for enhanced neutralization of SARS-CoV-2 VOC  
260 BQ.1.1, we tested the binding affinity of all variant antibodies to RBD as bivalent IgG using  
261 biolayer interferometry (BLI) to obtain the apparent dissociation constant ( $K_{D,app}$ ). For Ly-1404,  
262 all 23 variants with improved neutralization also have improved binding affinity up to ~27-fold.  
263 Interestingly, we found four additional inverse folding-recommended mutations, which were

264 neutral or deleterious to neutralization, also improved binding affinity. Across all variants there  
265 is a Spearman correlation of 0.47 between fold-change in  $IC_{50}$  and fold-change in  $K_{D,app}$  (**Figure**  
266 **4a**).

267 We similarly screened the SA58 variants for binding to the RBD of BQ.1.1. However,  
268 since the  $K_D$  of the wildtype antibody as IgG was already sub-picomolar, further improvements to  
269 binding were below the limit of quantitation and indistinguishable using this measure. Given this  
270 strong binding affinity of wildtype SA58 to BQ.1.1 RBD, we also screened this same set of  
271 variants against emerging VOC XBB.1.5 and observe improvements in  $K_{D,app}$  up to 37-fold  
272 (**Figure 4c**).

273 By testing several top affinity-matured designs in a polyspecificity assay, we also  
274 confirmed that improvements in binding are not mediated by generalized enhancements of non-  
275 specific interactions (**Supplementary Figure 5a**). In this assay, we observed no substantial  
276 changes in off-target binding of the evolved antibodies to membrane soluble proteins,  
277 particularly within a therapeutically viable range (as defined by controls of clinically approved  
278 antibodies with recorded high and low polyspecificity). Furthermore, we found no correlation  
279 between fold-change in polyspecificity and affinity fold-change (**Supplementary Figure 5b**).

280

### 281 *Analysis of evolutionary exploration*

282 Confronted by the large number of possible mutations, traditional experimental-based  
283 methods for antibody affinity maturation often restrict the mutational search space to only a few  
284 regions of the antibody. Specifically, binding optimization efforts are typically focused within  
285 the complementarity determining regions (CDR), which are hotspots for natural somatic  
286 hypermutation. However, using our unbiased approach to consider all regions of the variable

287 domain allows for many discoveries that may be less intuitive to a rational designer. For  
288 example, the most beneficial substitutions to Ly-1404, VH F24Y and VH V90S, are located  
289 within framework regions and positioned distally from the binding interface (**Supplementary**  
290 **Figure 6, Supplementary Table 2**). Interestingly, they both improve neutralization of BQ.1.1  
291 by over 3-fold and are not deleterious to Wuhan neutralization. In other cases, inverse folding  
292 also successfully predicts beneficial substitutions using residues rarely observed among human  
293 antibody sequences. Substitution VL N95V in SA58, which improves neutralization  
294 approximately 7-fold against BQ.1.1, is mediated by the incorporation of a valine observed in  
295 only 0.7% of human antibody sequences at that position and enhances antibody-antigen contact.  
296 While inverse folding is capable of successfully making novel predictions, in some instances it  
297 also does suggest reverting residues to ones frequently selected for in natural somatic  
298 hypermutation. Mutation VL F51Y in Ly-1404 changes a phenylalanine observed in just 5% of  
299 sequences to a tyrosine observed in 86% of sequences. However, this variant results in no change  
300 to Wuhan neutralization. Overall, these results highlight the novelty and value in augmenting a  
301 language model with structural information to evolve antibodies and proteins complexes.

302

## 303 **Discussion**

304 The discovery of mutations that improve protein function is inherently challenging due to  
305 the large sequence search space and complex rules that govern the relationship between sequence  
306 and function, such as stability or environmental selection pressures. We show that a general  
307 inverse folding protein language model informed with the sequence and backbone structural  
308 coordinates of a protein can considerably improve directed evolution efforts by serving as an  
309 improved prior compared to sequence-only deep learning methods. Importantly, we highlight

310 that inverse folding can interrogate protein fitness landscapes indirectly, without needing to  
311 explicitly model individual functional tasks or properties, making it broadly applicable to  
312 proteins across diverse settings ranging from enzyme catalysis to antibiotic and chemotherapy  
313 resistance (**Figure 1d**). We also demonstrate inverse folding generalizes to multimeric proteins,  
314 despite being trained only on single-chain proteins, through its ability to implicitly learn features  
315 of binding. This result is particularly remarkable considering inverse folding has no access to  
316 amino acid side chain atoms, coordinates, or bond information.

317 Equipped with these capabilities, we use inverse folding to evolve clinical therapeutic  
318 antibodies and identify several mutations which act synergistically to improve antibody potency  
319 and resilience against emerging variants of concern. In the context of pandemics and emergency-  
320 use situations, where monoclonal antibody therapies are limited in supply and vulnerable to  
321 resistance from viral evolution, the ability to rapidly make improvements in potency with a  
322 general method could have major clinical and economic implications.

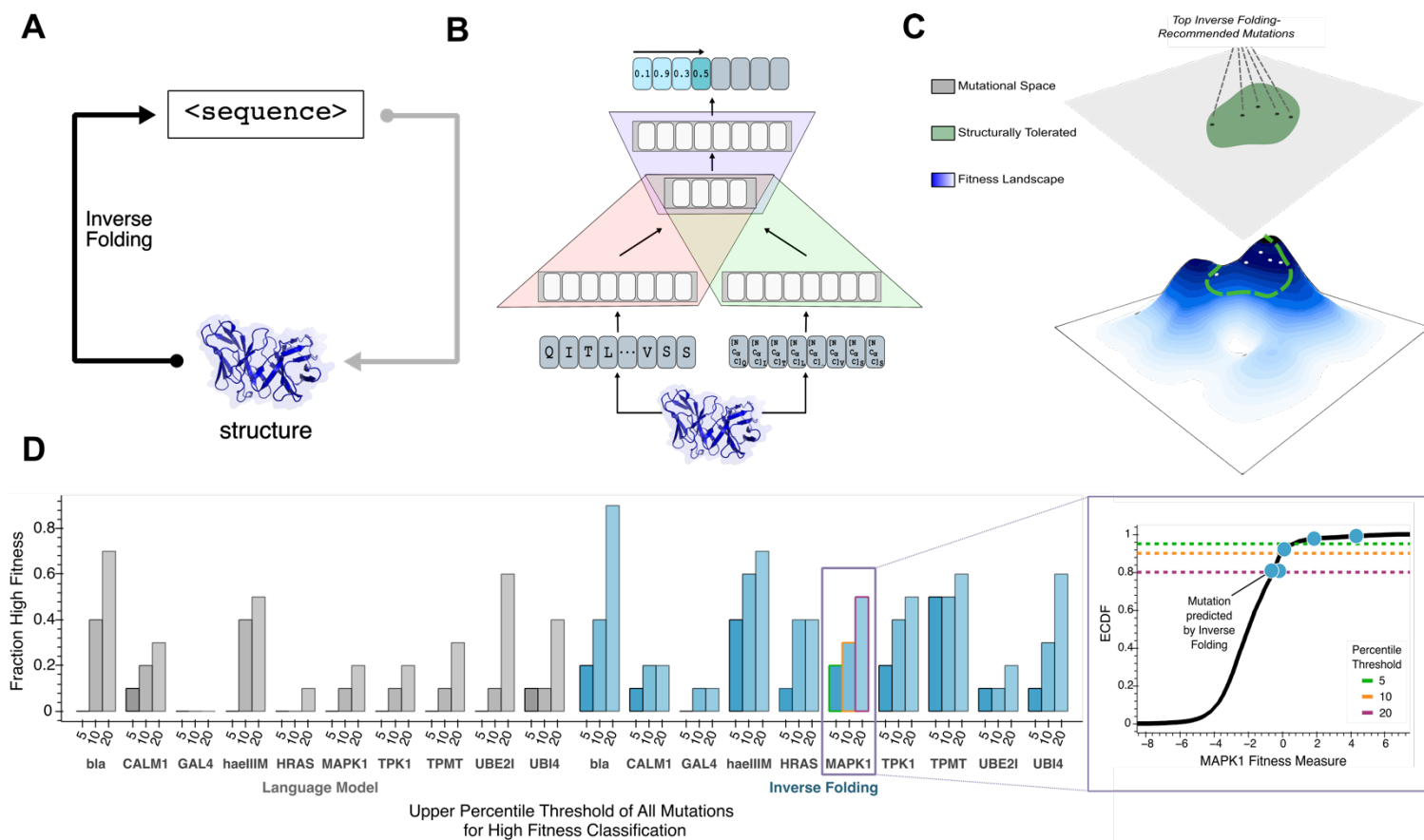
323 In comparison to fourteen other promising machine learning-guided protein design  
324 methods<sup>8,16–28</sup>, we find that inverse folding has the strongest performance to date, even without  
325 requiring any assay-labeled fitness data to use as training data for task-specific model supervision  
326 (**Figure 5, Supplementary Data 5**). By eliminating the reliance on any initial data collection,  
327 inverse folding has the potential to accelerate entire evolutionary campaigns.

328 Computational methods like the one we propose have the opportunity to democratize  
329 protein engineering efforts. Not only is our approach more efficient than conventional resource-  
330 intensive techniques that experimentally test the effects of all single-residue changes on  
331 biochemical functions like binding affinity, but consequently it enables directed evolution based  
332 on properties that are not easily measured at scale or that are incompatible with high-throughput

333 screening. Overcoming these limitations, we anticipate our structure-based paradigm will be  
334 useful for evolving proteins across many domains.

335





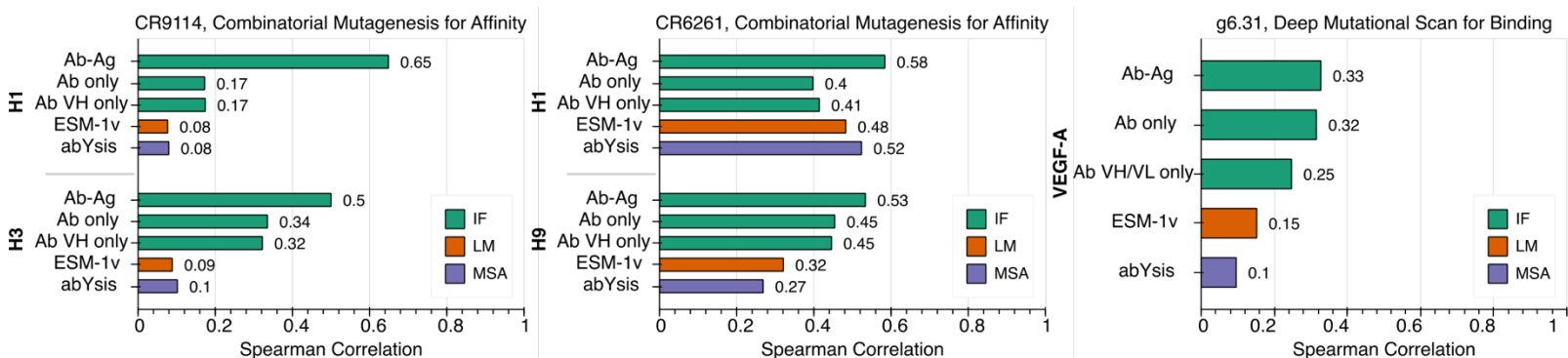
336

### 337 **Figure 1: Guiding evolution of diverse proteins via inverse folding**

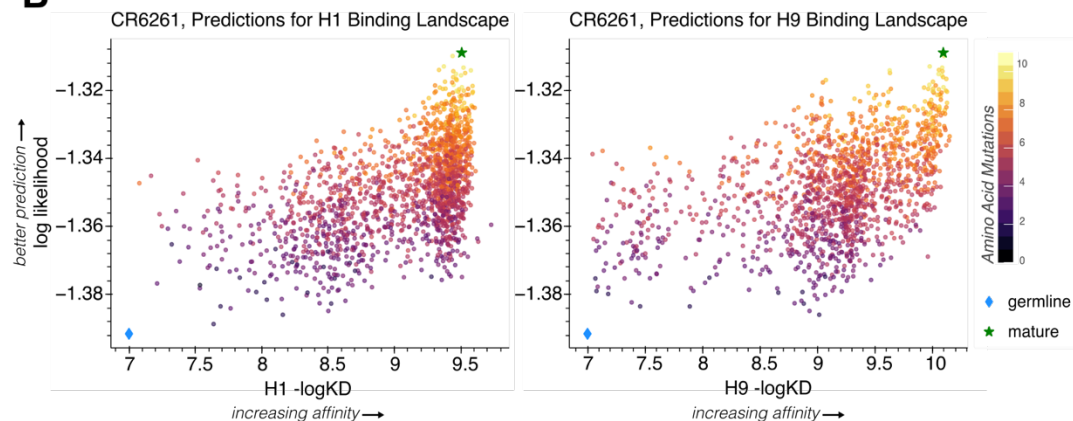
338 **(A)** The inverse folding problem refers to the prediction of a protein's native amino acid  
 339 sequence, given its three-dimensional backbone structure, which is conceptually analogous to the  
 340 opposite problem solved by structure prediction tools like AlphaFold<sup>12</sup>. **(B)** A hybrid  
 341 autoregressive model<sup>11</sup> integrates amino acid values and backbone structural information to  
 342 evaluate the joint likelihood over all positions in a sequence. Amino acids from the protein  
 343 sequence are tokenized (red), combined with geometric features extracted from a structural  
 344 encoder (green), and modeled with an encoder-decoder transformer (purple). Sequences assigned  
 345 high likelihoods by the model represent high confidence in folding into the input backbone  
 346 structure. **(C)** Our structure-guided framework for protein design indirectly explores the  
 347 underlying fitness landscape, without modeling a specific definition of fitness or requiring any

348 task-specific training data, by constraining the search space to regions where the backbone fold  
349 preserved. **(D)** High fitness sensitivity analysis reveals that multimodal input improves language  
350 model performance compared to sequence-only input across 10 proteins from diverse protein  
351 families (left). 'Fraction High fitness' is the fraction of the top ten single amino acid substitutions  
352 recommended by each model that are ranked in the top indicated percentile of all experimentally  
353 screened variants. A representative plot (right) demonstrates this metric for assessing enrichment  
354 of high-fitness MAPK1 mutations, with successfully predicted mutations highlighted (blue) on  
355 the empirical cumulative density function (ECDF) of the experimental data (black). The three  
356 different thresholds, as defined by percentiles, are also shown as dashed lines. Inverse folding  
357 predictions are more enriched, on average, for high fitness variants across various tested  
358 thresholds for high fitness classification. bla, Beta-lactamase TEM; CALM1, Calmodulin-1;  
359 haeIIIM, Type II methyltransferase M.HaeIII; HRAS, GTPase HRas; MAPK1, Mitogen-  
360 activated protein kinase; TMPT, Thiopurine S-methyltransferase; TPK1, Thiamin  
361 pyrophosphokinase 1; UBI4, Polyubiquitin; UBE2I, SUMO-conjugating enzyme UBC9  
362

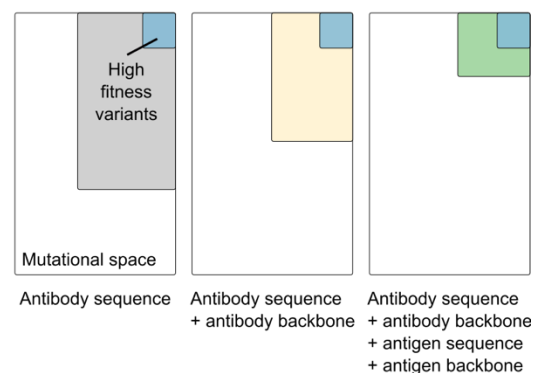
**A**



**B**



**C**



363

364 **Figure 2: Inverse folding of antibody-antigen complexes resolves mutational landscapes by**  
 365 **implicitly learning features of binding and protein epistasis**

366 (A) Spearman correlation using inverse folding as well as sequence-based modeling approaches

367 ESM-v<sup>49</sup> and abYsis<sup>55</sup> reported for three antibodies screened with corresponding influenza A HA

368 subtypes H1, H3, and H9. Bars are colored by the type of model used: IF, Inverse Folding

369 (green); LM, Language Model (orange); and MSA, Multiple Sequence Alignment (purple).

370 Inverse folding was evaluated in three different settings: i) providing the entire antibody variable

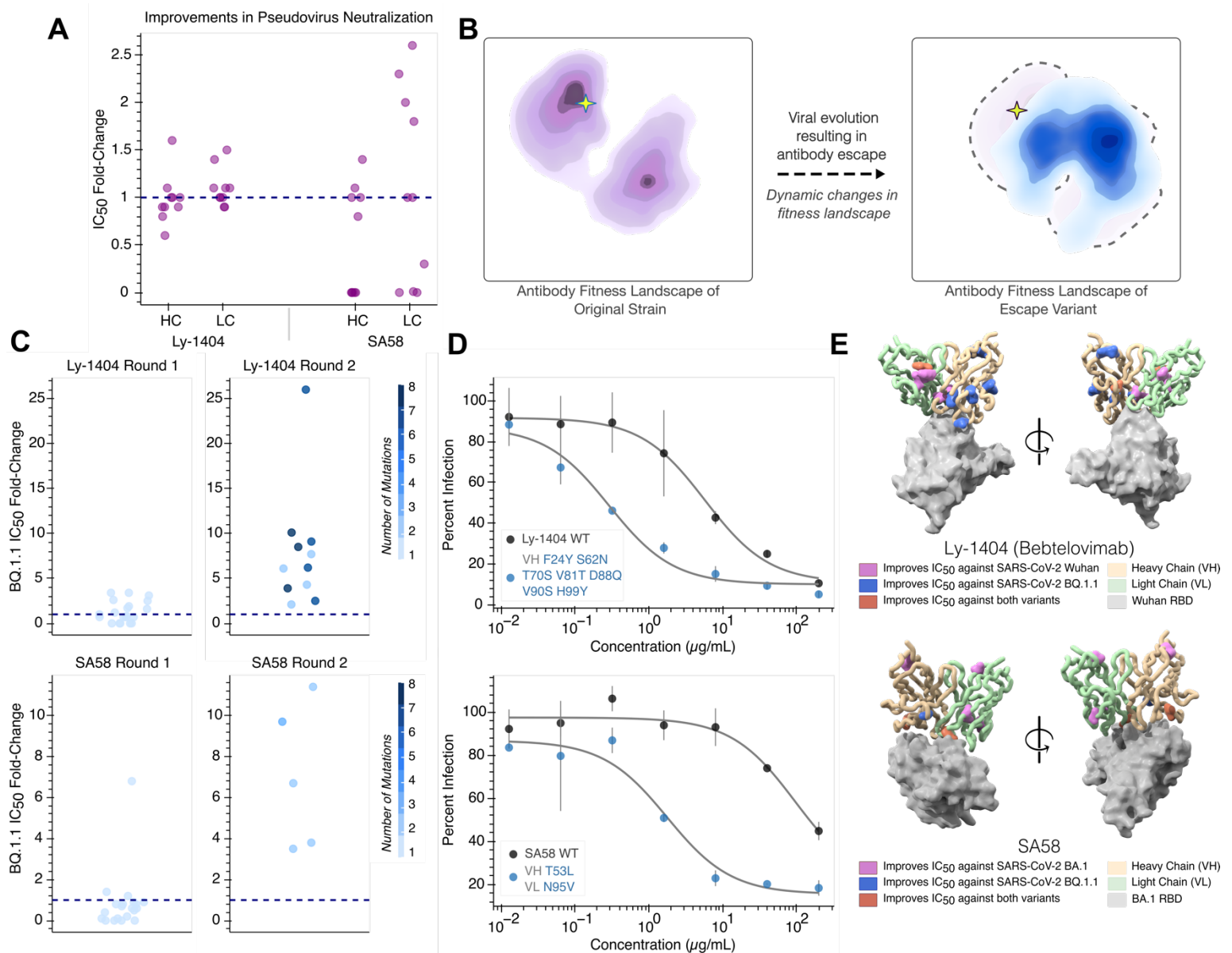
371 region and antigen complex (Ab-Ag) ii) providing only the antibody variable region (Ab only),

372 and iii) providing only the single antibody variable region of the chain responsible for binding or

373 being mutated (Ab VH only or Ab VH/VL only). Inverse folding implicitly learns features of

374 binding and protein epistasis. For example, when scoring combinatorial mutations to CR9114

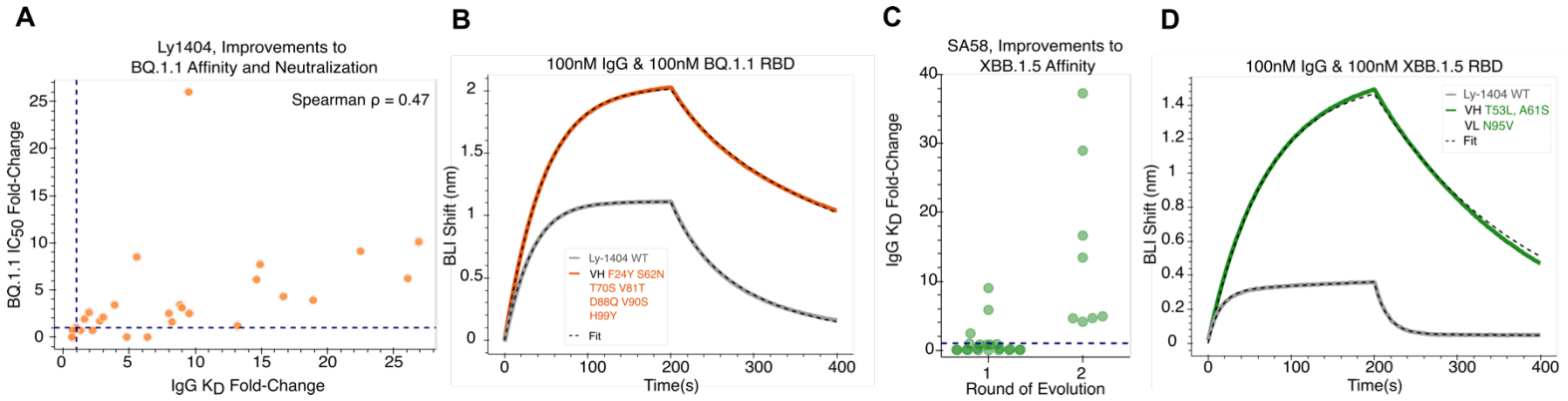
375 against H1, we find that the model has much higher performance (Spearman  $\rho = 0.65$  for H1, 0.5  
376 for H3) than a masked language model ESM-1v (Spearman  $\rho = 0.08$  for H1, 0.09 for H3) and a  
377 site-independent, alignment-based model abYsis (Spearman  $\rho = 0.08$  for H1, 0.1 for H3). This  
378 performance improvement is also consistent across the other combinatorial landscapes tested. **(B)**  
379 Scatter plots showing inverse folding predictions against experimentally determined dissociation  
380 constants of CR6261 against HA-H1(left) and HA-H9 (right). The germline and mature  
381 sequences are highlighted on all plots as indicated in the legend. For visualization, all scatter  
382 plots omit points on the lower limit of quantitation. Further analysis of assay limit on predictive  
383 performance is shown in **Supplementary Figure 2**. **(C)** Conceptual schematic representation of  
384 protein language performance improvements with improved priors. Providing sequence and  
385 structural information of both the antibody and antigen enables inverse folding to most  
386 efficiently identify complex destabilizing mutations and enrich for high fitness antibody variants.  
387



388 **Figure 3. Inverse folding-guided evolution of antibodies improves neutralization potency**  
 389 **and resilience**

390 (A) Each point represents the fold-change in IC<sub>50</sub> of pseudovirus neutralization for antibody  
 391 variants with single amino acid mutations. Antibodies are tested against the viral strain  
 392 represented in the input structure (Ly1404- Wuhan, SA58-BA.1 Omicron). A dashed line is  
 393 shown at fold-change of 1 corresponding to no change. 35% of Ly-1404 variants and 30% of  
 394 SA58 variants improved antibody potency (defined as 1.1-fold or higher improvement in IC<sub>50</sub>)

395 compared to wild-type). Among this subset of beneficial mutations, we identify single amino  
396 acid mutations that provide a 1.6-fold improvement in Ly-1404 IC<sub>50</sub> and a 2.6-fold  
397 improvement in SA58 IC<sub>50</sub>. **(B)** Conceptual representation of viral evolution. Selection for  
398 immune evasion drives antibody escape, which fundamentally represents a dynamic change in  
399 the underlying fitness landscape for the antibody. This antigenic drift displaces a potent antibody  
400 from a peak on the previous fitness landscape (left) to a new starting point at lower activity  
401 (right). **(C)** Strip plots visualizing antibody evolution across two rounds. Each point shows the  
402 corresponding fold-change in IC<sub>50</sub> of pseudovirus neutralization for a designed variant and is  
403 colored according to the number of mutations it has (1-8). Consistent with preserving backbone  
404 fold, all 55 designed variants across both antibody evolutionary campaigns could be expressed.  
405 All round 1 variants are only composed of only single amino acid changes while beneficial  
406 mutations are combined in round 2. All round 2 variants have improved neutralization activity  
407 compared to their respective wild-type antibody (dotted line). **(D)** Pseudovirus neutralization  
408 curves are shown for the most potent evolved antibody variant, consisting of mutations annotated  
409 to the left. The top Ly-1404 variant, bearing seven amino acid substitutions in VH, achieves a  
410 26-fold improvement in neutralization against BQ.1.1 (top). The top SA58 variant, bearing single  
411 amino acid mutations in both VH and VL, achieves an 11-fold improvement in neutralization  
412 against BQ.1.1 (bottom). **(E)** Residues at which mutations improve neutralization against either  
413 the structure-encoded strain, BQ.1.1, or both viral strains are highlighted with spheres for  
414 antibodies Ly-1404 (PDB 7MMO) and SA58 (PDB 7Y0W). Notably, beneficial mutations are  
415 identified both within the binding interface as well distal to the antigen. Neutralization enhancing  
416 mutations are labeled in **Supplementary Figure 6**.  
417



418

419 **Figure 4: Antibodies evolved for high potency also exhibit improved affinity**

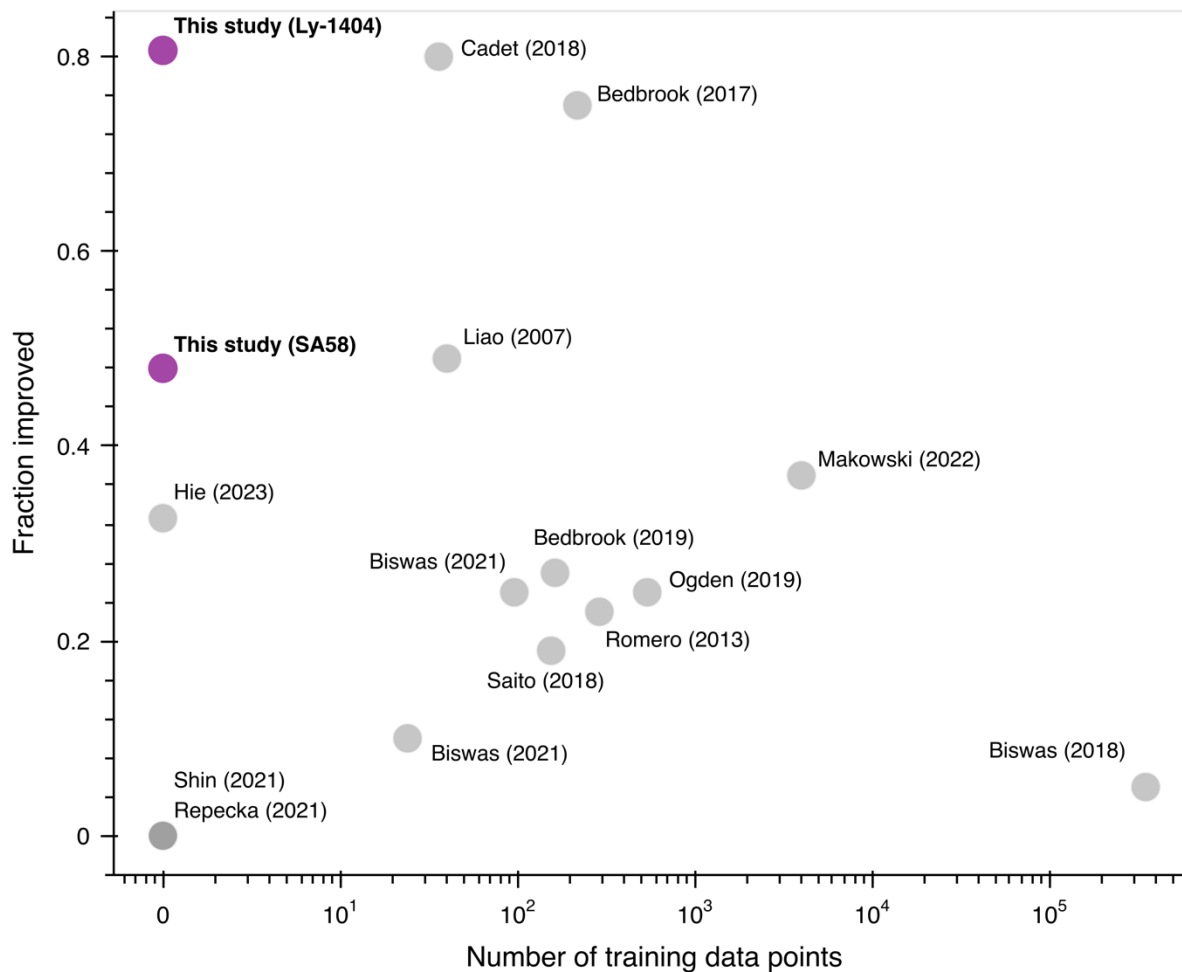
420 (A) Ly-1404 antibody variants show a Spearman correlation of 0.47 between apparent affinity  
421 fold-change and potency fold-change. Improved affinity is observed to be necessary but not  
422 sufficient for improved neutralization activity. Four variants exhibit improved affinity but do not  
423 enhance neutralization. All variants with improved neutralization also display improved affinity.  
424 The top inverse folding Ly-1404 design with a 27-fold improvement in neutralization has a 9.5-  
425 fold improvement in affinity to BQ.1.1 RBD, as measured using BLI. (C) SA58 antibodies  
426 evolved for improved potency against BQ.1.1 also exhibit improved affinity against VOC  
427 XBB.1.5, up to 37-fold. (B, D) Representative traces of BLI binding assays for Ly-1404 and  
428 SA58 variants with improved affinity.

429

430

431

432



433 **Figure 5: Comparison to other machine learning-guided directed evolution methods**

434 'Fraction improved' refers to the hit rate of variants tested which are improved relative to a  
435 wildtype protein used as a starting point for directed evolution or a reference protein used as a  
436 design template. Higher hit rates indicate more efficient experimental exploration. Inverse  
437 folding achieves the highest hit rate with the lowest number of assay-labeled training data points  
438 to-date<sup>8,16-28</sup>.



## 439 **Methods**

### 440 *Inverse folding model description and scoring of sequences*

441 As input to the inverse folding model, we provide a protein structure  $\mathbf{Y} \in \mathbb{R}^{N \times 3 \times 3}$ , where  
442  $N$  is the number of amino acids, and each amino acid is featurized by the three-dimensional  
443 physical coordinates of all three atoms in the protein backbone: the  $\alpha$ -carbon,  $\beta$ -carbon, and  
444 nitrogen atoms in the protein backbone (hence the dimensionality  $N \times 3 \times 3$ ). The inverse  
445 folding model learns the probability distribution  $p$  of a protein sequence  $\mathbf{x} = (x_1, \dots, x_N) \in \mathcal{X}^N$   
446 (where  $\mathcal{X}$  is the alphabet of amino acids) given a structure  $\mathbf{Y}$  via the chain rule of probability

$$447 \quad p(\mathbf{x}|\mathbf{Y}) = p(x_1|\mathbf{Y})p(x_2|x_1, \mathbf{Y}) \dots p(x_N|x_1, \dots, x_{N-1}, \mathbf{Y}).$$

448 The probability distribution at each position is defined over  $\mathcal{X}$ , such that it is a 20-  
449 dimensional vector with all constituent entries summing to 1.

450 Thus, for a given sequence  $\hat{\mathbf{x}} = (\hat{x}_1, \dots, \hat{x}_N)$  and its corresponding given structure  $\hat{\mathbf{Y}}$ , we  
451 can score the probability of  $\hat{\mathbf{x}}$  folding into  $\mathbf{Y}$  under the inverse folding model by computing the  
452 value of  $p(\mathbf{x} = \hat{\mathbf{x}}|\mathbf{Y})$ , which we can do autoregressively as

$$453 \quad p(\mathbf{x} = \hat{\mathbf{x}}|\hat{\mathbf{Y}}) = p(x_1 = \hat{x}_1|\hat{\mathbf{Y}}) \dots p(x_N = \hat{x}_N|\hat{x}_1, \dots, \hat{x}_{N-1}, \hat{\mathbf{Y}}).$$

454 This is evaluated output is a likelihood between 0 and 1, inclusive. The computed score  
455  $p(\mathbf{x} = \hat{\mathbf{x}}|\hat{\mathbf{Y}})$  is used as prediction for “fitness” (e.g., binding affinity or enzymatic activity).  
456 Importantly, the inverse folding model does not have any explicit access to “fitness” during  
457 either training or evaluation, which we refer to as “zero shot” fitness prediction.

458 We use the inverse folding model checkpoint of ESM-IF1 GVP-Transformer as of April  
459 10, 2022<sup>11</sup>.

460

461 *Diverse proteins benchmarking experiment with scanning mutagenesis data*

462 We analyzed the effectiveness of using the inverse folding language model, ESM-IF1  
463 model to identify high fitness variants from protein mutational scans as a proxy for the ability to  
464 guide evolution without explicitly modeling a protein's function. We also compared its  
465 performance to ESM-1v, a sequence-only general protein language model. To do so, we used all  
466 deep mutational scanning (DMS) datasets from the benchmarking study by Livesey and Marsh<sup>29</sup>  
467 profiling over 100 variants and reported to have 90% or higher coverage of DMS results across  
468 the corresponding curated PDB structure (**Supplementary Table 1**). From this set of 12  
469 proteins, Cas9 was excluded because its sequence length was larger than the maximum allowable  
470 length of 1024 amino acids by ESM-1v and ccdB was excluded because the experimental values  
471 were discretized within a small range. For each of the 10 mutagenesis datasets, all the sequence  
472 likelihood of all variants with coverage in the structure were determined using inverse folding.  
473 For ESM-1v, the average masked marginals likelihood score across all five models in the ESM-  
474 1v group was used. The experimental data distribution was binarized for high-fitness  
475 classification using a percentile-based threshold. The enrichment of high fitness variants was  
476 then determined by using the metric of fraction high fitness as defined by the fraction of the top  
477 10 model-predicted variants with experimental values above the high fitness threshold. The  
478 analysis was performed at three different percentile thresholds, top 5<sup>th</sup> percentile (95<sup>th</sup> percentile),  
479 top 10<sup>th</sup> percentile (90<sup>th</sup> percentile), and top 20<sup>th</sup> percentile (80<sup>th</sup> percentile), to determine  
480 sensitivity of the result based on the stringency of the selected cutoff parameter.

481

#### 482 *Benchmarking of antibody mutagenesis*

483 We use three antibody mutagenesis datasets<sup>50,51</sup> to benchmark the performance of  
484 modeling variant effects on antibody binding using inverse folding against two sequence-only

485 methods, ESM-1v<sup>49</sup> and abYsis<sup>55</sup>. Variant sequences were scored using the inverse folding model  
486 with three different forms of structure input: i) variable region of mutated antibody chain only ii)  
487 variable regions of both antibody chains iii) variable regions of both antibody chains in complex  
488 with antigen. The autoregressive scoring of sequences with inverse folding enables evaluation of  
489 sequences with multiple mutations. The Spearman correlation was determined between the log  
490 likelihood scores across all sequences and corresponding reported experimental binding  
491 measurements:  $-\log(K_D)$  for CR9114 and CR6261;  $\log(\text{binding enrichment})$  g6.31. The following  
492 structures were used for input backbone coordinates of the VH, VL, and antigen: PDB 4FQI<sup>52</sup>,  
493 CR9114-H5; PDB 3GBN<sup>53</sup>, CR6261-H1; PDB 2FJG, g6.31-VEGF.

494 ESM-1v and abYsis were scored using the variant sequence of the antibody variable  
495 region. For variants with multiple mutations, the average effect of all mutant amino acids in the  
496 sequence was considered, namely

$$497 \quad p(\mathbf{x}) = \frac{1}{|\mathcal{M}|} \sum_{i \in \mathcal{M}} [\log p(\mathbf{x}_i = \mathbf{x}_i^{\text{mt}}) - \log p(\mathbf{x}_i = \mathbf{x}_i^{\text{wt}})]$$

498 where  $\mathcal{M}$  is defined as the set of all mutations in the input sequence  $\mathbf{x}$ . For abYsis, individual  
499 mutation likelihoods were determined using the frequency of amino acids at each position based  
500 on multiple sequence alignment provided by the webtool version 3.4.1

501 (<http://www.abysis.org/abysis/index.html>). We aligned VH and VL protein sequences using the  
502 default settings provided in the ‘Annotate’ tool, with the database of ‘Homo sapiens’ sequences  
503 as of April 1, 2023.

504

### 505 *Acquisition of antibody amino acid substitutions using inverse folding*

506 We select amino acid substitutions recommended by the inverse folding model to test in  
507 our directed evolution campaigns for Ly-1404 and SA58. For a given wild-type antibody

508 variable region sequence,  $\mathbf{x} = (x_1, \dots, x_N) \in \mathcal{X}^N$ , where  $\mathcal{X}$  is the set of amino acids and  $N$  is the  
509 sequence length, we score all possible single amino acid substitutions against a corresponding  
510 structure of the variable regions of both antibody chains in complex with the RBD of SARS-  
511 CoV-2 Spike protein,  $\hat{\mathbf{Y}}$  by computing  $p(\mathbf{x} = \hat{\mathbf{x}}|\hat{\mathbf{Y}})$ . Protein structures used are reported in  
512 Supplementary Table 1. We then select the set of top ten predicted single amino acid  
513 substitutions at unique residues in each antibody variable region to test in the first round of  
514 evolution.

515         After testing individual amino acid mutations in a pseudovirus neutralization screen, in  
516 Round 2, beneficial mutations (defined as  $IC_{50}$  fold-change  $> 1.1$ ) were combined to assess the  
517 combinatorial effects and potential for further neutralization improvement. We tested up to four  
518 combinations of single amino acid mutations on each chain (two total mutations to the antibody).  
519 We also used the inverse folding model to score a library of all possible combinations of the  
520 beneficial mutations to an antibody chain (For example, VH Ly-1404 has 8 beneficial mutations  
521 resulting in 255 total candidate sequences), and selected the top five scoring designs (or less if  
522 there were a fewer number of total possible combinations). Lastly, we tested a maximum of two  
523 variants consisting of the best single-chain designs together. In total, 31 variants were tested for  
524 Ly-1404 and 25 variants were tested for SA58.

525

### 526 *Antibody cloning*

527         We cloned the antibody sequences into the CMV/R plasmid backbone for expression  
528 under a CMV promoter. The heavy chain or light chain sequence was cloned between the CMV  
529 promoter and the bGH poly(A) signal sequence of the CMV/R plasmid to facilitate improved  
530 protein expression. Variable regions were cloned into the human IgG1 backbone; Ly-1404

531 variants were cloned with a lambda light chain, whereas variants of SA58 were cloned with a  
532 kappa light chain. The vector for both heavy and light chain sequences also contained the  
533 HVM06\_Mouse (UniProt: [P01750](#)) Ig heavy chain V region 102 signal peptide  
534 (MGWSCIIILFLVATATGVHS) to allow for protein secretion and purification from the  
535 supernatant. VH and VL segments were ordered as gene blocks from Integrated DNA  
536 Technologies and were cloned into linearized CMV/R backbones with 5× In-Fusion HD Enzyme  
537 Premix (Takara Bio).

538

#### 539 *Antigen cloning*

540 RBD sequences were cloned into a pADD2 vector between the rBeta-globin intron and β-  
541 globin poly(A). All RBD constructs contain an AviTag and 6×His tag. RBD sequences were  
542 based off wild-type Wuhan-Hu-1 (GenBank: [BCN86353.1](#)), Omicron BA.1  
543 (GenBank: [UFO69279.1](#)), BQ.1.1 (GenBank: [OP412163.1](#)), XBB.1.5 (GenBank: [OP790748.1](#)).

544

#### 545 *DNA preparation*

546 Plasmids were transformed into Stellar competent cells (Takara Bio), and transformed  
547 cells were plated and grown at 37 °C overnight. Colonies were mini-prepped per the  
548 manufacturer's recommendations (GeneJET, K0502, Thermo Fisher Scientific) and sequence  
549 confirmed (Sequetech) and then maxi-prepped per the manufacturer's protocols (ZymoPure II  
550 Plasmid Maxiprep Kit, Zymo Research). Plasmids were sterile filtered using a 0.22-µm syringe  
551 filter and stored at 4 °C.

552

#### 553 *Protein expression*

554 All proteins were expressed in Expi293F cells (Thermo Fisher Scientific, A14527).  
555 Proteins containing a biotinylation tag (AviTag) were also expressed in the presence of a BirA  
556 enzyme, resulting in spontaneous biotinylation during protein expression. Expi293F cells were  
557 cultured in media containing 66% FreeStyle/33% Expi media (Thermo Fisher Scientific) and  
558 grown in TriForest polycarbonate shaking flasks at 37 °C in 8% carbon dioxide. The day before  
559 transfection, cells were pelleted by centrifugation and resuspended to a density of  $3 \times 10^6$  cells  
560 per milliliter in fresh media. The next day, cells were diluted and transfected at a density of  
561 approximately  $3\text{--}4 \times 10^6$  cells per milliliter. Transfection mixtures were made by adding the  
562 following components: maxi-prepped DNA, culture media and FectoPRO (Polyplus) would be  
563 added to cells to a ratio of 0.5 µg: 100 µl: 1.3 µl: 900 µl. For example, for a 100-ml transfection,  
564 50 µg of DNA would be added to 10 ml of culture media, followed by the addition of 130 µl of  
565 FectoPRO. For antibodies, we divided the transfection DNA equally among heavy and light  
566 chains; in the previous example, 25 µg of heavy chain DNA and 25 µg of light chain DNA would  
567 be added to 10 ml of culture media. After mixing and a 10-min incubation, the example  
568 transfection cocktail would be added to 90 ml of cells. The cells were harvested 3–5 days after  
569 transfection by spinning the cultures at 10,000g for 10 min. Supernatants were filtered using a  
570 0.45-µm filter.

571

### 572 *Antibody purification*

573 We purified antibodies using a 5-ml MabSelect Sure PRISM column on the ÄKTA pure  
574 fast protein liquid chromatography (FPLC) instrument (Cytiva). The ÄKTA system was  
575 equilibrated with line A1 in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid  
576 (HEPES) pH 7.4, 150 mM sodium chloride (NaCl), line A2 in 100 mM glycine pH 2.8, line B1

577 in 0.5 M sodium hydroxide, Buffer line in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic  
578 acid (HEPES) pH 7.4, 150 mM sodium chloride (NaCl) and Sample lines in water. The protocol  
579 washes the column with A1, followed by loading of the sample in the Sample line until air is  
580 detected in the air sensor of the sample pumps, followed by five column volume washes with A1,  
581 elution of the sample by flowing of 20 ml of A2 directly into a 50-ml conical containing 2 ml of  
582 1 M tris(hydroxymethyl)aminomethane (Tris) pH 8.0, followed by five column volumes of A1,  
583 B1 and A1 and then a wash step of the fraction collector with A1. We concentrated the eluted  
584 samples using 50-kDa cutoff centrifugal concentrators, followed by buffer exchange using a PD-  
585 10 column (Sephadex) that had been pre-equilibrated into 20 mM 4-(2-hydroxyethyl)-1-  
586 piperazineethanesulfonic acid (HEPES) pH 7.4, 150 mM sodium chloride (NaCl). Purified  
587 antibodies were used directly in experiments or flash-frozen and stored at  $-20^{\circ}\text{C}$ .

588

### 589 *Antigen purification*

590 All RBD antigens were His-tagged and purified using HisPur Ni-NTA resin (Thermo  
591 Fisher Scientific, 88222). Cell supernatants were diluted with 1/3 volume of wash buffer (20 mM  
592 imidazole, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4,  
593 150 mM sodium chloride (NaCl), and the Ni-NTA resin was added to diluted cell supernatants.  
594 For all antigens, the samples were then incubated at  $4^{\circ}\text{C}$  while stirring overnight.  
595 Resin/supernatant mixtures were added to chromatography columns for gravity flow purification.  
596 The resin in the column was washed with wash buffer (20 mM imidazole, 20 mM HEPES pH  
597 7.4, 150 mM NaCl), and the proteins were eluted with 250 mM imidazole, 20 mM HEPES pH  
598 7.4, 150 mM NaCl. Column elutions were concentrated using centrifugal concentrators at 10-  
599 kDa cutoff, followed by size-exclusion chromatography on an ÄKTA pure system (Cytiva).

600 ÄKTA pure FPLC with a Superdex 200 Increase (S200) gel filtration column was used for  
601 purification. Then, 1 ml of sample was injected using a 2-ml loop and run over the S200, which  
602 had been pre-equilibrated in degassed 20 mM HEPES, 150 mM NaCl before use and flash-frozen  
603 before storage at  $-20^{\circ}\text{C}$ .

604

### 605 *BLI binding experiments*

606 All reactions were run on an Octet RED96 at  $30^{\circ}\text{C}$ , and samples were run in  $1\times$  PBS  
607 with 0.1% BSA and 0.05% Tween 20 (Octet buffer). IgGs were assessed for binding to  
608 biotinylated antigens using streptavidin biosensors (Sartorius/ForteBio). Antigen was loaded at a  
609 concentration of 200nM. Tips were then washed and baselined in wells containing only Octet  
610 buffer. Samples were then associated in wells containing IgG at 100 nM concentration. A control  
611 well with loaded antigen but that was associated in a well containing only 200  $\mu\text{l}$  of Octet buffer  
612 was used as a baseline subtraction for data analysis. Association and dissociation binding curves  
613 were fit in Octet System Data Analysis Software version 9.0.0.15 using a 1:2 bivalent model for  
614 IgGs to determine apparent  $K_d$ . Fold-change in apparent  $K_d$  were determined by computing the  
615 ratio of wildtype  $K_d$  to variant  $K_d$ . Averages of  $K_d$  fold-change values from at least two  
616 independent experiments are reported to two significant figures in **Supplementary Data 2**. To  
617 estimate measurement error, we computed the standard deviation for each  
618 antibody–antigen  $K_d$  pair.

619

### 620 *Polyspecificity Particle assay*

621 Polyspecificity reagent (PSR) was obtained as described by Xu et al<sup>63</sup>. Soluble membrane  
622 proteins were isolated from homogenized and sonicated Expi 293F cells followed by



623 biotinylation with Sulfo-NHC-SS-Biotin (Thermo Fisher Scientific, 21331) and stored in PBS at  
624  $-80^{\circ}\text{C}$ . The PolySpecificity Particle (PSP) assay was performed as described in Makowski et  
625 al.<sup>64</sup>. Protein A magnetic beads (Invitrogen, 10001D) were washed three times in PBSB (PBS  
626 with  $1\text{ mg ml}^{-1}$  BSA) and diluted to  $54\text{ }\mu\text{g ml}^{-1}$  in PBSB. Then,  $30\text{ }\mu\text{l}$  of the solution containing  
627 the beads was incubated with  $85\text{ }\mu\text{l}$  of antibodies at  $15\text{ }\mu\text{g ml}^{-1}$  overnight at  $4^{\circ}\text{C}$  with rocking.  
628 The coated beads were then washed twice with PBSB using a magnetic plate stand (Invitrogen,  
629 12027) and resuspended in PBSB. We then incubated  $50\text{ }\mu\text{l}$  of  $0.1\text{ mg ml}^{-1}$  PSR with the washed  
630 beads at  $4^{\circ}\text{C}$  with rocking for 20 min. Beads were then washed with PBSB and incubated with  
631  $0.001\times$  streptavidin-APC (BioLegend, 405207) and  $0.001\times$  goat anti-human Fab fragment FITC  
632 (Jackson ImmunoResearch, 109-097-003) at  $4^{\circ}\text{C}$  with rocking for 15 min. Beads were then  
633 washed and resuspended with PBSB. Beads were profiled via flow cytometry using a Sony  
634 SH800 cell sorter. Data analysis was performed with FlowJo software version 10.9.0 to obtain  
635 median fluorescence intensity (MFI) values, which are reported for each antibody across three or  
636 more replicate wells. Elotuzumab (Fisher Scientific) and ixekizumab (Fisher Scientific) are also  
637 included in each assay as controls.

638

### 639 *Lentivirus production*

640 We produced SARS-CoV-2 Spike (Wuhan-Hu-1, BA.1, and BQ.1.1 variants)  
641 pseudotyped lentiviral particles. Viral transfections were done in HEK293T cells (American  
642 Type Culture Collection, CRL-3216) using BioT (BioLand) transfection reagent. Six million  
643 cells were seeded in D10 media (DMEM + additives: 10% FBS, L-glutamate, penicillin,  
644 streptomycin and 10 mM HEPES) in 10-cm plates one day before transfection. A five-plasmid  
645 system was used for viral production, as described in Crawford et al<sup>65</sup>. The Spike vector

646 contained the 21-amino-acid truncated form of the SARS-CoV-2 Spike sequence from the  
647 Wuhan-Hu-1 strain of SARS-CoV-2 (GenBank: [BCN86353.1](#)), BA.1 variant of concern  
648 (GenBank: [OL672836.1](#)), or BQ.1.1 variant of concern (GenBank: [OP412163.1](#)). The other viral  
649 plasmids, used as previously described<sup>65</sup>, are pHAGE-Luc2-IRS-ZsGreen (NR-52516), HDM-  
650 Hgpm2 (NR-52517), pRC-CMV-Rev1b (NR-52519) and HDM-tat1b (NR-52518). These  
651 plasmids were added to D10 medium in the following ratios: 10 µg pHAGE-Luc2-IRS-ZsGreen,  
652 3.4 µg FL Spike, 2.2 µg HDM-Hgpm2, 2.2 µg HDM-Tat1b and 2.2 µg pRC-CMV-Rev1b in a  
653 final volume of 1,000 µl.

654 After adding plasmids to medium, we added 30 µl of BioT to form transfection  
655 complexes. Transfection reactions were incubated for 10 min at room temperature, and then 9 ml  
656 of medium was added slowly. The resultant 10 ml was added to plated HEK cells from which the  
657 medium had been removed. Culture medium was removed 24 h after transfection and replaced  
658 with fresh D10 medium. Viral supernatants were harvested 72 h after transfection by spinning at  
659 300g for 5 min, followed by filtering through a 0.45-µm filter. Viral stocks were aliquoted and  
660 stored at -80 °C.

661

### 662 *Pseudovirus neutralization*

663 The target cells used for infection in SARS-CoV-2 pseudovirus neutralization assays are  
664 from a HeLa cell line stably overexpressing human angiotensin-converting enzyme 2 (ACE2) as  
665 well as the protease known to process SARS-CoV-2: transmembrane serine protease 2  
666 (TMPRSS2). Production of this cell line is described in detail by Rogers et al<sup>66</sup>. with the addition  
667 of stable TMPRSS2 incorporation. ACE2/TMPRSS2/HeLa cells were plated 1 day before

668 infection at 8,000 cells per well. Ninety-six-well, white-walled, white-bottom plates were used  
669 for neutralization assays (Thermo Fisher Scientific).

670 On the day of the assay, purified IgGs in 1× PBS were made into D10 medium (DMEM +  
671 additives: 10% FBS, L-glutamate, penicillin, streptomycin and 10 mM HEPES). A virus mixture  
672 was made containing the virus of interest (for example, SARS-CoV-2) and D10 media. Virus  
673 dilutions into media were selected such that a suitable signal would be obtained in the virus-only  
674 wells. A suitable signal was selected such that the virus-only wells would achieve a  
675 luminescence of at least >1,000,000 relative light units (RLU). Then, 60 µl of this virus mixture  
676 was added to each of the antibody dilutions to make a final volume of 120 µl in each well. Virus-  
677 only wells were made, which contained 60 µl of D10 and 60 µl of virus mixture. Cells-only wells  
678 were made, which contained 120 µl of D10 media.

679 The antibody/virus mixture was left to incubate for 1 h at 37 °C. After incubation, the  
680 medium was removed from the cells on the plates made one day prior. This was replaced with  
681 100 µl of antibody/virus dilutions and incubated at 37 °C for approximately 48 h. Infectivity  
682 readout was performed by measuring luciferase levels. Medium was removed from all wells, and  
683 cells were lysed by the addition of 100 µl of BriteLite assay readout solution (PerkinElmer) into  
684 each well. Luminescence values were measured using an Infinite 200 PRO Microplate Reader  
685 (Tecan) using i-control version 2.0 software (Tecan) after shaking for 30 sec. Each plate was  
686 normalized by averaging the cells-only (0% infection) and virus-only (100% infection)  
687 wells. Neutralization titer was defined as the sample dilution at which the RLU was decreased by  
688 50% as compared with the RLU of virus-only control wells after subtraction of background  
689 RLUs in wells containing cells only. Normalized values were fitted with a three-parameter  
690 nonlinear regression inhibitor curve in GraphPad Prism 9.1.0 to determine the half-maximal

691 inhibitory concentration (IC<sub>50</sub>) and are reported in **Supplementary Data 1**. Neutralization assays  
692 were performed in biological duplicates with technical duplicates.

### 693 *Computing frequency of changes to antibody protein sequences*

694 We computed the frequency of residues involved in affinity-enhancing substitutions using  
695 the abYsis webtool, which also computes the frequency of amino acids at each position based on  
696 a multiple sequence alignment. We aligned VH and VL protein sequences using the default  
697 settings provided in the ‘Annotate’ tool, using the database of ‘All’ sequences as of April 1,  
698 2023. We also used the Kabat region definition provided by abYsis webtool version 3.4.1 to  
699 annotate the framework regions and CDRs within the VH and VL sequences which are reported  
700 in **Supplementary Table 2**.

701

### 702 *Comparing efficiency of machine learning-guided directed evolution methods*

703 To compare inverse folding against other machine learning methods for protein  
704 evolution, we compared the fraction of variants tested in the protein engineering campaign to the  
705 number of assay-labeled training data points used to inform the predictions. Data was sourced  
706 from Biswas et al.<sup>17</sup> and made contemporaneous by the addition of recently published studies as  
707 indicated in **Supplementary Data 5**. The fraction improved, or hit rate, refers to experimentally  
708 tested predictions which have improved functional activity relative to either a wildtype protein  
709 that is used as a starting point for directed evolution or the protein used as a reference template  
710 for design.

711

## 712 **Acknowledgments**

713 We would like to thank D. Xu, S. Kim, and the members of the Kim lab for helpful discussions  
714 on this project. We are also grateful for assistance from D. Xu with protein graphics. V.R.S  
715 acknowledges the support of the Stanford University Medical Scientist Training Program grants  
716 (T32-GM007365 and T32-GM145402). V.R.S. and T.U.J.B. are both supported by the Sarafan  
717 ChEM-H Chemistry/Biology Interface Training Program. T.U.J.B. is also supported by the  
718 Knight-Hennessy Graduate Scholarship Fund and a CIHR Doctoral Foreign Study Award  
719 (FRN:170770). B.L.H is supported by the Stanford Science Fellows Program. This work was  
720 supported by the Virginia & D.K. Ludwig Fund for Cancer Research and the Chan Zuckerberg  
721 Biohub.

## 722 **Author contributions**

723 Conceptualization, methodology, interpretation: V.R.S., B.L.H., P.S.K.; Computational  
724 experiments and software development: V.R.S.; Antibody and antigen cloning, expression, and  
725 purification: V.R.S., T.U.J.B.; Lentivirus production and pseudovirus neutralization: T.U.J.B;  
726 Binding assays: V.R.S.; Writing (original draft): V.R.S with assistance from B.L.H and P.S.K.;;  
727 Writing (final draft): all authors

## 728 **Competing interests**

729 V.R.S., B.L.H., and P.S.K. are named as inventors on a patent application applied for by  
730 Stanford University and the Chan Zuckerberg Biohub entitled “Antibody Compositions and  
731 Optimization Methods”.

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884 **Supplementary Figures, Tables, Information, & Data**

885

886 **Supplementary Table 1:** List of proteins, protein structures, and assay information for deep  
887 mutational scanning experiments

888 **Supplementary Table 2:** Analysis of neutralization-enhancing mutations

889

890 **Supplementary Information:** Antibody sequences

891

892 **Supplementary Data 1:** Neutralization data with  $IC_{50}$  values of evolved antibodies across both  
893 evolutionary campaigns

894 **Supplementary Data 2:** Binding data with IgG  $K_D$  values of evolved antibodies

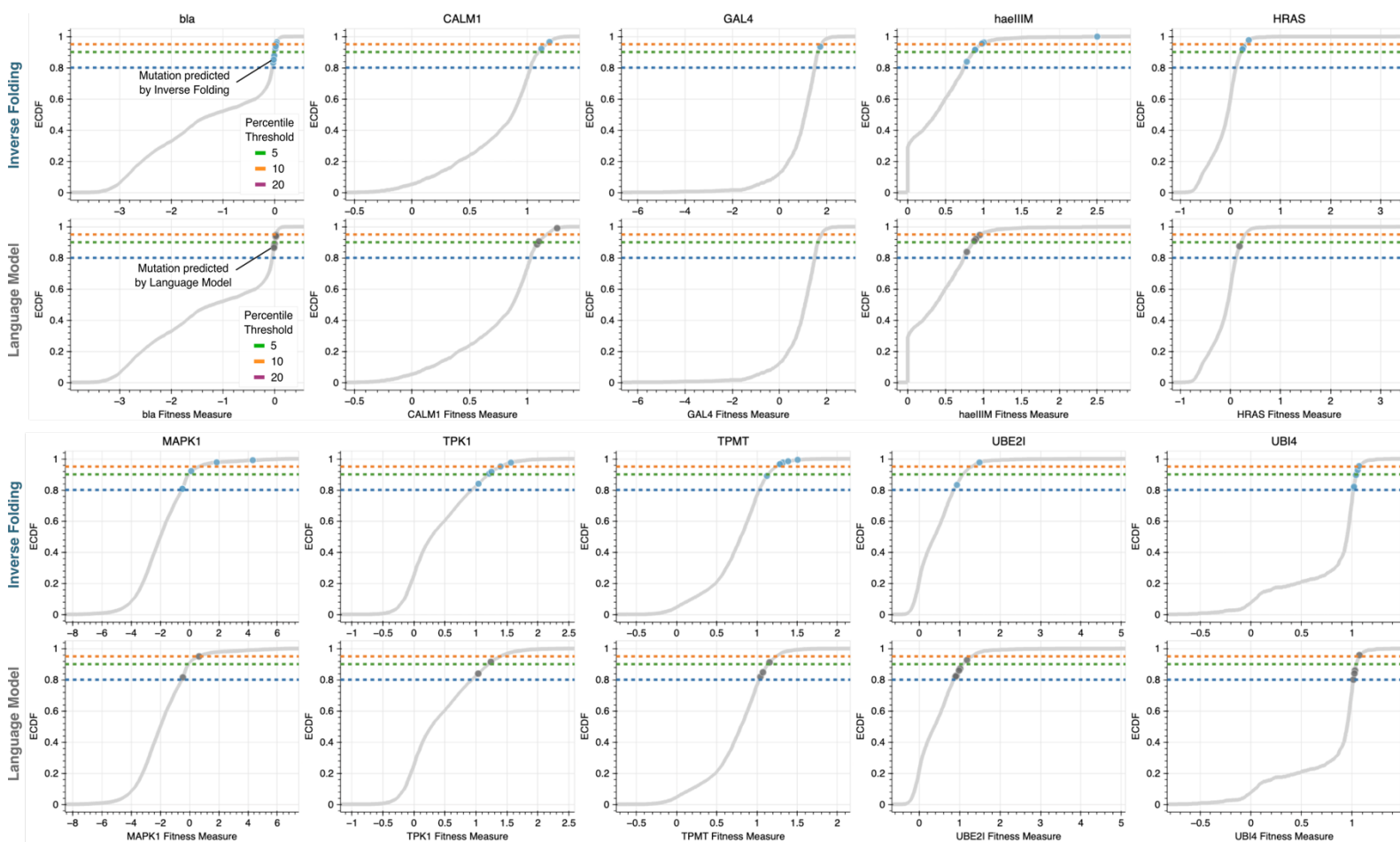
895 **Supplementary Data 3:** Antibody variant prediction benchmarking results

896 **Supplementary Data 4:** MFI values for polyspecificity experiments

897 **Supplementary Data 5:** Efficiency comparison of machine learning-guided directed evolution  
898 methods

899

900



901

902 **Supplementary Figure 1: Inverse folding identifies high fitness variants across proteins**

903 **with diverse functions**

904 In addition to higher hit rates of high fitness variants, inverse folding generally identifies variants

905 with greater magnitude of improvements in fitness. The top ten predicted variants with

906 experimental fitness values ranking in the 20<sup>th</sup> percentile of all variants profiled in the deep

907 mutational screen are shown. The grey curve shows the empirical cumulative distribution

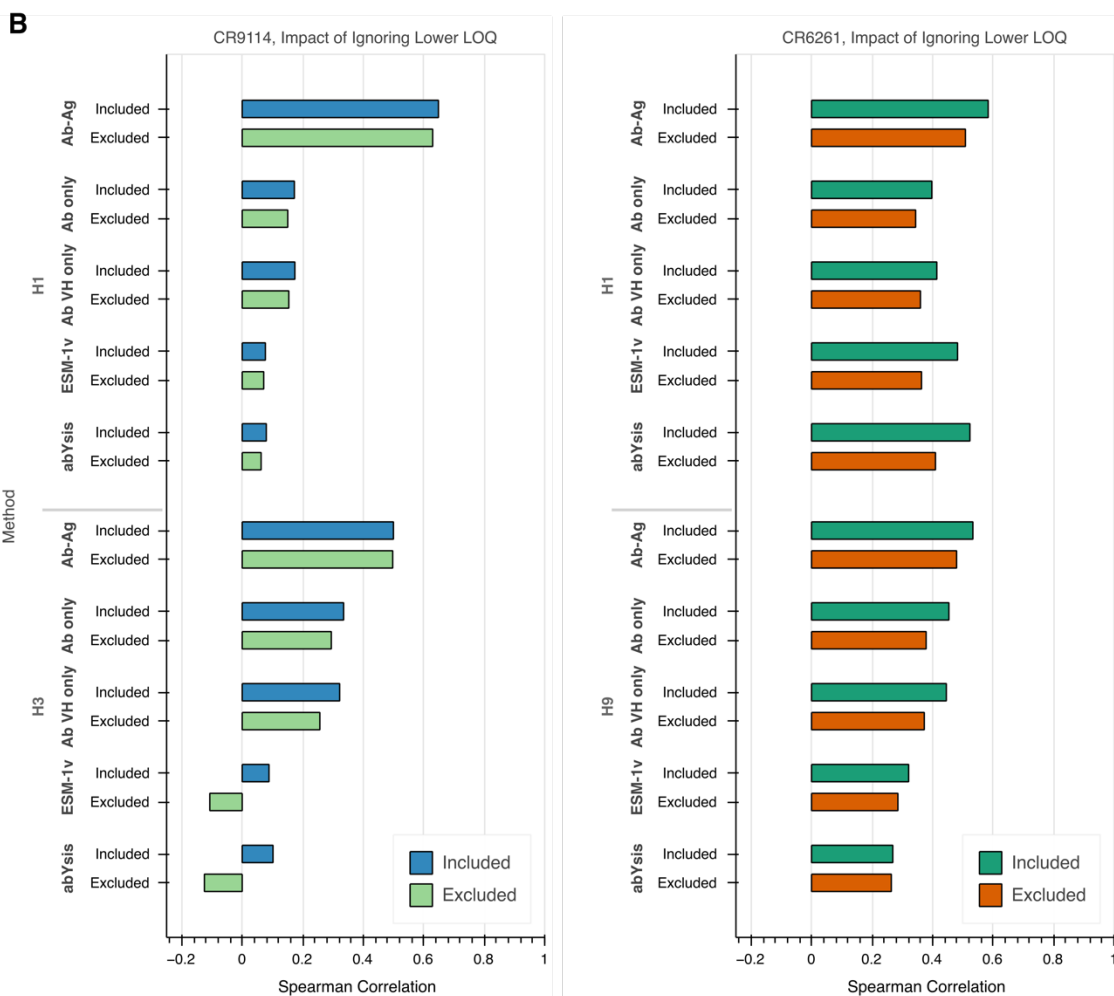
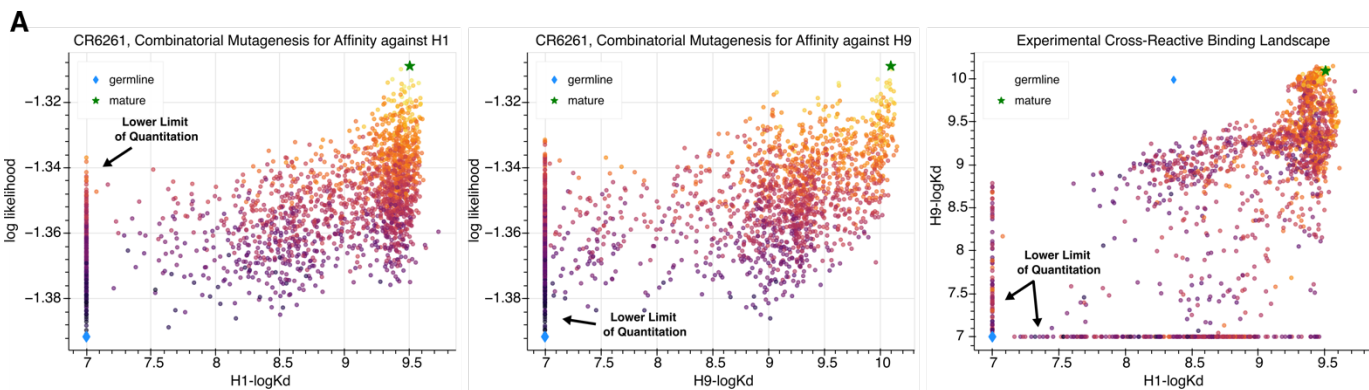
908 function (ECDF) of all experimental fitness values determined in the screen. The dotted lines

909 correspond to the three percentile-based thresholds used in the sensitivity analysis (**Figure 1d**) to

910 classify high fitness variants. bla, Beta-lactamase TEM; CALM1, Calmodulin-1; haeIIIIM, Type

911 II methyltransferase M.HaeIII; HRAS, GTPase HRas; MAPK1, Mitogen-activated protein

- 912 kinase; TMPT, Thiopurine S-methyltransferase; TPK1, Thiamin pyrophosphokinase 1; UBI4,
- 913 Polyubiquitin; UBE2I, SUMO-conjugating enzyme UBC9



914 **Supplementary Figure 2: Impact of lower limit of quantitation of binding assay on**

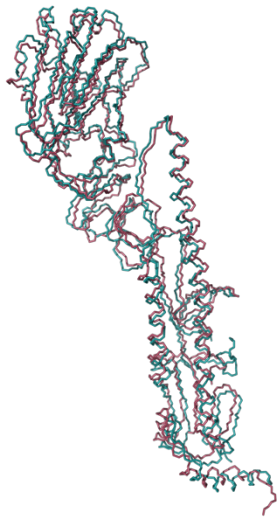
915 **predictive performance**

916 **(A)** Scatter plots showing CR6261 variant sequences scored with inverse folding compared to

917 experimental binding data and inclusive of the assay's lower limit of quantitation, which is



918 omitted for visualization in **Figure 3b. (B)** Comparative bar plots showing the impact of  
919 removing sequences with experimental measurements bounded artificially by the assay to  
920 dataset-wide correlation. While Spearman correlations shown in Figure 3a are computed without  
921 any modification to the data, trends in prediction and comparison among modeling methods are  
922 robust to filtering sequences affected by this assay artifact.  
923



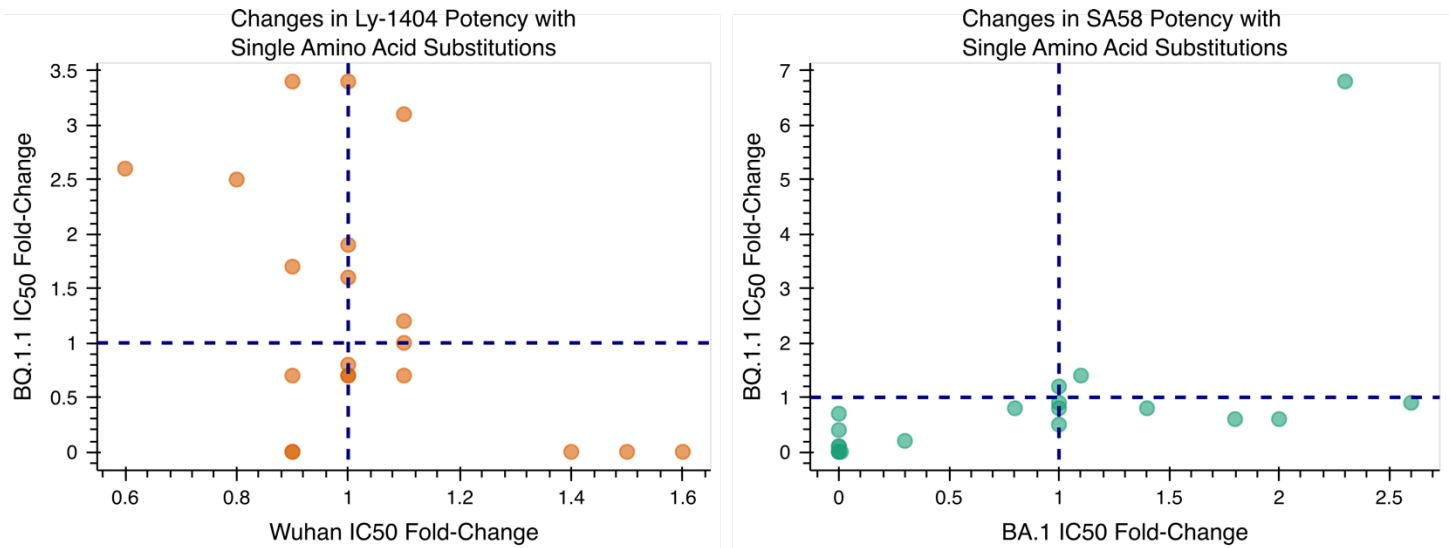
**HA of H5N1 influenza, PDB 4FQI**  
**HA of H1N1 influenza, PDB 7SCO**

Backbone RMSD = 2.1 Å (1416 atoms)  
Hamming Distance = 183/499 Amino Acids

924 **Supplementary Figure 3: Structural and sequence similarity of H5 and H1**

925 For cross-reactive antibodies, inclusion of the antigen structure is informative even for predicting  
926 binding to a different antigen. In Figure 3a, we report a correlation of 0.65 between inverse  
927 folding log likelihoods of CR9114 variants and experimental affinity measurements to H1  
928 despite using a structure solved with CR9114 in complex with H5. Inverse folding uses both the  
929 protein sequence and backbone structure coordinates as input. Across both HA subunits, H5 and  
930 H1 have considerable sequence differences and a 2.1 Å root mean square deviation (RMSD)  
931 across the entire protein backbone.

932



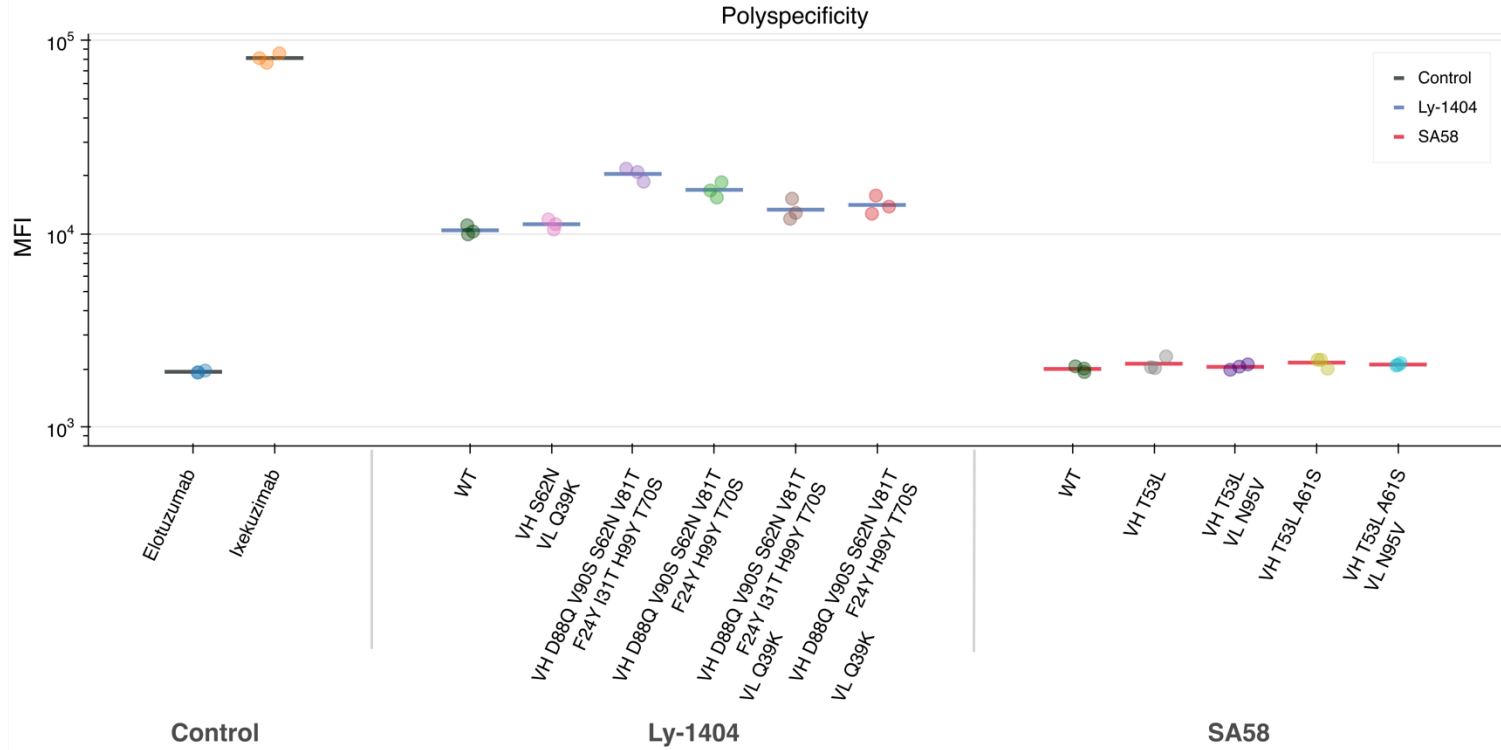
933

934 **Supplementary Figure 4: Functional diversity of inverse folding-recommended mutations**

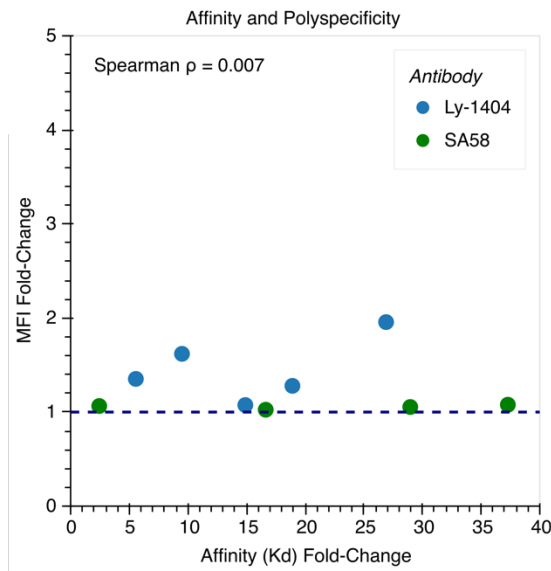
935 Among the 20 single amino acid substitutions tested for Ly-1404, 14 of 20 = 70% improve  
936 neutralization against at least one of the two strains tested. Similarly, 7 of 20 = 35% of the single  
937 amino acid substitutions tested for SA58 improve neutralization. While some variants improve  
938 function against both pseudovirus strains, others overwhelmingly only improve against one. This  
939 suggests that focusing sequence exploration to structurally compatible mutations does not  
940 compromise functional diversity.

941

**A**



**B**

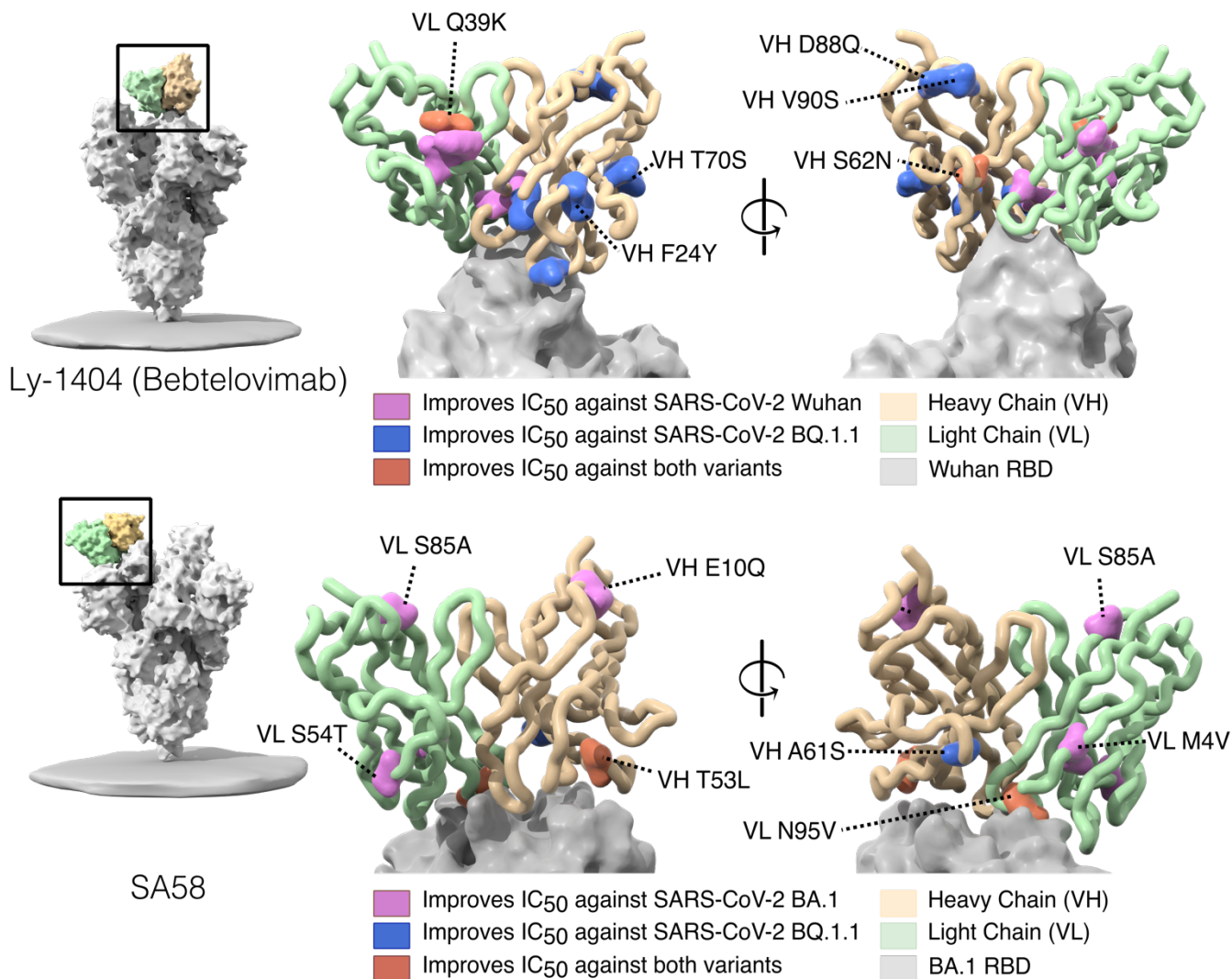


942 **Supplementary Figure 5: Polyspecificity of evolved antibodies**

943 (A) The median fluorescence intensity (MFI) signal obtained from flow cytometry is shown for

944 several evolved antibodies with improved affinity and compared to two clinical monoclonal

945 antibodies with high and low polyspecificity used to define a clinically viable range. **(B)** Fold-  
946 change in polyspecificity signal is plotted against fold-change in affinity as IgG against BQ.1.1  
947 for Ly-1404 and XBB.1.5 for SA58. There is no correlation between the improvements in on-  
948 target improvements in affinity and off-target nonspecific changes in polyspecificity (Spearman  $\rho$   
949 = 0.007).  
950



951

952 **Supplementary Figure 6: Mapping neutralization-enhancing substitutions**

953 Neutralization-enhancing mutations are labeled on the structure of the wild-type antibody in  
 954 complex with the RBD of SARS-CoV-2 spike protein (Ly-1404: PDB 7mmo; SA58: PDB  
 955 7y0w). Notably, several mutations are identified to have significant beneficial impacts on binding  
 956 neutralization and affinity (**Supplementary Data 1 & 2**) despite located away from the binding  
 957 interface.

958

**Supplementary Table 1.** Summary of the DMS datasets used in this analysis, including functional assay, method of mutagenesis, and structure used for inverse folding scoring. We also note the specific DMS assay from each study we use for calculating correlation with inverse folding log likelihoods.

Protein(s) (Uniprot ID)	Organism	Functional Assay	Mutagenesis Method	Utilized assay	PDB Structure	Total coverage of DMS (%)	Access date*	Reference
UBE2I (P63279)	Human	POPCode, a variant of multiple-site directed mutagenesis.	Competitive growth assay in yeast.	score	5F6E chain A	100	12/10/2018	(Weile <i>et al</i> , 2017)
TPK1 (Q9H3S3)				score	3S4Y chain A	92.46		
CALM1 (P0DP23)				score	5V03 chain R	100		
HRas (P01112)	Human	Systematic site-directed mutagenesis.	Two-hybrid assay.	unregulated	2CE2 chain X	100	12/10/2018	(Bandaru <i>et al</i> , 2017)
MAPK1 (P28482)	Human	Systematic site-directed mutagenesis.	Competitive growth assay.	VRT	4ZZN chain A	99.44	12/10/2018	(Brenan <i>et al</i> , 2016)
TPMT (P51580)	Human	Systematic site-directed mutagenesis.	Fluorescence of a GFP fusion protein.	score	2BZG chain A	92.9	12/10/2018	(Matreyek <i>et al</i> , 2018)
UBI4(b) (P0CG63)	Yeast	Site directed mutagenesis by cassette ligation.	Fluorescence activated cell sorting (FACS).	Relative_E1-activity_limiting	4Q5E chain B	100	12/10/2018	(Roscoe & Bolon, 2014)
GAL4 (P04386)	Yeast	Systematic site-directed mutagenesis.	Two-hybrid assay.	Nonselection_24	3COQ chain B	90.64	12/10/2018	(Kitzman <i>et al</i> , 2015)
bla(b) (P62593)	E. coli	Systematic site-directed mutagenesis.	Antibiotic resistance.	Ampicillin_2500	1M40 chain A	100	12/10/2018	(Stiffler <i>et al</i> , 2015)
haeIIIM (P20589)	H. aegyptius	Random mutagenesis.	Competitive growth assay.	DMS_G3	3UBT chain B	99.37	12/10/2018	(Rockah-Shmuel <i>et al</i> , 2015)

\*Access date is as reported in *Livesey & Marsh*, 2020 study from which these data were sourced and this table was adapted

**Supplementary Table 2.** Single amino acid substitutions with beneficial effects on neutralization are reported alongside the region of the variable domain they are located within, as well as the wild-type and mutant amino acid frequencies in observed human antibody sequences.

**Ly-1404**

<b>Chain Mutated</b>	<b>Design</b>	<b>Region</b>	<b>WT Amino Acid Frequency</b>	<b>Mutant Amino Acid Frequency</b>
HC	D88Q	HFR3	0.03333	0.00382
HC	V90S	HFR3	0.03316	0.05155
HC	S62N	CDR-H2	0.13159	0.16299
HC	V81T	HFR3	0.03432	0.00205
HC	F24Y	HFR1	0.01738	0.00002
HC	I31T	CDR-H1	0.00933	0.09048
HC	H99Y	HFR3	0.01593	0.00138
HC	T70S	HFR3	0.88405	0.06153
HC	I105L	CDR-H3	0.02764	0.05760
LC	A98I	CDR-L3	0.02297	0.03198
LC	Q39K	LFR2	0.92316	0.00238
LC	T5Q	LFR1	0.89340	0.00933
LC	K47E	LFR2	0.52285	0.01490
LC	M49L	LFR2	0.05585	0.77076

**SA58**

<b>Chain Mutated</b>	<b>Design</b>	<b>Region</b>	<b>WT Amino Acid Frequency</b>	<b>Mutant Amino Acid Frequency</b>
HC	T53L	CDR-H2	0.03814	0.00963
HC	A61S	CDR-H2	0.59797	0.13159
HC	E10Q	HFR1	0.24182	0.01366
LC	N95V	CDR-L3	0.13399	0.00685
LC	S85A	LFR3	0.01109	0.00698
LC	S54T	CDR-L2	0.65138	0.05372
LC	M4V	LFR1	0.29424	0.03348