

1 **Antibody escape and cryptic cross-domain stabilization in the SARS-** 2 **CoV-2 Omicron spike protein**

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37 **Summary:**

38 The worldwide spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to the
39 repeated emergence of variants of concern. The Omicron variant has two dominant sub-lineages, BA.1 and
40 BA.2, each with unprecedented numbers of nonsynonymous and indel spike protein mutations: 33 and 29,
41 respectively. Some of these mutations individually increase transmissibility and enhance immune evasion,
42 but their interactions within the Omicron mutational background is unknown. We characterize the
43 molecular effects of all Omicron spike mutations on expression, human ACE2 receptor affinity, and
44 neutralizing antibody recognition. We show that key mutations enable escape from neutralizing antibodies
45 at a variety of epitopes. Stabilizing mutations in the N-terminal and S2 domains of the spike protein
46 compensate for destabilizing mutations in the receptor binding domain, thereby enabling the record number
47 of mutations in Omicron sub-lineages. Taken together, our results provide a comprehensive account of the
48 mutational effects in the Omicron spike protein and illuminate previously unknown mechanisms of how
49 the N-terminal domain can compensate for destabilizing mutations within the more evolutionarily
50 constrained RBD.

51

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53 cytometry

54

55 **Introduction**

56
57 The continuous evolution and spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)
58 has produced variants of concern (VOCs) and variants of interest (VOIs) with enhanced immune evasion,
59 transmissibility, and occasionally increased disease severity¹⁻⁵. Omicron (or B.1.1.529 in the PANGO
60 nomenclature) rapidly displaced the Delta VOC globally⁶⁻⁸. The BA.1 sub-lineage of Omicron caused
61 record numbers of infections and breakthrough cases in fully vaccinated and previously infected
62 individuals^{9,10}. As of February 2022, the BA.2 lineage has displaced BA.1 in many countries and shows
63 additional enhanced transmissibility over all prior VOCs^{11,12}.

64 The SARS-CoV-2 spike protein is key to both transmissibility and immune evasion¹³. This
65 homotrimeric protein is displayed on the SARS-CoV-2 capsid surface, mediates virus binding and entry
66 into host cells, and elicits a strong immune response that gives rise to neutralizing antibodies and a robust
67 T-cell response¹⁴⁻¹⁶. The spike protein ectodomain (ECD) is the primary immune target and consists of
68 three main functional units: the N-terminal domain (NTD), receptor binding domain (RBD), and the
69 fusogenic stalk (S2)¹⁷. Because of its importance in cell entrance and immune escape, spike mutations
70 accumulate rapidly in circulating viral variants (**Figure 1A**). The NTD appears to tolerate the most
71 mutations, harboring 31% of all amino acid substitutions and 84% of indels found in circulating variants
72 (GISAID database accessed on 18/December/2021) (**Figure 1B**), while the RBD and S2 regions are more
73 restricted in the structural changes that they can tolerate, likely due to conserved functional constraints of
74 host-cell receptor binding and membrane fusion. The functional consequences of most of these mutations—
75 and the molecular epistasis of multiple mutations—is key for understanding viral evolution and interactions
76 with our immune system.

77 Omicron BA.1 and BA.2 sub-lineages have an unprecedented 33 and 29 nonsynonymous changes
78 relative to the ancestral Wuhan-Hu-1 (WHU1) lineage. These include four distinct amino acid (AA)
79 deletions (del), one insertion (ins), and 36 substitutions distributed across the ECD (**Figure 1C**). BA.2

80 shares 21 of the mutations in BA.1, but also contains eight unique substitutions and deletions (**Figure 1D**).
81 Some of these mutations increase the evasion of neutralizing monoclonal antibodies (mAb), rendering most
82 mAb therapies ineffective¹⁸. Similarly, the mutations significantly reduced authentic virus neutralization by
83 convalescent and vaccinated/boosted sera^{19,20}. While recent studies have probed small numbers of
84 individual amino acid changes within BA.1 and BA.2^{21,22}, questions about the synergistic and contextual
85 effects of most Omicron mutations remain unanswered.

86 Here, we leverage mammalian cell surface display of the spike protein to rapidly characterize the
87 expression, antibody binding, and cell receptor affinity of coronavirus spike ECDs²³. We characterize the
88 effects of all Omicron mutations with respect to human ACE2 (hACE2) binding, spike protein stability,
89 and escape from multiple distinct classes of mAbs. We compare the effects of individual spike protein
90 mutations between the WHU1 and Omicron mutational contexts to reveal how these mutations alter
91 antigenicity and hACE2 affinity. These results also explain how Omicron evades neutralizing mAbs,
92 including those with quaternary binding between adjacent spike protomers, via changing both the surface
93 epitopes and RBD conformational dynamics. Finally, we show that NTD mutations potentiate new RBD
94 mutations, expanding the ability of the RBD to further evolve under increased evolutionary pressure from
95 our adaptive immune response.

96

97 **Results**

98 **Omicron spike proteins have distinct antigenic features**

99 We used mammalian cell surface display to compare the antigenicity and expression of the BA.1 and BA.2
100 ECDs (residues 1-1208) to earlier VOCs (**Figure 1E**). We transiently express spike variants on the surface
101 of human embryonic kidney (HEK293T) cells. Surface-displayed spikes are then immunostained to
102 measure expression, antibody binding, and hACE2 affinity by two-color flow cytometry (**Figures S1A-B**,
103 **see Methods**)²³.

104 We first cloned spike proteins from five VOCs (Alpha, Beta, Gamma, Delta, and Omicron BA.1 &
105 BA.2) and one VOI (Epsilon), representing the dominant variants during different surges of the pandemic.
106 The globally-dominant D614G mutation and six prefusion stabilizing prolines were incorporated into all
107 variants to increase surface expression and maintain the prefusion conformation²⁴⁻²⁶. We assayed each
108 variant for expression and antibody escape potential with a set of 21 mAbs, many with known high-
109 resolution structures (**Figures 1F and S1C-D**)²⁷⁻²⁹. Of these, nine are NTD-targeting mAbs^{28,30,31}, which
110 we previously classified based on their binding epitopes (classes I-IV)²³. The remaining 12 target all four
111 classes of neutralizing RBD epitopes^{27,32}. We include the clinically-used REGN10933 (casirivimab) and
112 REGN10987 (imdevimab)^{33,34}, LY-CoV555 (bamlanivimab)³⁵, and S309 (sotrovimab)^{36,37} antibodies. We
113 also tested four mAbs with quaternary RBD binding modes^{27,31,38} (C002, C144, 2-43, and N3-1) and the
114 pan-variant mAb S2H97^{31,32,37,38}. Together, this panel provides a comprehensive overview of neutralizing
115 antibody escape by variant spike proteins.

116 Consistent with previous reports^{20-22,39,40}, BA.1 and BA.2 show enhanced antibody escape
117 compared to all other variants (**Figure 1F**). BA.1 escapes the majority of antibodies in our panel, including
118 nearly all classes of NTD binding mAbs tested here (the only exception is the class II antibody 5-7). BA.1
119 spike is also refractory to many RBD targeting mAbs, with strong escape from class II binders, half of the
120 class III binders, and all quaternary binders. BA.2 shows considerable antibody escape but remains
121 susceptible to the class I NTD binders and some class III RBD antibodies. In contrast, BA.2 escapes 5-7,
122 the noncanonical class I RBD binder S2X35, and the class III RBD binder S309 to a greater degree than
123 BA.1.

124 To test whether these results translated to live virus, we performed microneutralization assays with
125 authentic BA.1 virus and a subset of ten mAbs with known neutralization of WHU1. We tested four NTD-
126 binding mAbs, one from each of the four binding classes (**Figure 1G**). Consistent with the cell surface
127 display results, BA.1 completely escaped neutralization by 4A8 (class I), 4-8 (class III), and 4-18 (class
128 IV). Only the class II mAb 5-7 had a measurable neutralizing effect. Similarly, we tested a representative

129 of each of the four classes of RBD-targeting mAbs and two additional quaternary binding mAbs (**Figure**
130 **1H**). Again, BA.1 escaped the same antibodies as in the mammalian cell surface display assay. In the
131 aggregate, the data showed that our screening methodology recapitulated other *in vitro* and *in vivo*
132 observations and that the BA.1 and BA.2 spike proteins are antigenically distinct.

133

134 **BA.1 evades NTD-targeting mAbs better than BA.2**

135 We sought to investigate the molecular mechanism of mAb binding escape by Omicron, starting with the
136 effects of the NTD mutations. All VOCs contain at least one mutation in the NTD that increases viral
137 escape from NTD-targeting mAbs (**Figure 1F**)^{2,41-43}. Compared to previous VOCs, BA.1 and BA.2 have
138 the most mutated NTDs: 11 and 6 NTD mutations, respectively. The BA.1 NTD has four AA substitutions,
139 three deletions (del69-70, del143-145, del211), and a novel insertion (ins214EPE) (**Figure 2A**). The BA.2
140 NTD has four AA substitutions and one deletion (del25-27). Several of these mutations are located in the
141 intrinsically disordered NTD loops (N-loops) that comprise an antigenic supersite (**Figures S2A-E**)^{23,29,44}.
142 Strikingly, BA.1 and BA.2 only share one G142D substitution, which also appears in the Kappa and Delta
143 variants.

144 We first screened WHU1 spike protein variants containing each of the individual mutations found
145 in BA.1 and BA.2 (**Figure S2F**)²³. BA.1 completely escaped all NTD class I, III, and IV mAbs due to a
146 series of contiguous mutations (G142D, del143-145) in the N3-loop (**Figure 2B**). Binding by 5-7 (class II),
147 which interacts with the periphery of the NTD supersite, was reduced ~5-fold by these mutations^{31,45}. The
148 other BA.1 mutations (A67V, del69-70, T95I, del211, L212I, and ins214EPE) also had moderate effects
149 on binding of mAb 5-7, but the other mAbs were not impacted.

150 To further interrogate these antigenicity changes, we dissected the mutation clusters into different
151 combinations of mutations (**Figure S2G**). Class I mAbs, which predominately bind towards the apex of the
152 N3-loop, had decreased binding due to a shortening of the loop (deletions) and the Y145D mutation (**Figure**

153 **S2G-H**). Conversely, binding of class III and IV mAbs was more sensitive to mutations located at the base
154 of the N3-loop (G142D and del142) (**Figure S2I**). Mutations del211 and ins214EPE each decreased binding
155 of mAb 5-7 1.9-fold and moderately decreased pseudovirus neutralization (**Figures 2B and S2G**)²¹. These
156 results document the importance of both the G142D substitution and the 143-145 deletion for the ability of
157 BA.1 to escape different classes of NTD-targeting mAbs.

158 BA.2 still retained sensitivity to some NTD-targeting mAbs (**Figure 1F**). Mutations in the N1-loop
159 (T19I, L24R, del25-del27) moderately decreased binding for class II, III, and IV mAbs (**Figure 2C and**
160 **Figure S2J**) and in combination with G142D, BA.2 completely escaped antibody recognition at this site.
161 Notably, the V213G substitution, found distal from the N-loops and in the same location as the del211,
162 L212I, ins214EPE mutation cluster in BA.1, was inconsequential for mAb binding. However, these
163 mutations may influence virus fitness at the RNA or protein level in ways that cannot be accessed by our
164 platform.

165 Next, we tested whether BA.1 and BA.2 mutations impacted antibody escape at the NTD. Starting
166 with each of the BA.1 and BA.2 sequences, we systematically reverted each of the NTD mutations to WT
167 and measured the difference in mAb binding when compared with the full variant mutation set (**Figures**
168 **2D-E**). Reversion of the N3-loop mutations (G142D, del143-145) in BA.1 restored binding for all the
169 affected mAbs (**Figure 2F**). However, reversion of the del211, L212I, ins214EPE mutation cluster failed
170 to restore binding by mAb 5-7 to WHU1 levels. Reversion of N1-loop mutations (L24S, del25-27) and
171 G142D in the BA.2 context each partially restored binding, showing the additive effect of antibody escape
172 for the class II, III, and IV mAbs (**Figure 2G**). Surprisingly, reversion of T19I in the BA.2 context failed
173 to restore any binding for mAb CM30, due to the strong escape elicited by the other BA.2 NTD mutations.
174 Thus, BA.1 and BA.2 effectively evade class III and IV mAbs but show different binding of class I and II
175 mAbs. These observations highlight the continued immunological selection to evade potent mAbs elicited
176 by natural infection or immunization^{17,44}.

177

178 **Mutation context of Omicron RBDs impact mAb escape mechanisms**

179 Despite the significance of immunologic responses to the NTD, mucosal and systemic responses to SARS-
180 CoV-2 infection primarily target the RBD during the acute phase of natural infection⁴⁶⁻⁴⁸. Thus,
181 understanding the molecular mechanisms underlying RBD-targeting mAb escape is critical. BA.1 and BA.2
182 share 12 RBD substitutions, with three additional substitutions (S371L, G446S, and G496S) unique to BA.1
183 and four substitutions (S371F, T376A, D405N, and R408S) unique to BA.2 (**Figure 3A**). To study the
184 effects of these RBD substitutions on mAb binding we first screened WHU1 spike protein variants
185 containing each of the individual mutations found in BA.1 and BA.2, using the 12 previously described
186 RBD-targeting mAbs (**Figures 3B and S3A**). S2X35, a class I mAb, showed moderately reduced binding
187 due to the E484A mutation. However, each of the S371F, D405N, and R408S AA substitutions caused
188 substantial decreases in binding, likely accounting for the enhanced resistance of BA.2 to mAb S2X35.
189 Class II antibodies C002, C144, and LY-CoV555, and the quaternary binder 2-43, were most affected by
190 the E484A and Q493R single mutations. Class III mAb REGN10987 showed decreased binding with both
191 the N440K and G446S single mutations, and C135 binding was greatly affected by both N440K and Q498R
192 (**Figure 3B**). Two other class III mAbs, C110 and S309, were weakly affected by S317F and were not
193 escaped by the BA.1 and BA.2 spike proteins. Interestingly, S2H97, a class IV mAb, had 4.3-fold decreased
194 binding due to the S371F substitution, versus the 1.4-fold decrease observed with the full set of BA.2 spike
195 mutations. Conversely, no individual AA substitution greatly reduced N3-1 binding despite the strong
196 binding escape observed with the full BA.1 and BA.2 spike proteins.

197 Previous studies of Omicron mutations on mAb escape have been performed solely by adding
198 Omicron AA mutations to the WHU1 spike protein^{21,22}. These binding or escape measurements fail to
199 capture the nonadditive, epistatic interactions among the mutated sites^{49,50}. To explore these contextual
200 effects, we reverted each individual Omicron RBD mutation back to the WT AA in the corresponding
201 BA.1 and BA.2 spike proteins and assayed mAb binding (**Figures 3C-D**). Reversions associated with
202 improved binding, relative to the full set of BA.1 or BA.2 spike mutations, were interpreted to be

203 important for mAb escape in the BA.1 or BA.2 spikes. In the BA.2 spike, no single AA reversion restored
204 mAb S2X35 binding, suggesting an additive effect of S371F, D405N, and R408S mutations together are
205 responsible for escape. LyCoV555 appeared to escape by contributions from E484A and Q493R, which
206 restored binding to different levels in BA.1 and BA.2. REGN10933 binding was greatly restored by both
207 S477N and Q493R reversion in BA.1 and BA.2. However, the K417N reversion substantially restored
208 mAb binding for the BA.1 spike protein but not BA.2. REGN10987 retained affinity to BA.2 due to the
209 absence of the G446S substitution and dampened sensitivity to N440K in the BA.2 context. C135's
210 escape in BA.2 appears driven by N440K, which completely restored binding when reverted. In BA.1,
211 reverting N440K, G446S, and N501Y all restored binding, suggesting that they all contribute to escape.
212 No reversion in either the BA.1 or BA.2 contexts fully restored binding for the quaternary mAbs C144 or
213 2-43. Lastly, N3-1 binding improved upon reverting S375F in the BA.1 spike and, to a lesser degree,
214 S373P in BA.2. While these individual mutations do not directly clash with the N3-1 Fabs in the WHU1
215 structure, there are likely direct or allosteric perturbations to N3-1's engagement of the Omicron spike
216 (**Figure S3B**), highlighting the importance of mutation context in studying antibody escape pathways.

217 When comparing the effects of adding and reverting mutations in different spike protein contexts,
218 two interesting cases emerge: (1) mutations that are sufficient for escape in the WHU1 spike but not
219 necessary in the BA.1 or BA.2 spikes, and (2) mutations that are insufficient for escape by the WHU1
220 spike but necessary for the BA.1 or BA.2 spikes (**Figures 3E-F**). Sufficient but not necessary mutations
221 (below the diagonal line) may indicate examples where BA.1 or BA.2 have stacked multiple mutations
222 that each break a mAb-spike protein binding interaction. For example, C144 binding was strongly
223 disrupted by either E484A or Q493R when these mutations were made to the WHU1 spike but reverting
224 E484A in the BA.1 or BA.2 spike failed to restore binding (**Figure 3G**). Necessary but not sufficient
225 mutations (above the diagonal line) may indicate mutational clusters that have a synergistic or epistatic
226 effect on antibody binding. As an example, N440K reduced, and G446S and N501Y slightly reduced the
227 binding of antibody C135 when made in the WHU1 context (**Figure 3H**). BA.1, which has these three

228 mutations, nearly eliminated C135 binding, and this binding was partially restored when any of the three
229 mutations are reverted. The restoration effect from BA.1 is considerably larger than the reduction in
230 binding from WHU1 suggesting that N440K, G446S, and N501Y have synergistic effects greater than
231 their individual effects. In contrast, BA.2, which lacks G446S, does not show such clear synergy between
232 N440K and N501Y. Overall, these results highlight antigenic differences between the BA.1 and BA.2
233 spike RBDs and how the overall genetic context impacts antibody escape (**Figure S3C**).

234

235 **BA.1 and BA.2 RBDs balance antibody escape and human ACE2 binding**

236 Nearly half of the BA.1 and BA.2 RBD mutations are in the ACE2-binding receptor binding motif (RBM).
237 We screened each single RBD amino acid substitution in the WHU1 spike protein and observed increased
238 hACE2 binding with the S477N and N501Y mutations (**Figure 3I**). Conversely, mutations S371F, S375F,
239 G496S, and Y505H decreased hACE2 affinity. These results are consistent with previously reported RBD
240 DMS measurements⁵¹, although S371F was more detrimental to hACE2 binding in our assay. We speculate
241 this is due to contextual effects of the full spike glycoprotein.

242 Reverting each individual RBD mutation in the BA.1 and BA.2 spike proteins showed the
243 deleterious effects of S371F, S375F, K417N, Q496S, and Y505H on hACE2 binding to be less severe in
244 the Omicron contexts. The critical role of N501Y for hACE2 binding by the BA.1 and BA.2 spikes was
245 also shown, as its removal nearly abrogated hACE2 binding, in our assays (**Figures 3I and S3D-E**).
246 Although Q498R mildly reduced hACE2 binding in the WHU1 context, it improved hACE2 binding for
247 the BA.1 spike, and, to a lesser extent the BA.2 spike. Cooperative hACE2 binding due to the Q498R and
248 N501Y substitutions has been previously noted, but to our knowledge the mutation-specific effects had not
249 been measured in the full BA.1 and BA.2 spike proteins^{50,52}. We validated these results via biolayer
250 interferometry (BLI). Using dimeric hACE2 we see similar changes in binding (**Figures 3J and S3H**).
251 Together, these results highlight the starkly different molecular basis for hACE2 engagement for BA.1 and
252 BA.2, and the importance of the spike genetic context in understanding these interactions⁵³⁻⁵⁵.

253

254 **Cryptic cross-domain interactions in the BA.1 spike contribute to mAb escape**

255 Reversion of single RBD mutations in the BA.1 and BA.2 spikes broadly failed to fully restore mAbs with
256 quaternary binding modes (C002, C144, 2-43, N3-1) (**Figures S3F-G**). These mAbs simultaneously engage
257 two or three RBDs to enhance their binding via avidity effects. We reasoned that these antibodies can't
258 bind Omicron relative to the WHU1 spike because of subtle changes in the RBD conformation dynamics.
259 In support of this hypothesis, structures of the BA.1 spike revealed a strict 1-RBD-up, 2-RBD-down
260 conformation⁵⁴. To identify potential cross-domain interactions that may contribute to the extent of escape
261 measured for the full set of BA.1 and BA.2 spike mutations, we created spike proteins containing
262 combinations of NTD, RBD, and S2 mutation sets from the WHU1, BA.1 or BA.2 variants. We then
263 assayed these spike proteins for mAb binding using 12 RBD-targeting mAbs (**Figure 4A**). For most RBD-
264 targeting mAbs, such as C144, the set of BA.1- and BA.2-RBD mutations alone decreased binding to the
265 level of the complete set of BA.1 or BA.2 spike mutations (**Figure 4B**). Interestingly, only the combination
266 of BA.1-RBD and -S2 mutations recapitulate the loss of N3-1 binding measured for the full BA.1 spike
267 (**Figure 4C**). In contrast, the BA.2-RBD mutations alone were adequate for reduced N3-1 binding.

268 To determine if differences in the ability of BA.1 and BA.2 to escape of N3-1 was intrinsic to their
269 RBD and S2 mutations, we created chimeric spikes by swapping the BA.1 and BA.2 mutation sets (NTD,
270 RBD, and S2) (**Figure 4D**). We observed a further 1.4-fold reduction in N3-1 binding when the BA.1-RBD
271 was replaced with the BA.2-RBD, in the BA.1 spike. Furthermore, the BA.2-S2 mutations did not
272 synergistically reduce N3-1 binding when combined with the BA.1-NTD and -RBD mutations. Conversely,
273 replacing BA.2-RBD mutations with BA.1-RBD mutations dampened N3-1 escape. By swapping the BA.1-
274 S2 mutations into the BA.2 spike, we did observe a marginal (1.1-fold) reduction in N3-1 binding. We
275 postulate that the N856K and L981F mutations, which comprise the only differences between the BA.1-
276 and BA.2-S2 mutations sets, alter the RBD-up vs -down equilibrium or spike conformation, thus further

277 reducing N3-1 binding. These data highlight possible routes of quaternary-binding mAb evasion by
278 Omicron through altering antigenic epitopes and RBD dynamics (**Figure 4E**).

279

280 **Omicron spike domains provide stability compensation to immune evasive RBD**

281 Expression—a proxy for spike stability—correlates with improved infectivity and viral fitness^{56,57}. BA.1
282 spikes express 1.2-fold greater than WHU1 spikes while BA.2 express 2.1-fold lower (**Figure S1D**). We
283 determined the combination of mutations that are responsible for this increased expression by establishing
284 the effect of each individual BA.1 and BA.2 mutation. Spike expression was monitored via fluorescent
285 signals from two discrete epitopes: a triple FLAG in the linker between the ectodomain and transmembrane
286 domain and the foldon trimerization domain (**Figure S4A-B**) (See Methods). Most of the BA.1 and BA.2,
287 NTD and S2 mutations enhanced spike expression. For example, NTD mutations del69-70 and ins214EPE
288 in BA.1, and G142D shared by both BA.1 and BA.2 improved WHU1 spike expression. Reversion of either
289 del69-70 or ins214EPE in the BA.1 spike only modestly decreased expression (**Figure S4C**). Interestingly,
290 the G142D mutation is more central to the overall stability of the BA.2 spike, as its reversion reduced BA.2
291 spike expression 5.3-fold. Surprisingly, we also saw extremely destabilizing mutations in the BA.1 and
292 BA.2 RBDs. The addition of S375F to the WHU1 spike dramatically reduced expression 12.3-fold.
293 Mutations N440K and E484A, which are responsible for mAb escape, and N501Y, which enhances hACE2
294 affinity are also mildly destabilizing (**Figure S4D**).

295 Next, we assayed each set of domain-specific mutations (NTD, RBD, and S2) in the context of
296 WHU1 spike (**Figure 4F**) to determine their relative effects on spike expression. We found that both the
297 BA.1- and BA.2-RBD mutation sets greatly reduced spike protein expression relative to WHU1, whereas
298 the NTD and S2 mutation sets increased expression. The fully mutated BA.1 spike had greater expression
299 than the WHU1 spike, suggesting that the destabilizing mutations in the RBD are compensated for primarily
300 by mutations in the NTD and S2 domains. Further domain exchanges showed that the BA.1 NTD mutations
301 are sufficient to stabilize the BA.1 RBD mutations, with the S2 mutations contributing, but insufficient on

302 their own. BA.2 follows the same general pattern, however the BA.2-NTD and -S2 were less effective at
303 offsetting the relatively milder decreased expression associated with the BA.2-RBD. This finding is
304 consistent with the relatively poor spike expression in BA.2, compared to expression by the WHU1 and
305 BA.1 variants. Lastly, we swapped BA.1 and BA.2 domains for the BA.1 and BA.2 spikes to determine if
306 stabilizing effects were transferrable. Interestingly, the exchange of BA.2 NTD mutations into the BA.1
307 spike greatly reduce spike expression (**Figure 4G**). The exchange of BA.1 RBD mutations into the BA.2
308 spike also reduced BA.2 spike expression.

309 To test how expression relates to spike stability, we tested the thermal denaturation of a subset of spike
310 variants via differential scanning fluorimetry (DSF) (see Methods). Soluble SARS-CoV-2 spike trimers
311 generate two distinct denaturation peaks, denoted here as Tm1 and Tm2. Compared to other VOCs, the
312 BA.1 and BA.2 spike proteins have poor thermostability, as shown by their respective 7 °C and 6 °C shifts
313 in Tm1, relative to WHU1 (**Figures S4A-B**). DSF measurements of spike variants reveals that these effects
314 are driven by RBD mutations in BA.1 (**Figures S4C-F**) and BA.2 (**Figures S5G-J**). In sum, both BA.1 and
315 BA.2 spikes are destabilized by their highly mutated RBDs. Although the BA.1 NTD mutations don't
316 improve the thermostability of the spike protein, they compensate for poor spike expression. Taken
317 together, these results reveal that the expression loss due to nAb-evasive but otherwise destabilizing RBD
318 mutations is offset by otherwise stabilizing NTD & S2 mutations. These mutations work synergistically
319 with a particular spike background, as swapping mutation sets between BA.1 and BA.2 leads to overall
320 reduced expression.

321

322 **Discussion:**

323 This work dissects the effects of individual mutations in different spike protein contexts to understand how
324 these mutations evade neutralizing antibodies and impact spike stability. The G142D, del143-145 mutation
325 cluster in the BA.1-NTD is both necessary and sufficient for the comprehensive escape of class I, III, and

326 IV NTD-targeting mAbs (**Figure 2F**). All BA.2-NTD mutations, except for V213G, collectively contribute
327 to the escape of class II, III, and IV NTD-targeting mAbs (**Figure 2G**). These results highlight antigenic
328 differences between the BA variants and the original variant, as well as between the BA.1 and BA.2 spike
329 NTDs themselves. Moreover, our work reveals that multiple NTD mutations additively evade potent NTD-
330 directed mAbs.

331 In contrast to the NTD, mutations in the BA.1- and BA.2-RBDs often resulted in non-additive
332 levels of escape from RBD-targeting mAbs. We identified instances where multiple mutations were
333 required to escape binding completely. For example, reversions of either N440K or G446S in the BA.1
334 spike largely restored the binding of REGN10933 and C135 (**Figure 3C**). We also identified several class
335 II mAbs (C002 and C144) that failed to show binding improvements after single mutation reversions in the
336 Omicron spike proteins, suggesting redundant mutations contributing to antigenic escape (**Figure 3B-D**).
337 We speculate that Omicron RBDs have undergone extensive mutation under continuous pressure to evade
338 diverse classes of RBD-targeting antibodies, outside of the predominant class II antibodies found in
339 polyclonal plasmas after immunization or natural infection³². This redundancy in escape may also arise
340 from mutation-induced alterations in RBD conformational equilibria and dynamics, as described in
341 previous structure studies^{53,54,58}.

342 Reverting individual BA.1 and BA.2 mutations back to WT (WHU1) in the BA.1 and BA.2 spikes
343 generally failed to restore binding for the four mAbs we tested that were capable of binding multiple RBDs
344 at once: C002, C144, 2-43, and N3-1 (**Figures S3F-G**). These results suggested that the virus was likely
345 not simply presenting different epitopes than the ancestral variant but presenting them in the context of
346 different proportions of RBD up vs down states. We showed that RBD-S2 cross-domain interactions in the
347 BA.1 spike led to reduced N3-1 binding beyond the BA.1-RBD mutations alone (**Figure 4C**). In further
348 support of this hypothesis, the removal of the N856K and L981F mutations from the BA.1 spike, which are
349 implicated in creating cross-domain interactions, partially restored N3-1 binding to the level of the BA.1-
350 RBD mutations alone (**Figure 4D**). Similarly, BA.2-RBD mutations proved even more effective than

351 BA.1-RBD mutations for escaping N3-1, in line with recently solved structures that show that the down
352 conformation of the RBD is largely stabilized in the BA.2-RBD mutations⁵⁸.

353 The accumulation of novel mutation clusters in Omicron, likely due to selection pressure to evade
354 immunity, came at the cost of destabilizing the RBD. Several RBD mutations, most notably S375F,
355 drastically reduce spike expression (**Figure S4D**). We propose that NTD mutations such as the 69-70
356 deletion offset protein folding/stability deficiencies associated with Omicron RBDs⁵⁷. Addition of BA.1-
357 NTD mutations compensated for the poor expression of the BA.1-RBD mutations (**Figure 4F**).
358 Interestingly, the same compensation effects were not seen with the BA.2-NTD and BA.2-RBD mutations,
359 resulting a lower net expression of BA.2 spike relative to WHU1 in our assays. Future studies will be
360 required to explore whether and how improvements in immune evasion have led to fitness costs for Omicron
361 relative to spike expression and fitness.

362 Our data suggest that the Omicron BA.1 and BA.2 subvariants retain high affinity for hACE2.
363 Previous directed evolution⁵² and YSD-DMS⁵⁰ studies have shown N501Y to greatly improve hACE2
364 binding and Q498R to moderately reduce it; these studies also determined that the co-occurrence of N501Y
365 and Q498R synergistically boosted hACE2 affinity. We confirm that the full Omicron BA.1 and BA.2
366 spikes also benefit from these substitutions, with N501Y and Q498R playing critical roles in the molecular
367 engagement of hACE2 (**Figure 3I-J**). Intriguingly, this effect appears distinct in BA.1 versus BA.2 (**Figure**
368 **S3D-E**). As compared to BA.1, N501Y has a much greater impact on ACE2 binding in BA.2 but Q498R is
369 not as critical, possibly due to the absence of BA.1 mutations G446S and G496S. Taken together, these
370 results reveal evolutionary features of Omicron spikes that enable the accrual of immune evasive
371 mutations without sacrificing hACE2 affinity and infectivity.

372 Our study has several limitations. First, we used a prefusion stabilized spike protein that does not
373 precisely mimic the dynamics of the native Omicron spike protein²⁶. Second, our binding assays use a set
374 of potent neutralizing mAbs which only serve as proxies for the antibodies found in patient antibody
375 repertoires after immunization or natural infection. Third, our work only touches on antibody recognition

376 and hACE2 binding; T-cell immunity plays a critical role in protecting against SARS-CoV-2 disease.
377 Additional studies focused on the perturbations of spike variants on T-cell response will continue to bridge
378 the gap in the understanding of immune escape between humoral and cell-mediated immunity.

379 In the aggregate, the data presented here add critical new information about key features of
380 Omicron spike protein mutations and how these mutations synergize to create spike variants that
381 successfully evade antibodies while maintaining high affinity hACE2 binding. Our binding maps largely
382 complement prior structure-based studies of binding escape, but now provide new insights into the role of
383 compensatory substitutions in the NTD that impact both expression/stability and conformation. We
384 conclude that the continuing accumulation of NTD mutations will further alter the conformational
385 equilibrium and stability of the spike protein to allow for the accumulation of new, more virulent
386 mutations in the RBD. Further, our study also highlights the importance of rapidly analyzing novel
387 variant spike proteins in near-native contexts. As SARS-CoV-2 continues to evolve and new variants
388 inevitably arise and spread, it is critical that these mutations can be understood in their native genetic
389 contexts to better inform future therapeutic targets and vaccine formulations. Finally, the strategy we have
390 used here will be applicable to future zoonotic outbreaks and other heretofore unrelated microbial
391 pathogens. Mammalian cell-display will continue to serve as a powerful platform for investigating
392 evolutionary trajectories of infectious agents and engineering conformational vaccine candidates.

393 **Figure Captions**

394 **Figure 1: Omicron spike protein ectodomain has dozens of mutations contributing antibody escape.**

- 395 A. The number of mutated amino acid positions for each VOI and VOC. Spike NTD mutations, light
396 blue; spike RBD mutations, yellow; spike S2 mutations, dark blue; other mutations, white.
- 397 B. Distribution of all non-synonymous mutations (substitutions = 42,077,816; insertions = 31,063;
398 deletions = 15,664,146, colored as in Figure 1A) found in GISAID (accessed on
399 18/December/2021). The NTD has the majority of insertions and deletions (81% and 84%
400 respectively).
- 401 C. SARS-CoV-2 spike ectodomain structure (PDB: 7DDN⁵⁹) with mutations found in BA.1 and BA.2
402 colored by domain as in Figure 1A.
- 403 D. Mutations found in the Omicron spike variants. Shading indicates the percentage of BA.1 or BA.2
404 strains containing these mutations, as analyzed on outbreak.info (accessed on 12/February/2022).
- 405 E. Spike Display overview. Spike protein ectodomains with different mutation sets are constructed
406 using a semi-automated Golden Gate-cloning pipeline, displayed on the surface of HEK293T cells,
407 and assayed in high-throughput with flow cytometry. Biophysical characterization is performed
408 with spikes cleaved from cell surfaces.
- 409 F. Relative monoclonal antibody binding to spikes from VOCs (Alpha, Beta, Gamma, Delta, and
410 Omicron) and a VOI (Epsilon). Red, decreased binding; blue, increased binding; normalized to the
411 original WHU1 spike with D614G mutation (top row). Mean \pm SD of log-transformed values from
412 at least two biological replicates. Spike domain targets and epitope classifications of antibodies
413 shown on top, * = quaternary binding.
- 414 G. Authentic BA.1 virus neutralization for selected NTD-directed monoclonal antibodies. Mean \pm SD
415 of two biological replicates. Curves are a Sigmoidal (4PL, X), Least squares fit, IC₅₀ values listed
416 in Figure S1E.
- 417 H. Authentic BA.1 virus neutralization for selected RBD-directed monoclonal antibodies as in Figure
418 1G.

419 **Figure 2: NTD indels and substitutions enable mAb binding escape**

- 420 A. An enlarged NTD structure (PDB: 7DDN⁵⁹) with nonsynonymous mutations from BA.1 (brown),
421 BA.2 (grey), or both (black) indicated.
- 422 B. Relative mAb binding to WHU1 spike proteins containing BA.1-NTD mutations. Red, decreased
423 binding; blue, increased binding; normalized to the WHU1 spike. Mutations color coded as in
424 Figure 2A.
- 425 C. Relative mAb antibody binding to WHU1 spike proteins containing BA.2-NTD mutations. Colored
426 as in B, normalized to the WHU1 spike. Mutations color coded as in Figure 2A.
- 427 D. Relative mAb binding to BA.1 spike proteins containing reversions of the BA.1-NTD mutations to
428 the WHU1 sequence. Colored as in Figure 2B, normalized to the BA.1 spike.
- 429 E. Relative mAb binding to BA.2 spike proteins containing reversions of the BA.2-NTD mutations to
430 the WHU1 sequence. Colored as in Figure 2B, normalized to the BA.2 spike protein.
- 431 F. Comparison of log₂(normalized binding) measurements for adding (+1) BA.1-NTD mutations to
432 the WHU1 spike versus reverting (-1) the corresponding mutations from the BA.1 spike protein.
433 Mutations with equal antigenic effects in both spike contexts are expected to fall on the diagonal
434 line $y = -x$.
- 435 G. Comparison of the effect of adding and reverting BA.2-NTD mutations as in Figure 2F.
- 436 For all plots, mean \pm SD of log-transformed values from at least two biological replicates

437 **Figure 3: Omicron-RBD mutations enable antibody evasion and preserve hACE2 affinity**

- 438 A. An enlarged RBD structure (PDB: 7DDN⁵⁹) with mutations in BA.1 (brown), BA.2 (grey), or both
439 (black) indicated.
- 440 B. Relative monoclonal antibody binding to WHU1 spike proteins containing BA.1- and BA.2-RBD
441 mutations. Red, decreased binding; blue, increased binding; relative to the WHU1 spike.
- 442 C. Relative monoclonal antibody binding to BA.1 spike proteins containing reversions of the BA.1-
443 RBD mutations to the WHU1 sequence. Colored as in Figure 3B, normalized to the BA.1 spike.
- 444 D. Relative monoclonal antibody binding to BA.2 spike proteins containing reversions of the BA.2-
445 RBD mutations to the WHU1 sequence. Colored as in Figure 3B, normalized to the BA.2 spike.
- 446 E. Comparison of the effect of adding (+1) BA.1-RBD mutations to the WHU1 spike protein versus
447 reverting (-1) the corresponding mutations from the BA.1 spike. Mutations with equal antigenic
448 effects in both spike contexts are expected to fall on the diagonal line $y = -x$.
- 449 F. Comparison of the effect of adding and reverting BA.2-RBD mutations as in Figure 3E.
- 450 G. Relative monoclonal antibody C135 binding to the WHU1, BA.1, and BA.2 spike proteins and
451 spike proteins containing the N440K, G446S, or N501Y mutations or reversions as appropriate,
452 normalized to the level of binding to the WHU1 spike.
- 453 H. Relative monoclonal antibody C144 binding to the WHU1, BA.1, and BA.2 spike proteins and
454 spike proteins containing the E484A or Q493R mutations or reversions, normalized to the level of
455 binding to the WHU1 spike. BA.2 does not contain G446S.
- 456 I. Relative monomeric hACE2 binding to WHU1 spikes containing BA.1- and BA.2-RBD mutations
457 (WHU1 base), and BA.1 / BA.2 spike proteins containing reversions of the RBS BA.1 / BA.2
458 mutations (BA.1 base / BA.2 base). Red, decreased binding; blue, increased binding; relative to the
459 WHU1, BA.1, and BA.2 spike proteins as appropriate.
- 460 J. Monomeric hACE2 binding to WHU1 and BA.1 spike proteins and spike proteins containing the
461 Q498R or N501Y mutations or reversions as appropriate, measured by BLI. All values are
462 normalized to the binding of the WHU1 spike.

463 For all plots, mean \pm SD of log-transformed values from at least two biological replicates

464

465 **Figure 4: Cross-domain interactions contribute to mAb escape and stabilize the Omicron spike**
466 **protein**

- 467 A. Differences in monoclonal antibody binding of RBD-directed antibodies against spike proteins
468 containing combinations of the NTD, RBD, and S2 mutation sets from the BA.1 and BA.2 variants.
469 Red, decreased binding; blue, increased binding, relative to the WHU1 spike.
- 470 B. Monoclonal antibody binding of C144 to spike proteins containing combinations of the NTD, RBD,
471 and S2 BA.1 and BA.2 mutation sets relative to WHU1.
- 472 C. Monoclonal antibody binding of N3-1 to spike proteins containing combinations of the NTD, RBD,
473 and S2 BA.1 and BA.2 mutation sets relative to WHU1.
- 474 D. Additional N3-1 binding data as in Figure 4C.
- 475 E. Proposed escape mechanism for biparatropic antibody (N3-1) by Omicron spike proteins. Antibody
476 binding reduced via mutations at the N3-1 binding epitope on the RBD (top). Top-down view of
477 spike protein trimers with annotated RBD up vs down positions (bottom). Violet circles, N3-1
478 binding epitope; green dot, preserved binding; red “x,” escaped binding.
- 479 F. Differences in expression of spike proteins containing combinations of the NTD, RBD, and S2
480 BA.1 and BA.2 mutation sets. Data normalized to WHU1 spike expression.

481 G. Additional expression data as in Figure 4F.

482 For all plots, mean \pm SD of log-transformed values from at least two biological replicates

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520 **Author Contributions**

521 KJ, THSS, IJF, and JDG designed the research. KJ performed the flow experiments. KJ, DRB, AA, and
522 JDG purified antibodies and other reagents. KJ and THSS cloned spike variants. KJ and CWC performed
523 BLI DSF experiments. RJO and JMM isolated and sequenced Omicron. XX, HX, PS, and SW provided
524 the authentic virus neutralization data. KJ, THSS, CDJ, and JDG analyzed the data. KJ, THSS, ADE, IJF,
525 and JDG wrote the paper with editorial assistance from all co-authors.

526 **Declaration of Interests**

527 The authors declare competing financial interests. DRB, ADE, and JDG have filed patent applications
528 monoclonal antibodies targeting SARS-CoV-2. KJ, C.-W.C., and I.J.F. have filed patent applications on
529 spike 6p (HexaPro). The authors declare no competing non-financial interests.

530 **Inclusion and Diversity**

531 One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science.
532 The author list of this paper includes contributors from the location where the research was conducted
533 who participated in the data collection, design, analysis, and/or interpretation of the work.

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541 **MATERIALS & METHODS DETAILS**

542 **Spike variant cloning**

543 We assembled protein variants using a Golden Gate / TypeIIIs cloning framework. Generally, segments of
544 the spike protein coding sequence were ordered as synthetic DNA fragments (IDT, eBlocks), cloned into
545 a plasmid backbone, and sequence verified. Verified DNA parts were transferred with plasmid backbone
546 into 96-well PCR plates using an Echo 525 liquid handler (Beckman Coulter). To each well, we added the
547 following Golden Gate reaction mixture: 0.25 μ L of T4 or T7 DNA Ligase (NEB, M0202 / M0318), 0.25
548 μ L of AarI (Thermo Fisher, ER1582), 0.2 μ L AarI Oligo (Thermo Fisher), 1 μ L T4 DNA Ligase Buffer
549 (NEB B0202A), and nuclease-free water to bring the final volume to 10 μ L per reaction. We incubated
550 the reaction mixtures on a thermocycler using the following settings: 25 digestion and ligation cycles (1
551 min at 37°C and 2 min at 16°C), a final digestion step (30 min at 37°C), and heat inactivation (20 min at
552 80°C). For assemblies with 4+ parts, we increased the cycled digestion and ligation steps to 3 and 5 min,
553 respectively, to improve assembly efficiencies.

554 We transformed each unique reaction mixture into NEB10-beta cells (NEB, C3019) using the
555 Mix & Go! E.coli Transformation Buffer Set (Zymo Research, T3002). Cell were outgrown with SOC at
556 37°C and drop-plated onto LB agar + carbenicillin in Nunc OmniTrays (5 μ L per spot, 96-spots per plate)
557 (Thermo Fisher, 140156). We allowed the drops to dry at room temperature before transferring the plates
558 to an incubator at 37°C for growth overnight. After growth, we picked colonies into deep well grow
559 blocks (Axygen P-2ML-SQ-C-S or Greiner Bio-One, 780270) containing 1 mL of Superior Broth
560 (AthenaES, 0105) media + carbenicillin and grew them overnight at 37°C with shaking. Overnights were
561 miniprepmed using the Wizard SV 96 Plasmid DNA Purification Kit (Promega A2250) and sequence-
562 verified before further characterization.

563

564 **Expression and purification of neutralizing anti-spike monoclonal antibodies**

565 We cultured Expi293 cells in Expi293 Expression Medium (Sigma-Aldrich A1435101) and used a
566 humidified cell culture incubator to maintain cells at 37°C and 8% CO₂ with continuous shaking at 125
567 rpm. For transfection, we used an Expi293 Transfection Kit (Sigma-Aldrich L3287) according to the
568 manufacturer's instructions. Briefly, we transfected cells with VH and VL expression vectors at a 1:3
569 molar ratio. Five days after transfection, we collected the protein-containing supernatant using a two-step
570 centrifugation protocol. First, we separated cells and supernatant by centrifuging cultures at 4°C and 300
571 g for 5 min. Next, we separated cell debris and supernatant by centrifuging at 4°C and 3,000 g for 25 min.
572 To purify human IgGs, we washed Protein G magnetic beads (Promega G7471) with PBS buffer and
573 added the beads to the separated supernatant in a 1:10 volumetric ratio. After a 1h incubation with
574 gyration at room temperature, we used a magnetic peg stand to pellet bead-bound antibodies, which we
575 washed before final elution with a 100 mM glycine-HCl solution at pH 2.5. Finally, we passed the elute
576 through a 0.22- μ m syringe filter to clarify residual beads before neutralization with 2 M Tris buffer at pH
577 7.5. We kept purified antibodies at 4°C or -20°C for short and long-term storage, respectively.

578

579 **Expression and purification of human ACE2-Fc**

580 We recombinantly expressed human ACE2-Fc in Expi293 cells using a previously described method with
581 minor modification⁶². Briefly, we transfected the ACE2-Fc expression vector into Expi293T cells (Sigma-

582 Aldrich). Five days after transfection, we centrifuged the cultures at 4°C and 300 g for 5 min and
583 collected the supernatant. We further separated cell debris and supernatant by centrifuging at 10,000 g
584 and 4°C for 20 min. After resuspending it in PBS, we purified ACE2-Fc over Protein A Agarose (Thermo
585 Fisher 15918014). We next equilibrated the Protein A Agarose in PBS buffer, ran through the supernatant
586 three times, and used 10 bed volumes of PBS buffer for washing. Finally, we used 100 mM glycine-HCL
587 at pH 2.4 to elute ACE2-Fc into 0.1x volume Tris buffer at pH 8.5 and 100 mM NaCl. We kept purified
588 ACE2-Fc at 4°C and -20°C for short and long-term storage, respectively.

589

590 **Expression and purification of SARS-CoV-2 spike proteins**

591 We transfected and expressed plasmids using Expi293 cells using previously described methods²⁶.
592 Briefly, we purified variants from 40 mL of cell culture. We filtered the supernatant using a 0.22-µm filter
593 and ran it through a StrepTactin Superflow column (IBA 2-1206-025). We further purified spikes via
594 Superose 6 Increase 10/300 (GE GE29-0915-96) size-exclusion column chromatography with a buffer
595 containing 2 mM Tris at pH 8.0, 200 mM NaCl, and 0.02% NaN₃. We kept purified samples at 4°C and -
596 20°C for short and long-term storage, respectively.

597

598 **HEK293T transfection**

599 We seeded cells into 6 or 12-well polystyrene-coated plates (VWR 10861-696, 10861-698) at a density of
600 0.3×10^6 cells mL⁻¹ or 0.1×10^6 cells mL⁻¹, respectively, one day before transfection. At 60-80%
601 confluence, we used Lipofectamine 3000 (Thermo Fisher L3000015) and Opti-MEM (Gibco 51985091)
602 to transfect cells with expression plasmids (3 µL of lipofectamine per µg of plasmid DNA) according to
603 manufacturer instructions. At 48 h post-transfection, we assayed or collected the cells.

604

605 **Flow cytometry and data analysis**

606 At 48 h post-transfection, we collected HEK293T cells containing surface-displayed spike. We washed
607 cells once with PBS and used gentle pipetting to resuspend them in PBS. We used the Logos Biosystems
608 (L40002) cell counter to determine cell density and spun cells down at 200 g for 1 min. We next decanted
609 the supernatant and resuspended cells to a density of $\sim 3 \times 10^6$ cells mL⁻¹ in chilled PBS-BSA using 1%
610 BSA (Sigma-Aldrich A3294), 1X PBS, and 2 mM EDTA at pH 7.4.

611 We used Axygen deep well grow blocks (P-2ML-SQ-C-S) to prepare flow cytometric assays. We
612 added predetermined concentrations of primary antibody or chimeric cell receptor (ACE2-Fc) diluted in
613 PBS-BSA and 50 µL (1.5×10^5) of HEK293T cells to each well. We incubated the mixtures at room
614 temperature for 1 h with shaking at 950 rpm. To pellet cells, we spun plates at 500 g in a swinging bucket
615 rotor for 2 min. We then washed cells twice by decanting the supernatant and adding 500 µL of PBS-BSA
616 to each well. To each well, we added 500 µL total volume of 5 µM Alexa Fluor® 488 anti-mouse
617 secondary (SouthernBiotech 1031-30) and 10 µM Alexa Fluor® 647 anti-human secondary
618 (SouthernBiotech 2048-31) antibodies in PBS-BSA. We incubated plates in the dark for 25 min at 4°C
619 with shaking at 950 g. We washed each well twice with PBS-BSA and resuspended cells in PBS-BSA
620 (300 µL) before loading them onto the SA3800 Spectral Cell Analyzer (SONY).

621 To establish forward scatter-area (FSC-A) and side scatter-area (SSC-A) gating, we used
622 HEK293T cells. For singlet discrimination, we gated with forward scatter-height (FSC-H) vs forward

623 scatter-area (FSC-A) and side scatter-height (SSC-H) vs side scatter-area (SSC-A). For each assayed
624 sample, we acquired a minimum of 10,000 singlet events. We further analyzed singlet HEK293T cells
625 using Alexa Fluor 488 (AF-488) and Alexa Fluor 647 (AF-647) channels with excitation and detection
626 settings recommended by the manufacturer. To reduce spectral spill-over and autofluorescence effects, we
627 applied spectral unmixing to all data.

628 For each sample, we measured the median height (H) for the AF-488 and AF-647 channels. To
629 measure spike expression, we used the signal from the AF-488 channel (anti-FLAG). We used the
630 following equation to calculate the expression of spike variant (x) relative to WT ($6P$ -D614G):

$$631 \text{ } \\ 632 \text{ } \textit{Normalized expression} = \textit{Log}_2(\textit{Median: } 488_H_x / \textit{Median: } 488_H_{6P_D614G})$$

633
634 To correct for changes in transfection efficiency or spike expression in antibody or ACE2 binding
635 measurements, we also included anti-FLAG signal as an internal normalization control. We used the
636 following equation to calculate normalized binding measurements of spike variant (x) expression relative
637 to WT ($6P$ -D614G):

$$638 \text{ } \\ 639 \text{ } \textit{Normalized binding} = \textit{Log}_2\left(\frac{\textit{Median: } 647_H_x / \textit{Median: } 488_H_x}{\textit{Median: } 647_H_{6P_D614G} / \textit{Median: } 488_H_{6P_D614G}}\right)$$

640
641 We used FlowJo v9 for all flow cytometry data analyses.

642 643 **SARS-CoV-2 authentic virus neutralization assay**

644 To measure human sera and monoclonal antibody neutralization titers, we used a fluorescent focus
645 reduction neutralization test (FFRNT) with an mNeonGreen (mNG) reporter SARS-CoV-2 (strain USA-
646 WA1-2020) or SARS-CoV-2 (strain USA-WA1-2020) bearing a variant spike gene (e.g., Omicron BA.1).
647 The construction of the mNG USA-WA1-2020 SARS-CoV-2 bearing variant spikes has been reported
648 previously (Liu et al., 2021; Zou et al., 2022). For the FFRNT assay, we seeded 2.5×10^4 Vero E6 cells
649 into black, μ CLEAR flat-bottom 96-well plates (Greiner Bio-One™). We incubated plates at 37°C with 5%
650 CO₂ overnight. The next day, each sample was two-fold serially diluted in culture medium with an initial
651 dilution of 1:20. We incubated diluted serum or antibody with 100-150 fluorescent focus units (FFU) of
652 mNG SARS-CoV-2 at 37°C for 1 h before loading the serum-virus mixtures into 96-well plates pre-seeded
653 with Vero E6 cells. Following a 1 h infection period, we removed the inoculum and added overlay medium
654 (100 μ L DMEM + 0.8% methylcellulose, 2% FBS, and 1% penicillin/streptomycin). We then incubated the
655 plates at 37°C for 16 h and acquired raw images of mNG fluorescent foci using a Cytation™ 7 (BioTek)
656 cell imaging reader with a 2.5 \times FL Zeiss objective and wide field of view. We used GFP software settings
657 [469,525], a threshold of 4000, and an object selection size of 50-1000 μ m during image processing. For
658 relative infectivity calculations, we counted and normalized the foci in each well relative to non-
659 serum/antibody-treated controls. We plotted curves of relative infectivity versus serum dilution using Prism
660 9 (GraphPad). We used a nonlinear regression method to determine the dilution fold at which 50% of mNG
661 SARS-CoV-2 was neutralized, defined as FFRNT₅₀ in GraphPad Prism 9. Each antibody was tested in
662 duplicate.

663
664

665 **Biolayer Interferometry**

666 After 3C protease-mediated cleavage, we diluted supernatants containing spike variants two-fold with
667 BLI Kinetics Buffer (Fortébio) containing 10 mM HEPES at pH 7.5, 150 mM NaCl, 3 mM EDTA, 0.05%
668 v/v Surfactant P20 (Cytiva BR100054), and 1 mg mL⁻¹ BSA. We also serially diluted analytes with the
669 BLI buffer. We hydrated anti-mouse Fc capture (AMC) biosensors (FortéBio 18-5088) in BLI buffer for
670 10 min in an Octet RED96e (FortéBio) system and then immobilized mouse anti-FLAG M2 (Sigma-
671 Aldrich F3165) antibodies to the AMC sensor tips. For each assay, we performed the following steps: 1)
672 baseline: 60 s with BLI buffer; 2) IgG immobilization: 360 s with anti-FLAG IgG; 3) spike loading: 360 s
673 with diluted supernatants; 4) baseline: 300 s with BLI buffer; 5) association: 600 s with serially diluted
674 analytes (antibodies or ACE2); 6) dissociation: 600 s with BLI buffer. We used Octet Data Analysis
675 software v11.1 with steady-state analysis to reference-subtract and analyze the data.
676

677 **Differential Scanning Fluorimetry**

678 Sample solutions containing 5X SYPRO Orange Protein Gel Stain (Invitrogen S6651) and 0.15-0.20
679 mg/mL of purified spike protein were added to a 96-well qPCR plate (Azenta Life Sciences 4ti-0955).
680 Fluorescence measurements were obtained continuously using $\lambda_{ex}=465$ nm and $\lambda_{em}=580$ nm, using a
681 Roche LightCycler 480 II, and a temperature ramp rate of 4.5°C/minute increasing from 22 °C to 95 °C.
682 Fluorescence data were then plotted as the derivative of the melting curve as a function of temperature (-
683 dF/dT). SARS-CoV-2 spike proteins generate two local minimums that we report to as Tm1 and Tm2. All
684 data were visualized in GraphPad Prism 9. For simplicity, we normalize all melting curve traces by their
685 absolute min -df/dT value.
686

687 **Computational analysis of GISAID sequence data**

688 To investigate the clinical frequency of SARS-CoV-2 spike mutations and the probability of mutation co-
689 occurrences, we performed pairwise amino acid sequence alignments between the GISAID spike
690 reference sequence, GenBank number QHR63250.2, and all GISAID EpiCoV database SARS-CoV-2
691 spike amino acid sequences. We downloaded amino acid sequences from GISAID (accessed on
692 December 18, 2021) as a FASTA file. We performed semi-global amino acid sequence alignment using
693 MATLAB's Needleman-Wunsch alignment function as a part of its Bioinformatics Toolbox add-on. We
694 set alignment parameters to include no gap open penalty at the beginning and end of sequences, an
695 internal gap open penalty of 5, a gap extension penalty of 2, and the BLOSUM80 scoring matrix, as the
696 aligned sequences were similar.

697 We filtered alignment pairs to remove all sequences that were non-human in origin, sequences
698 containing over 1280 or fewer than 1260 amino acids, and sequences containing more than 800 unknown
699 ("X") amino acids. We identified non-synonymous mutations from the alignments. The following
700 equation was used to find frequencies for each mutation:

$$701 \text{Frequency} = \Sigma(\text{Sequences containing a specific mutation}) / \Sigma(\text{all mutations in all sequences})$$

702
703
704 We found mutations that occurred independently by removing all alignment pairs that did not
705 contain the target mutation and sequences that contained mutations other than the target mutation.
706 Additionally, we considered alignment pairs for which the only other mutation (other than the target) was

707 D614G were as independent, as D614G was highly prevalent in all strains after its initial appearance. The
708 following equation was used to calculate frequency for independent mutations:

709
710
$$\text{Frequency} = \frac{\Sigma(\text{Sequences containing only the specified mutation or the specified mutation and}$$

711
$$\text{D614G})}{\Sigma(\text{number of all sequences searched})}$$

712

713 **Structural analyses and data visualization**

714 We downloaded all structures (7DDN⁶¹, 7L2D²⁹, and 7L2E²⁹) as PDB files from the RCSB PDB and
715 imported them into ChimeraX 1.1. We rescaled the Log₂ (normalized binding) values (-7 to 0) and
716 converted them to monochromatic ChimeraX color codes representing changes in binding relative to 6P-
717 D614G spike. Dark red indicates decreased binding and white indicates no change in binding. For every
718 amino acid screened in our Spike Display assay, we superimposed these colors scales onto spike protein
719 structures. In figures showing grouped antibody epitopes, we averaged normalized binding values for all
720 mutations in each position for every antibody comprising that group. Finally, we converted the averaged
721 binding values to a single color that was then mapped onto spike structures.
722

723 **STATISTICAL ANALYSIS**

724 The means \pm S.D. log-transformed values, or non-transformed values were calculated and reported for all
725 appropriate data.

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742 **References**

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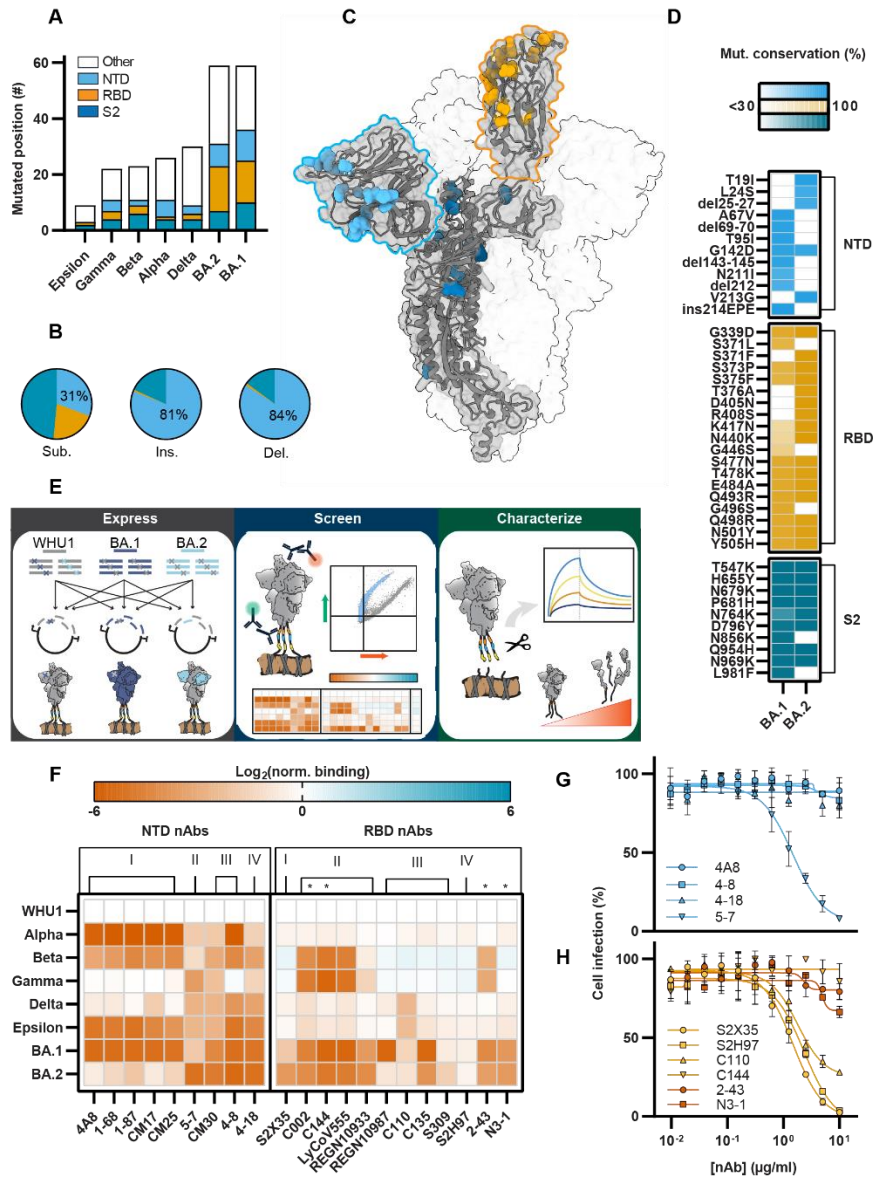
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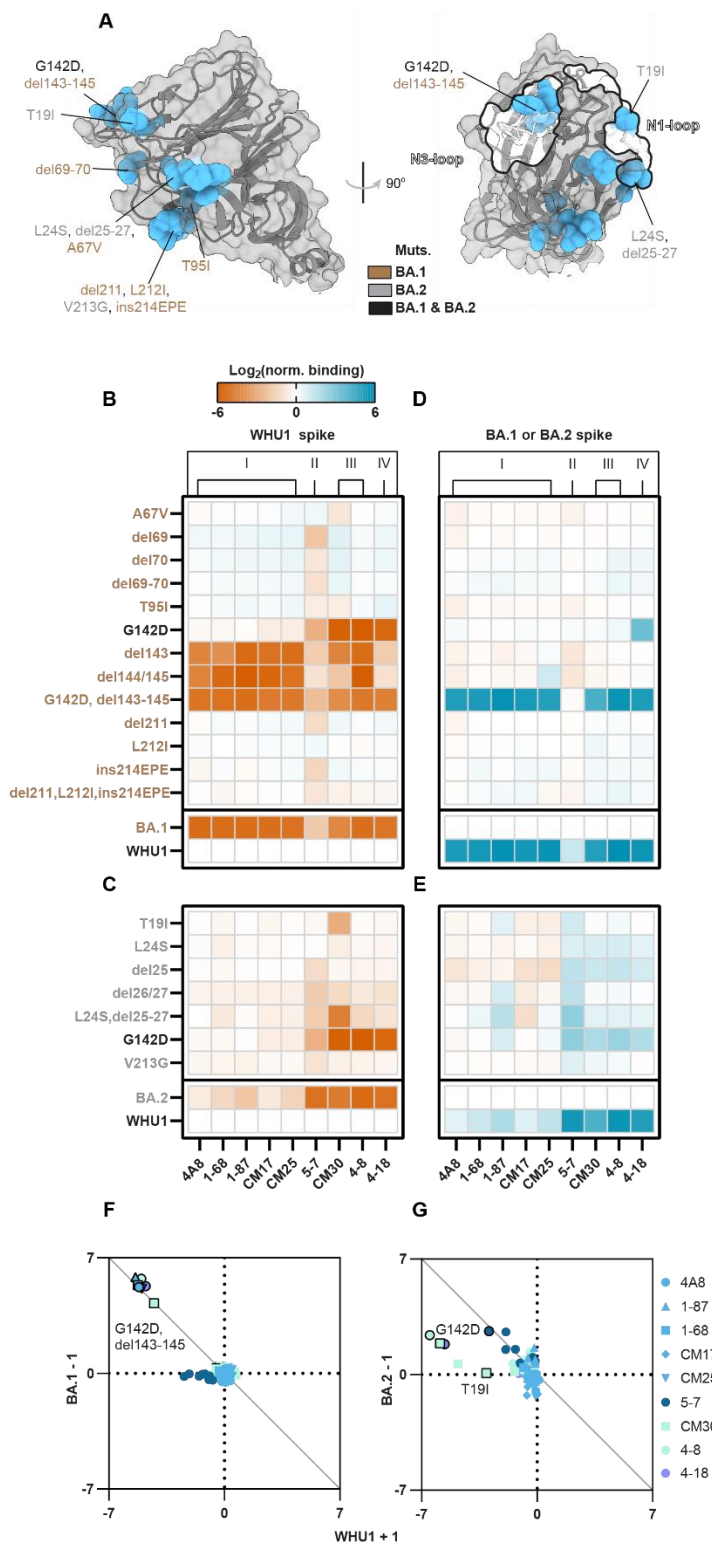
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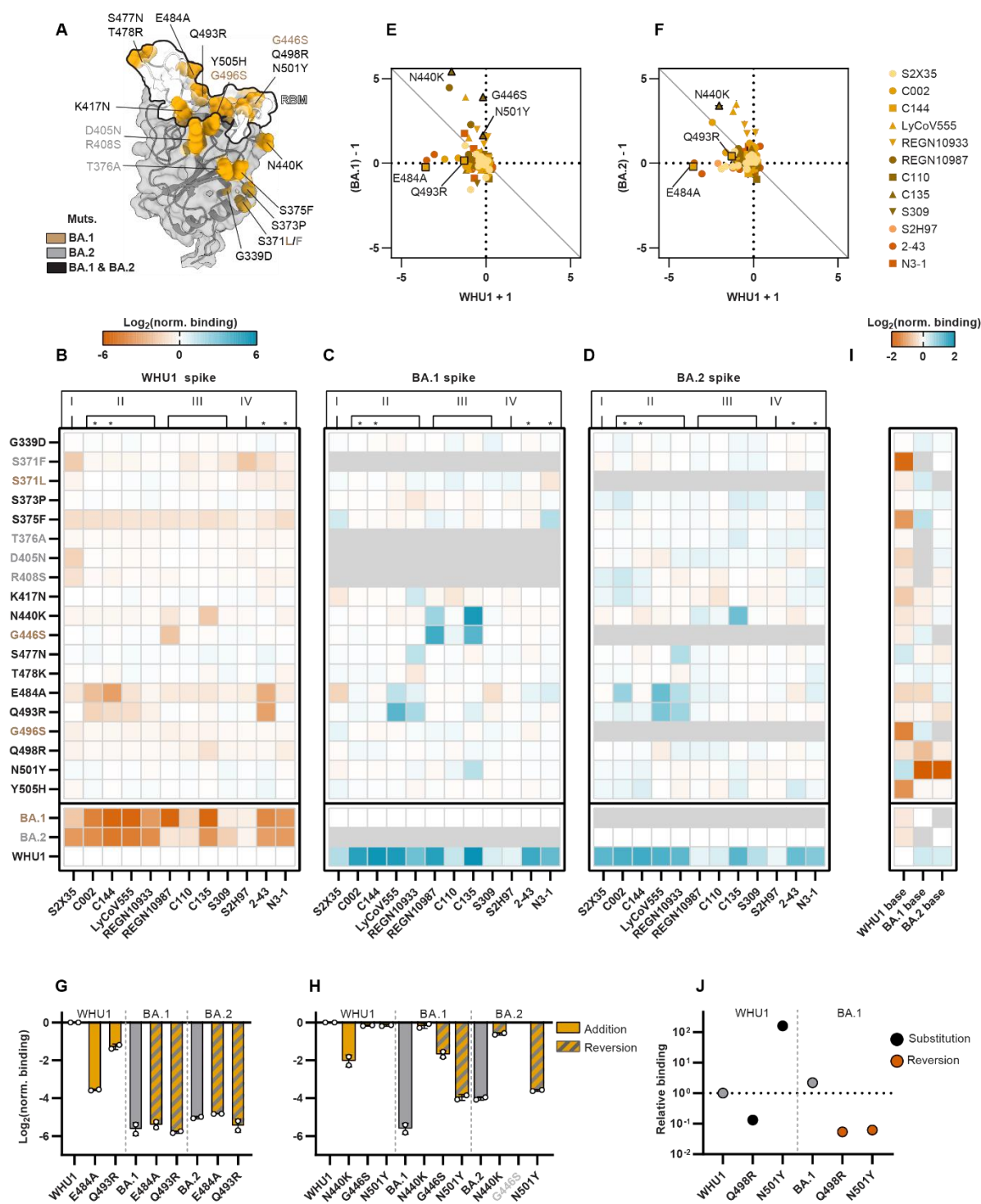
872 **Figure 1**



874 **Figure 2**



876 **Figure 3**



878 **Figure 4**

