Conserved sites on the influenza H1 and H3 hemagglutinin recognized by human antibodies

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ABSTRACT

Monoclonal antibodies (mAbs) targeting the influenza hemagglutinin (HA) have the potential to be used as prophylactics or templates for next-generation vaccines that provide broad protection. Here, we isolated broad, subtype-neutralizing mAbs from human B cells targeting the H1 or H3 HA head as well as a unique mAb targeting the stem. The H1 mAbs target the previously defined lateral patch epitope on H1 HAs and recognize HAs from 1933 to 2021 in addition to a swine H1N1 virus with pandemic potential. Using directed evolution, we improved the neutralization potency of these H1 mAbs towards a contemporary H1 strain. Using deep mutational scanning of four antigenically distinct H1N1 viruses, we identified potential viral escape pathways. For the H3 mAbs we used cryo-EM to define the targeted epitopes: one mAb recognizes the side of the H3 head, accommodating the N133 glycan and a pocket underneath the receptor binding site. The other H3 mAb recognizes an epitope in the HA stem that overlaps with previously characterized mAbs, but with distinct antibody variable genes and mode of recognition. Collectively, these mAbs identify common sites recognized by broad, subtype-specific mAbs that may be elicited by next-generation vaccines.

INTRODUCTION

Two influenza types cause human disease: influenza A viruses (IAVs) emerge from animal reservoirs and cause global pandemics while influenza B viruses (IBVs) only circulate in the human population. Antigenic and genetic differences of the major surface glycoprotein hemagglutinin (HA), which engages the sialic acid receptor and fuses the viral and endosomal membranes, further divide IAVs into two groups with 18 HA subtypes (H1–H18) total. In the last century, IAVs bearing H1 or H2 (group 1), or H3 (group 2) HAs have circulated in the human population; H1N1 and H3N2 viruses currently circulate.

Vaccine effectiveness against seasonal influenza viruses reaches modest levels around 50%[1] and updated vaccines are needed due to antigenic drift – the acquisition of amino acid mutations in HA that preserve function but escape population-wide antibody recognition. IAVs undergo antigenic drift more rapidly than IBVs[2], requiring continuous monitoring for new antigenic variants. Additional barriers to an effective vaccine include complex and variable immune histories within the human population. A complementary approach to seasonal vaccination is to identify monoclonal antibodies (mAbs) that recognize conserved HA sites for prophylactic use, particularly in high-risk populations. Characterizing such mAbs can also serve as templates to guide next-generation vaccine designs and inform how viral escape from vaccine-induced immunity may occur.

Antibodies that target the HA "head" and prevent sialic acid receptor engagement are an established correlate of protection but are usually subtype-specific[3-5]. mAbs with heterosubtypic breadth typically engage epitopes on the membrane proximal HA "stem" within group 1 or group 2, and, in some cases, both[6-10]. Although these stem-directed mAbs are protective in mice[6-10], they require Fc-effector functions for protection, suggesting that although stem-directed mAbs can neutralize *in vitro*, there is minimal neutralization activity *in vivo*[11]. Due to their reactivity to potentially pandemic subtypes of IAVs that have not circulated in humans, stem-directed mAbs may help protect from severe disease in the case of zoonotic spillover or viral escape from anti-head mAbs. However, while passive transfer of stem-directed antibodies offers some protection, head-directed antibodies better protect against acquiring infection even when normalized by binding titers[12].

In contrast to anti-stem mAbs, IAVs escape anti-head antibodies frequently at the population level, demonstrating that these mAbs provide a significant barrier for transmission that

75 the virus must overcome to persist in the population[2,13,14]. Although epitopes in the head are more
76 variable than the stem, it is nevertheless possible to identify broad, subtype-specific anti-head
77 mAbs[15-24]. Epitopes on the side of the HA head (*e.g.,* the lateral patch, vestigial esterase) are
78 conserved[15,21,22], affording mAbs targeting these sites considerable breadth and potency. If next-
79 generation vaccines can elicit antibodies targeting these conserved epitopes[25-27], it is unclear how
80 such a diverse polyclonal response would structurally recognize HA and ultimately how the virus
81 would evolve to escape.

82 Here, we isolated and characterized broad H1 and H3 human mAbs capable of neutralizing
83 human IAVs spanning several decades. For the broad H1 mAbs, which neutralize H1N1 IAVs
84 spanning ~100 years, we used four deep mutational scanning H1N1 viral libraries to map critical
85 residues within the targeted epitope. Although escape from broad H1 mAbs was readily selected
86 *in vitro*, human influenza H1N1 isolates have rarely contained these escape mutations. Broad H1
87 mAb neutralizing potency was improved against a contemporary H1N1 strain using directed
88 evolution. For the broad H3 mAbs, which neutralize human H3N2 IAVs spanning ~50 years, we
89 determined cryo-EM structures in complex with HA. One broad H3 mAb targets an epitope in the
90 head domain with regions of low mutational tolerance[28]. The other broad H3 mAb targets the group
91 2 stem site but engages with a distinct mode of recognition. The epitopes identified by these broad
92 H3 mAbs overlap with those targeted by previously characterized mAbs, but with different
93 antibody variable gene usage and paratopes. These data suggest that while antibodies targeting
94 these conserved sites could produce viral escape variants, mAbs targeting these sites may
95 nevertheless serve as prophylactics for high-risk populations.
96

97 **RESULTS**

98 **Isolation of broad H1 and H3 monoclonal antibodies**

99 To identify broad subtype neutralizing mAbs, we used a panel of reporter IAVs spanning
100 1933 to 2021 that includes distinct antigenic clusters[2], representing IAVs that have previously
101 escaped humoral immunity. We sorted single H1-reactive B cells from human PBMCs using
102 recombinant A/California/07/2009 HA (**Supplementary Fig. 1A**). We then sequenced the variable
103 genes, recombinantly produced mAbs[29], and screened for neutralization activity. H1-targeting
104 mAbs were screened against representative H1N1 strains A/WSN/1933, A/Solomon
105 Islands/3/2006, and A/Michigan/45/2015. We identified two clonally related mAbs, ADI-77470

106 and ADI-77474, that completely neutralized (>99%) all three H1N1 viruses at 1 μg/ml used for
107 screening (**Supplementary Fig. 1B**).
108 To isolate broad H3 mAbs, we sorted B cells using recombinant HA probes from H3N2s
109 A/Hong Kong/1/1968 (HK68) and A/Darwin/6/2021 (DAR21) coupled to different streptavidin
110 fluorophore conjugates to identify cross-reactive B cells (**Supplementary Fig. 1C**). The HK68
111 probe included the HA head appended to a foldon trimerization tag, allowing us to isolate head-
112 directed mAbs in addition to those that target full-length DAR21 HA. The frequency of cross-
113 reactive HK68/DAR21 head-reactive memory B cells was rare, ranging from 0.0049 to 0.016%
114 across five donors (**Supplementary Fig. 1C**). We further characterized two H3 mAbs, ADI-85647
115 and ADI-85666 after screening for broad neutralization activity (**Supplementary Fig. 1D**).
116 We characterized the subtypic neutralization potency and breadth of these broad H1 and
117 H3 mAbs against 11 H1N1 or 10 H3N2 IAVs. The broad H1 mAbs potently neutralized all H1N1
118 viruses, but not a representative H3N2; the broad H3 mAbs neutralized all H3N2 viruses, but not
119 a representative H1N1 (**Fig. 1**), demonstrating breadth within but not across subtypes. To further
120 test the breadth of these mAbs, we screened for binding by ELISA to the HA from a swine H1N1
121 with pandemic potential (A/Swine/HN/SN13/2018[30]) or an H3 HA from an avian-origin canine
122 IAV found to evolve mammalian influenza-like traits (A/Canine/Hainan/79/2019[31]). Although
123 these IAVs have never circulated in humans, these mAbs bound these strains, highlighting their
124 considerable breadth (**Fig. 1C**). We performed competition assays to crudely map the region on
125 HA targeted by these mAbs. The broad H1 mAbs competed with 047-09M-2F03[17], a mAb that
126 engages the lateral patch of H1 HAs. ADI-85647, a broad H3 mAb, competed with F005-126[32],
127 H3v-47[15], and c585[21], which target the H3 head, whereas the other H3 mAb, ADI-85666, competed
128 with MEDI8852[9], showing that it engages the stem region (**Fig. 1C**). Consistent with binding the
129 stem, ADI-85666 additionally neutralized a group 2 H7N9 (**Fig. 1B**).
130
131 **Viral escape from broad H1 antibodies targeting the head**
132 We next more finely mapped the recognition of the broad H1 mAbs by viral escape. We
133 previously generated deep mutational scanning viral libraries of three H1N1s containing all single
134 amino acid mutations and deletions in the HA head (residues 52–263, H3 numbering):
135 A/USSR/90/1977 (USSR77), A/Siena/10/1989 (SN89), and A/Solomon Islands/3/2006 (SI06)[33].
136 Here, we additionally generated a viral library on the A/Sydney/5/2021 (SYD21) background to

137 determine whether escape is different in a post-2009 pandemic H1N1 virus (**Supplementary Fig.**
138 **2A**). We determined neutralization potencies of ADI-77470, ADI-77474, and a V-gene germline-
139 reverted ADI-77474 against these libraries (**Supplementary Fig. 2B**). The germline-reverted
140 ADI-77474 showed undetectable neutralization activity, demonstrating the importance of the
141 somatic mutations in these mAbs (**Supplementary Fig. 2B**). We passaged the four viral libraries
142 in the presence of either ADI-77470 or ADI-77474 at 10x the IC_{99} concentration to identify escape
143 variants (**Fig. 2 and Supplementary Fig. 2B**). Mutation of P122 to acidic residues (e.g., Asp, Glu)
144 or deletion were the most selected in all virus backgrounds (**Fig. 2C and Supplementary Fig.**
145 **2F**). However, ADI-77474 was more sensitive to mutational escape in SYD21 compared to its
146 clonal relative ADI-77470 (**Fig. 2 and Supplementary Fig. 2**). Although escape primarily
147 occurred at position 122, mutation to Asn at 122, 123, and 124 in SYD21 were selected by both
148 mAbs; each of these Asn mutations introduces a predicted N-linked glycosylation sequon (**Fig. 2C**
149 **and Supplementary Fig. 2F**). Although 123N and 124N mutations in the historical strains
150 USSR77, SN89, and SI06 also create potential N-linked glycosylation sites, these mutations were
151 not enriched in these strains, demonstrating different barriers to escape for pre- and post-pandemic
152 H1s (**Fig. 2C and Supplementary Fig. 2F**). We mapped the escape mutations onto the structure
153 of an HA bound by a mAb engaging the same epitope and that uses the same V-genes, V_H3-
154 23/V_K1-33[17] (**Fig. 2B**). Residues 119, 122-125, and 172 cluster around the YxR motif often found
155 in this class of mAbs[17]; the mAbs we describe have a similarly charged YxK motif in the CDRH3
156 (**Supplementary Fig. 3C**). These escape mutations do not overlap with those of Ab6649[33], which
157 targets an overlapping region within the lateral patch.
158
159 **Affinity maturation of broad H1 mAbs increases neutralization potency**
160 To increase the affinity of the broad H1 mAbs, we generated yeast libraries encoding
161 mutations in the variable heavy and light chain genes and selected variants with increased binding
162 affinity using recombinant monomeric SYD21 head[34] (**Supplementary Fig. 3A**). We down-
163 selected affinity-matured mAbs based on a single replicate neutralization screen against SYD21
164 virus (**Supplementary Fig. 3B**) and further tested the most potent of these mAbs against the
165 historical H1N1 A/Beijing/262/1995 (BE95) (**Fig. 3A**). The *in vitro* affinity-matured mAb, ADI-
166 86325, has 2 heavy and 4 light chain mutations relative to ADI-77470 (**Supplementary Fig. 3C**),
167 which increased the potency against SYD21 by ~4-fold while maintaining BE95 neutralization

168 (**Fig. 3A**). We next assessed whether *in vitro* affinity maturation of ADI-77470 to ADI-86325
169 affected viral escape by using deep mutational scanning of the SYD21 virus. We found that
170 mutations that had limited escape effect from ADI-77470 had a reduced effect against ADI-86325,
171 but highly selected escape mutations were nevertheless similar suggesting these residues are
172 critical for HA recognition by these mAbs (**Fig. 2C and 3B**).
173 To determine the occurrence of escape mutations in IAV isolates, we used a previous
174 conservation analysis performed on 1222 unique H1 sequences representative of sequence clusters
175 from 1918–2019[35] to determine whether observed escape mutations have occurred naturally (**Fig.**
176 **3B-D**). Despite some positions with low ConSurf[36] scores, specific amino acid escape mutations
177 have rarely been observed in H1 sequences (**Fig. 3B-D**), suggesting that ADI-86325 is likely to
178 neutralize most H1N1 viruses and providing an explanation for the high degree of neutralization
179 breadth. The identification of escape mutations *in vitro* that have yet to appear naturally possibly
180 suggests that such antibodies have historically been rare within the human population.
181
182 **A broad H3 mAb targets the side of the H3 head**
183 As analogous deep mutational scanning libraries for historical and contemporary H3N2
184 viruses were unavailable, we used cryo-EM to structurally determine the epitopes targeted by the
185 broad H3 mAbs. ADI-85647, encoded by V_H3-9/V_K1-39, in complex with A/Darwin/6/2021
186 (DAR21) HA shows that it binds to the side of the HA head, consistent with competition with
187 H3v-47, c585, and F005-126 (**Figs. 1, 4A, and Supplementary Fig. 4**). During antigenic drift of
188 H3N2s, potential N-linked glycosylation sites on HA were acquired and maintained at this site at
189 positions 122, 126, and 133[37]. Although the cryo-EM map showed clear features of a glycan at
190 N133, we did not observe a glycan at N122, consistent with a previous report showing no and full
191 glycan occupancy at N122 and N133, respectively[38]. The N133 glycan sits within a cleft between
192 the variable heavy and light chains of ADI-85647, and the CDRH2 inserts between the N133 and
193 N126 glycans (**Fig. 4B**). The Y32 hydroxyl and V2 backbone in the heavy chain form hydrogen
194 bonds with the GlcNAc and one of the branching mannose moieties of the glycan, respectively,
195 and thus stabilize the glycan within this cleft. There are eleven hydrogen bonds, two of which are
196 with the N133 glycan; no salt bridges are present. The heavy chain contributes only two hydrogen
197 bonds in addition to interacting with HA through several aromatic residues (**Fig. 4C**); W102 in the
198 CDRH3 sits within a pocket underneath the 130-loop. While the ADI-85647 epitope significantly

199 overlaps with that of H3v-47 (**Fig. 4D**), ADI-85647 can also bind and neutralize H3N2 strains
200 prior to 1989 in contrast to H3v-47[15].
201
202 **Group 2 neutralization through a unique structural solution**
203 The anti-stem mAb ADI-85666 uses the germline genes V_H4-61 and V_K1-17, which have
204 not been previously reported for group 2 anti-stem mAbs; we therefore determined a cryo-EM
205 structure bound to DAR21 HA_0 to understand how recognition differs from previously reported
206 mAbs (**Fig. 5A**). The epitope overlaps with recognition of other group 2 neutralizing mAbs[10,39],
207 such as CR8020, but in contrast to other group 2 neutralizing mAbs, the CDRH3 of ADI-85666
208 extends into a groove spanning the β-sheet at the base of the stem located near the viral membrane
209 (**Fig. 5B**). The tyrosine at the tip of the CDRH3 makes a stabilizing aromatic-sulfur contact with
210 M133 (HA_2 numbering) (**Fig. 5B**). One side of the CDRH3 interacts with the hydrophobic groove
211 with I107 and F109 in the CDRH3 while the other side forms a Glu-Arg-Tyr triad with R25 of
212 HA. A second Glu-Arg-Tyr triad is formed with E15 of the fusion peptide and residues Y34 and
213 R99 in the CDRH2 and CDRH3, respectively (**Fig. 5B**). Comparison of the interfacing residues of
214 ADI-85666 with CR8020 shows overlap of the outermost β-strand but diverge towards the central
215 strands or G-helix for ADI-85666 or CR8020, respectively (**Fig. 5C**).
216
217 **Conservation and mutational tolerance of broad H3 mAb epitopes**
218 We next mapped conservation and mutational tolerance of the ADI-85647 and ADI-85666
219 epitopes onto the structure of the DAR21 HA (**Fig. 6**). The conservation scores were obtained from
220 a previous ConSurf[36] analysis of 3,408 unique H3 HA sequences representing historical sequence
221 clusters[35]. To assess the potential for viral escape, we also mapped mutational tolerance derived
222 from A/Perth/16/2009 onto the HA structure, which measures whether mutations at each site
223 impact viral fitness[28]. The ADI-85647 footprint contains some highly variable residues (**Fig. 6**),
224 explaining the variable neutralization potencies (**Fig. 1**); the CDRs of ADI-85647, however,
225 mainly interact with historically conserved residues (**Fig. 6**). Tryptophan 102 in the CDRH3
226 interacts with a pocket underneath the receptor binding site that is both historically conserved and
227 mutationally intolerant (**Fig. 6**). The groove contacted by ADI-85666 is moderately conserved, but
228 contains few highly variable residues, explaining the broad and consistent neutralization potency

229 (**Fig. 1**). However, several positions within the ADI-85666 footprint are highly tolerant to
230 mutations, suggesting mutation at these sites may result in viral escape (**Fig. 6**).
231
232 **DISCUSSION**
233 Here, we isolated broad H1 and H3 neutralizing mAbs from human donors and
234 characterized the targeted epitopes by viral escape and cryo-EM, respectively. Broad H1 mAbs
235 potently neutralize all tested H1N1 strains from 1933 to 2021 and bind to a pre-pandemic swine
236 HA. Despite conservation of the lateral patch recognized by broad H1 mAbs, viral escape occurred
237 readily, primarily at position 122. We engineered the broad H1 mAbs for increased affinity to a
238 contemporary HA, increasing neutralization potency ~4-fold. This affinity-matured mAb, ADI-
239 86325, selected for viral escape mutations P122D/E/-, 125D/E, and Asn mutations at 122-124,
240 which introduce potential N-linked glycosylation sites; however, these mutations are rarely
241 observed in human IAV isolates. Structural characterization of the broad H3 anti-head mAb, ADI-
242 85647, suggests that engagement of a conserved pocket underneath the 130-loop of the receptor
243 binding site is a target for broad H3 mAbs. The structure of the group 2 anti-stem mAb, ADI-
244 85666, shows the CDRH3 inserts into a groove not contacted by previously characterized group 2
245 anti-stem mAbs. Comparing these epitopes with previous deep mutational scanning data suggests
246 potential viral escape should this type of antibody response become dominant[28].
247 The 2009 H1N1 pandemic initially recalled cross-reactive B cell responses that targeted
248 the RBS and lateral patch[17,22,40]. However, upon subsequent exposures, these responses were
249 absent[17], which likely contributes to the continued conservation of the lateral patch epitope. Next-
250 generation vaccines using mosaic nanoparticles containing antigenically distinct H1 HA heads
251 elicit antibodies targeting the lateral patch in animal models[25,26]. In humans, initial recall of lateral
252 patch-directed mAbs after the pandemic selected for viral escape through an N-linked
253 glycosylation[22]. However, a subset of lateral patch-directed mAbs, primarily encoded by V_H3-
254 23/V_K1-33, can overcome this escape. It is possible that these mosaic nanoparticle vaccine
255 candidates in humans may preferentially elicit V_H3-23/V_K1-33 responses targeting the lateral
256 patch. The two clonally related mAbs encoded by V_H3-23/V_K1-33 genes we identified here elicited
257 escape at positions engaged by the common CDRH3 motif in this class of mAb (119, 122, 125,
258 and 172), mostly independent of the background strain. Despite further affinity maturation, these
259 mAbs were still readily escaped by mutations at the same positions in contrast to affinity

260 maturation of RBS-directed mAbs that we have previously shown can prevent escape at certain
261 positions[33]. These data suggest that next-generation vaccines eliciting antibodies targeting this site
262 may not provide long-term, broad protection. However, if population-wide immune pressure on
263 this site does not increase, lateral patch mAbs may nevertheless serve as potent prophylactics or
264 therapeutics for high-risk populations. Further studies are needed to understand whether there are
265 mAbs that target this site and are insensitive to the escape mutations described here while
266 maintaining their broad reactivity.

267 Comparison of the structures of ADI-85647 and H3v-47 show that both insert hydrophobic
268 residues into a conserved pocket underneath the 130-loop. ADI-85647, but not H3v-47, recognizes
269 strains prior to 1989[15], highlighting that these mAbs are sensitive to different mutations within this
270 epitope despite largely overlapping footprints. As shown previously, the H3 head domain appears
271 to be more mutationally intolerant than the stem; this contrasts with H1 HAs[28]. This conservation
272 and mutational intolerance within the pocket engaged by these mAbs may be a result of the
273 proximity to the conserved W153 in the receptor binding pocket; therefore, mAbs engaging this
274 pocket with different V-genes, angles of approach, and mutation sensitives may be an additional
275 way to target H3N2s with escape resistance; reminiscent of a polyclonal antibody response to the
276 RBS pocket[23]. Although ADI-85647 targets a site on H3 like that targeted by H1 lateral patch
277 mAbs, the neutralization potency is markedly lower. While *in vitro* affinity maturation of ADI-
278 85647 may increase potency, it is possible that H1N1 and H3N2 viruses are differentially
279 neutralized by broad mAbs targeting these sites or that the potency of mAbs targeting these sites
280 are dependent on the angle of approach.

281 The group 2 mAb, ADI-85666, targets an epitope that overlaps with that of other group 2
282 neutralizing mAbs, CR8020[10] and CR8043[39], but has an extended CDRH3 that reaches into a
283 groove not contacted by other mAbs; this is the third structurally characterized group 2 mAb that
284 engages this site, each with distinct epitopes. The varied gene usage and diversity of recognition
285 suggests that a polyclonal antibody response to this site may be resistant to escape. We note,
286 however, that a recent clinical trial of a prophylactic pan-influenza stem-targeting mAb, VIR2482,
287 failed to significantly reduce IAV illness (NCT05567783). Therefore, although we do not expect
288 group 2 stem-directed mAbs, or vaccines that elicit them, to prevent infection, they may afford an
289 important bridge during seasons of large antigenic change or in the case of spillover of a subtype
290 for which humans have limited immunity. Together, these broad subtypic neutralizing mAbs could

291 be used as prophylactics or therapeutics. Additionally, these mAbs, the viral escape mutations, and
292 structurally characterized epitopes can be used as tools for designing next-generation vaccines for
293 broad protection.
294
295 **MATERIALS AND METHODS**
296
297 **Human B cell sorting**
298 H1 and H3 antigen-specific B cells were detected using recombinant biotinylated H1 and
299 H3 antigens tetramerized with fluorophore-conjugated streptavidin (SA) as previously described[41].
300 Briefly, His-tagged H1N1 A/California/04/2009 full length trimer Y97F (Sino Biological Cat #
301 11055-V08B1) was mixed in 4:1 molar ratios with SA-Allophycocyanin (APC; Invitrogen) and
302 SA phycoerythrin (PE; Invitrogen) while A/Darwin/6/2021 full-length soluble trimer and A/Hong
303 Kong/1/1968 head trimer (see below) were mixed in 4:1 molar ratios with SA phycoerythrin (PE;
304 Invitrogen) and SA- Allophycocyanin (APC; Invitrogen), respectively, and were incubated for 20
305 min on ice. B cells were isolated using a Pan B cell isolation kit (Miltenyi Cat#130-101-638) from
306 approximately 10 million PBMCs and stained with tetramerized antigens (25 nM each); anti-
307 human antibodies anti-CD19 (PE-Cy7; Clone HIB19; Biolegend Cat # 302216), anti-CD3 (PerCP-
308 Cy5.5; CloneOKT3; Biolegend Cat # 317335), anti-CD8 (PerCP-Cy5.5; Clone SK1; Biolegend
309 Cat # 344710), anti-CD14 (PerCP-Cy5.5; Clone 61D3; Invitrogen Cat # 45-0149-42), and anti-
310 CD16 (PerCP-Cy5.5; Clone B73.1; Biolegend Cat # 360712); and 50 µl Brilliant Stain Buffer (BD
311 BioSciences) diluted in FACS buffer (2% BSA/1mM EDTA in 1X PBS). All antibodies were used
312 at 1:100 dilutions and cells were incubated for 15 min on ice. After one wash with FACS buffer,
313 cells were stained in a mixture of propidium iodide and anti-human antibodies anti-IgG (BV605;
314 Clone G18–145; BD Biosciences Cat # 563246), anti-IgA (FITC; Abcam Cat # Ab98553), anti-
315 CD27 (BV510; Clone MT271; BD Biosciences Cat # 740167), and anti-CD71 (APC-Cy7; Clone
316 CY1G4; Biolegend Cat # 334110). Following 15 min of incubation on ice, cells were washed two
317 times with FACS buffer and analyzed using a BD FACS Fusion (BD BioSciences). For sorting of
318 H1/H3-specific, class-switched B cells, B cells that react with H1N1 A/California/04/2009 full
319 length trimer Y97F tetramers or A/Hong Kong/1/1968 head trimer and A/Hong Kong/1/1968 head
320 trimer tetramers among CD19+CD3−CD8− CD14−CD16−PI− and IgG+ or IgA+ cells were
321 single-cell index sorted into 96-well polystyrene microplates (Corning) containing 20 µl lysis

322 buffer per well [5 µl of 5X first strand SSIV cDNA buffer (Invitrogen), 1.25 µl dithiothreitol (0.1
323 M, Invitrogen), 0.625 µl of NP-40 (Thermo Scientific), 0.25 µl RNaseOUT (Invitrogen), and 12.8
324 µl dH2O]. Plates were briefly centrifuged and then frozen at −80 °C before PCR amplification.
325
326 **Cell lines and media**
327 Cell lines HEK293T, HEK293T-PB1, MDCK-SIAT1-PB1, and MDCK-SIAT1-H7 (gifts
328 from Jesse Bloom and Masaru Kanekiyo) were maintained in DMEM (Gibco) with 10% heat-
329 inactivated FBS (Peak), and penicillin/streptomycin (Gibco). For the growth of MDCK-SIAT1-
330 PB1 and MDCK-SIAT1-H7 cells, 1 mg/ml geneticin (Gibco) and 0.25 µg/ml puromycin (Gibco)
331 were additionally added. For neutralization assays, "flu media" was used, which contains Opti-
332 MEM (Gibco), 0.3% BSA (Sigma), penicillin/streptomycin, 0.1 mg/ml $CaCl_2$, and 0.01% heat-
333 inactivated FBS (Peak).
334
335 **Generation of influenza reporter viruses**
336 Influenza reporter viruses were generated as described previously[42] based on the methods
337 from Creanga et al.[35]. Briefly, reverse genetics bidirectional pHW plasmids (some of which were
338 gifts from Jesse Bloom and Masaru Kanekiyo) encoding the HA and NA of each strain, the internal
339 segments (except PB1) from A/WSN/1933 (for H1N1 viruses) or A/Netherlands/009/2010 (for
340 H3N2 viruses), tdKatushka2 with PB1 packing sequences, and a plasmid that drives expression of
341 human TMPRSS2 were transfected into a co-culture of HEK293T-PB1 (a gift from Jesse Bloom)
342 and MDCK-SIAT1-PB1 cells (a gift from Masaru Kanekiyo) in 6-well plates using TransIT-LT1
343 (MirusBio) at a 2:1 ratio. For H1N1s, the non-coding regions from A/WSN/1933 were used and
344 for H3N2s, the non-coding regions from A/Netherlands/009/2010 were used. The virus-containing
345 supernatant was harvested after three days, clarified by centrifugation, and added to a confluent
346 monolayer of MDCK-SIAT1-PB1 cells seeded the day prior. Once cytopathic effect was evident
347 (two to three days), the supernatant was clarified and stored at -80 °C. To safely generate an H7N9
348 A/Shanghai/02/2013 virus in a BSL2 setting, we used pHW plasmids encoding tdKatushka with
349 HA packing sequences, the A/Shanghai/02/103 NA, the internal genes from mouse-adapted PR8,
350 and a plasmid that expresses full-length H7 HA from A/Shanghai/02/2013, but lacks non-coding
351 regions. This virus was propagated using the MDCK-SIAT-H7 cell line (a gift from Masaru
352 Kanekiyo) that constitutively expresses cell-surface H7 A/Shanghai/02/2013 HA.

353

354 **Microneutralization assays**

355 Neutralization assays were performed as previously reported[43] based on a previously
356 developed assay[35]. Briefly, MDCK-SIAT1-PB1 (or MDCK-SIAT1-H7 for H7N9 neutralization
357 assays) cells were seeded in 96-well plates (Cellvis, #P96-1.5P) at 20,000-30,000 cells per well in
358 100 µl of flu media. The next day, viruses were diluted in flu media containing 2 µg/ml TPCK-
359 trypsin and antibodies were diluted separately in flu media at 2-20 µg/ml for screening or titrated
360 to determine IC_{50} values. The virus and antibody dilutions were mixed at a 1:1 ratio (60 µl : 60 µl)
361 and incubated (37 °C / 5% CO_2) for 1 hour. Then, the flu media was removed from the MDCK-
362 SIAT1-PB1 (or -H7) cells and 100 µl of the antibody/virus mixture was overlayed on top of the
363 cells. Approximately eighteen hours later, the plate was imaged for tdKatushka2 fluorescence
364 using a Zeiss CellDiscoverer7 and fluorescent cells were counted using Zeiss Zen software. The
365 average counts of the virus control wells (no antibody added) determined 0% neutralization and
366 cell only control wells (no virus added) determined 100% neutralization. The neutralization values
367 were plotted in GraphPad Prism and IC_{50} values were determined by a sigmoidal curve fit.

368

369 Antibodies used for deep viral escape selections were also titrated on the libraries to
370 determine concentrations to use in selections as described previously[33]. Briefly, 20,000-30,000
371 humanized MDCK (hCK) cells[44] were seeded in 96-well plates overnight in flu media. Viruses
372 were then diluted in half-log dilutions for titering or to 200 TCID50 / 50 µl for neutralization
373 assays. For neutralization assays, four-fold antibody dilutions were then added and complexed
374 with virus (1 hour / 37 °C / 5% CO_2). After 1 hour, the antibody/virus mixture was removed and
375 the cells were washed with PBS before adding fresh flu media with 1 µg/ml TPCK-trypsin. After
376 18 hours, the cells were fixed with cold 80% acetone in PBS for 10 minutes and air-dried. The
377 wells were washed with PBS + 0.3% Tween20 and blocked with PBS + 0.1% Tween20 + 1% BSA
378 (1 hour, room temperature). The wells were again washed and a mixture of anti-nucleoprotein
379 mAbs (Sigma) were added (1 hour, room temperature). The wells were washed again before adding
380 anti-mouse antibody HRP conjugate (seracare) (1 hour, room temperature). Prior to adding Ultra
381 TMB (ThermoFisher), the wells were washed 5 times. The development of TMB was stopped by
382 addition of 2M sulfuric acid and the resulting absorbance was read at 450 nm. $TCID_{50}$ values were
383 determined by the Reed and Muench method[45] using 4-8 replicates. Neutralization IC_{50} values

384 were calculated by sigmoidal curve fit in GraphPad Prism and IC_{99} values were then calculated by
385 $IC_{99} = (99/(100-99))^{(1/\text{hill slope})} \times IC_{50}$.
386
387 **ELISA**
388 Recombinant full-length soluble ectodomain HA (see below) was coated on high binding
389 96-well plates (Corning) at a concentration of 2 µg/ml overnight at 4 °C. The antigen was then
390 removed and wells were blocked with PBS + 1% BSA + 0.1% Tween20 (1 hour, room
391 temperature). The blocking buffer was removed and 40 µl of antibody was added at 10 or 1 µg/ml
392 to screen for binding (1 hour, room temperature). The antibodies were removed, and the wells were
393 washed three times with PBS + 0.1% Tween20. Anti-human IgG HRP conjugate (Abcam) was
394 then added (1 hour, room temperature). The wells were then washed three times with PBS + 0.1%
395 Tween20 and One Step ABTS was added before reading absorbance at 405 nm. The average of
396 multiple wells with PBS added instead of primary antibody was used to as a background
397 measurement.
398
399 **BLI competition assay**
400 Epitope competition binning of mAbs was assessed using the ForteBio Octet HTX (Sartorious).
401 All protein reagents were diluted in PBSF (PBS+0.1% BSA). Anti-human capture (ACH) sensor
402 tips were loaded for 180 seconds with test IgGs at 300 nM. Next, tips were transferred to wells for
403 180 seconds containing 300 nM soluble HA trimer. After antigen binding, tips were transferred to
404 wells containing test IgGs at 100 nM for 180 seconds to ensure complete blocking of the antigen
405 epitopes of these mAbs. In the last step, sensor tips were transferred to wells containing control
406 IgGs at 100 nM for 180 seconds. An observable binding response in the last step indicates non-
407 competition whereas no observable binding response indicates competition. Data were analyzed
408 using ForteBio Octet BLI analysis software version 12.2.13.5.
409
410 **Influenza HA plasmid library generation**
411 Influenza HA library cloning and viral generation was performed as previously described[33].
412 A deep mutational scanning library encoding all possible single amino acid mutations (Twist
413 Bioscience), including deletions, in the HA head (H3 numbering 52-263) of A/Sydney/5/2021 was
414 assembled using golden gate cloning (BsmBI-v2 Golden Gate Mix, NEB) with a barcoded segment

415 into pDM_RevGen068, a modified pHW2000 containing a GFP insert to detect background
416 colonies during library cloning. After assembly, the reaction was dialyzed prior to electroporation
417 into NEB 10-beta cells. After 1 hour of recovery, the cells were plated on large agar plates and
418 ~200,000 colonies were scraped, cultured in LB supplemented with carbenicillin for 2 hours before
419 isolating the plasmid library by maxiprep (Macherey-Nagel).
420
421 **PacBio sequencing**
422 To link the barcodes with individual amino acid variants, PacBio sequencing of SalI-
423 digested plasmid was performed by the Bauer core using a SMRTbell v3.0 kit and Pacbio Sequel
424 IIe sequencer. Computational methods to link barcodes with amino acid variants was performed
425 exactly as described in Maurer et al.[33], which is based on previous SARS-CoV-2 RBD deep
426 mutational scanning efforts[46,47].
427
428 **Deep viral escape assay**
429 In T150 flasks with hCK cells (50,000 cells per cm^2 seeded the day prior in flu media),
430 virus libraries were combined with antibody at 10x the calculated IC_{99} value in 28 mls of flu media
431 with 1 µg/ml TPCK-trypsin and incubated (1 hour / 37 °C / 5% CO_2). The media was then removed
432 from hCK cells and the virus/antibody mixture was added for 1 hour (37 °C / 5% CO_2) before
433 removing the inoculum, washing with PBS, and adding fresh flu media with 1 µg/ml TPCK-
434 trypsin. The next day (~20-24 hours), dead cells and cell debris were removed from the supernatant
435 by centrifugation and 20 mls of clarified supernatant was concentrated with a 100K MWCO
436 concentrator (ThermoFisher). The viral RNA was then extracted with a QIAamp viral RNA mini
437 prep (Qiagen).
438
439 **Viral barcode amplification**
440 Barcode amplification was performed as previously described[33]. One µl ezDNase buffer
441 and 1 µl ezDNase was added to 8 µl of viral RNA and incubated at 37 °C for 2 minutes. Then, 1
442 µl dNTPs (10 mM), 1 µl water, and a primer specific for the 3' NCR (prDM370) was added. The
443 mixture was incubated for 5 minutes at 65 °C followed by ice for 1 minute. To this mixture, 1 µl
444 SuperScript IV, 4 µl SuperScript IV buffer, 1 µl RNaseOUT, and 1 µl DTT were added and the
445 reaction was incubated at 50 °C for 30 minutes and then 80 °C for 10 minutes. The cDNA was

446 purified by a 1.6X AMPure XP bead cleanup (Beckman). The cDNA was amplified in two PCR
447 steps. The first PCR step combined 20 µl cDNA, 10 µl Q5 buffer, 1 µl dNTPs, 13.5 µl water, 2.5
448 µl each primer, and 0.5 µl Q5 polymerase. The cycling conditions for the first PCR step were 98
449 °C for 30 seconds; four cycles of 98 °C for 10s, 67 °C for 10s, 72 °C for 10s; 72 °C for 2 minutes.
450 The first PCR was purified by a 1.6X AMPure XP bead cleanup. The second PCR step combined
451 20 µl PCR1 product, 10 µl Q5 buffer, 1 µl dNTPs, 13.5 µl water, 5 µl NEBNext unique dual index
452 primers, and 0.5 µl Q5 polymerase. The cycling conditions for the second PCR step were 98 °C
453 for 30s; 14 cycles of 98 °C for 10s, 72 °C for 15s; 72 °C for 2 minutes. After purification with
454 AMPure XP beads, the product was analyzed by a D1000 tapestation, and combined at equimolar
455 concentrations before quantifying with Qubit and submitting for sequencing on a NovaSeq SP
456 (Bauer Core).
457
458 **Protein production**
459 Antibodies used in selections and recombinant HAs (head or full-length soluble
460 ectodomain) were cloned into a pVRC8400 (Vaccine Research Center, NIAID, Bethesda, MD)
461 backbone. All proteins contained a His-tag and were purified by cobalt chromatography followed
462 by FPLC purification on S200 column using an AKTA Pure. All proteins, except for the
463 A/Darwin/6/2021 HA trimer, were produced in HEK293F by transfecting with PEI (3:1 ratio) or
464 Expi-293F (ThermoFisher) cells following the manufacturer's recommended transfection
465 protocol. Full-length soluble ectodomain trimers, except for the A/Darwin/6/2021 HA trimer used
466 in cryo-EM, contained a foldon trimerization tag. The A/Darwin/6/2021 HA trimer used for cryo-
467 EM was produced in Expi293F GnTI- cells and contained three stabilizing mutations in the stem:
468 H26W, K51I, and E103I[48]. Proteins used for B cell sorting and yeast-based affinity maturation
469 were biotinylated by amine coupling and quantified using the QuantTag biotin kit (Vector
470 Laboratories, #BDK-200). Antibodies used for screening, as well as ADI-86325 used in deep
471 mutational scanning selections, were produced in yeast. Expression and purification of IgGs in
472 yeast were produced as full-length IgG1 proteins in S. cerevisiae cultures, as previously
473 described[49]. Briefly, yeast cultures were incubated in 24-well plates placed in Infors Multitron
474 shaking incubators at 30 °C, 650 rpm, and 80% relative humidity. After 6 days, the supernatants
475 containing the IgGs were harvested by centrifugation and purified by protein A-affinity

476 chromatography. The bound IgGs were eluted with 200 mM acetic acid with 50 mM NaCl (pH
477 3.5) into 1/8 [v/v] 2 M HEPES (pH 8.0) and buffer-exchanged into PBS (pH 7.0).
478
479 **Yeast-based affinity maturation**
480 Affinity maturation libraries were generated as described previously[50]. Overlap extension
481 PCR was used to incorporate NNK codons into the complementary determining regions (CDRs)
482 of the variable heavy- and light-chains. Variable heavy and light chain libraries were generated
483 separately and recombined in heavy chain or light chain libraries in yeast by homologous
484 recombination with a linearized plasmid to create yeast libraries of ~1 x 10^7 by electroporation. To
485 prepare S. cerevisiae yeast for electroporation, cells were first grown overnight in YPD media and
486 refreshed the morning of electroporation to 0.5 OD600 in YPD for 4 hours. Yeast cells were then
487 collected and washed twice with sterile water before incubating in 0.1 M lithium acetate / 10 mM
488 DTT for 10 minutes. The yeast cells were then washed twice with ice-cold water and resuspended
489 in ice-cold 10 mM lithium acetate. The insert and plasmid DNA were added to the yeast cells and
490 electroporated at 0.5 kV / 2000 µF. YPD media was used to recover the cells before incubating at
491 30 °C. The yeast cells were then resuspended in SDCAA media (6.7 g/L Yeast Nitrogen Base, 4.0
492 g/L drop out amino acid mix, 7.3 g/L NaH2PO4, 5.4 g/L Na2HPO4, 4% w/v dextrose) and grown
493 at 30 °C. The heavy- and light-chain libraries were selected over two rounds for binding to
494 recombinant A/Sydney/5/2021 HA head monomer. The number of yeast cells stained in each
495 selection was at least 10-fold the library diversity. Each library was incubated with either 10 or 1
496 nM of biotinylated SYD21 HA head monomer under equilibrium conditions. The yeast cells were
497 washed twice in PBSF (1x PBS, 0.1% [w/v] BSA) before staining with anti-human LC-FITC
498 (Southern Biotech), Streptavidin 633 (Invitrogen), and propidium iodide (Invitrogen) for 15
499 minutes on ice. The yeast cells were washed twice and resuspended in PBSF before sorting on a
500 BD FACS Aria II. Yeast cells showing improved antigen binding over parental clones were sorted
501 in each round. After two rounds of sorting, the outputs from the heavy- and light-chain libraries
502 were combined to generate a library of clone with diversity across all CDRs. This library was also
503 sorted for two rounds before plating the yeast on SDCAA agar plates for single clone isolation and
504 sequencing.
505
506 **HA conservation and mutational tolerance analyses**

507 For the pairwise identity maps shown in Figure 1, HA sequences of reporter viruses used
508 in this study were aligned and pairwise identity was calculated in Geneious. For conservation
509 analyses of H1 and H3 HAs we used a previous analysis[35] of 1222 unique H1 or 3408 unique H3
510 HA sequences from the GISAID database[51], removing duplicates or sequences with poor
511 sequencing data, performing clustering using CD-HIT EST[52], and selecting representative
512 sequences from these clusters. Cluster representatives were then analyzed for conservation using
513 ConSurf[36]. For mutational tolerance analyses, we used the entropy values from Lee et al.[28]. Color
514 spectrums were obtained from colorbrewer2.org. Antibody footprints were determined by
515 interfacing residues from PISA[53].
516
517 **Cryo-EM sample preparation and data collection**
518 The recombinant HA (rHA) full length soluble ectodomain (FLsE) of A/Darwin/6/2021
519 (H3N2) was incubated with ADI-85647 or ADI-85666 Fab at a molar ratio 1:1 and a final protein
520 concentration of 2.5 mg/ml for 40 minutes on ice. Immediately before depositing the sample onto
521 Quantifoil R0.6/1, 3400 mesh gold grids coated with 2nm continuous carbon, octyl-beta-glucoside
522 was added to the pre-formed complex at 0.083% (w/v) final concentration. Plasma cleaning of the
523 grids was performed with a Solarus plasma cleaner machine (Gatan) for 30 seconds (H2, O2).
524 Three microliters of the sample were applied on the continuous carbon face of the grids, blotted
525 for 5.5 seconds on the same face of the grid, at 20˚ C and 80% humidity, then plunge-frozen in
526 100% liquid ethane using a Leica EM GP2 plunge freezer. The grids were first screened on an
527 Artica Talos microscope equipped with a Gatan K3 camera and operated at 200 kV. The highest
528 quality grid was then imaged using a Titan Krios microscope operated at 300 kV and equipped
529 with a Gatan K3 Summit direct electron detector. A total of 7,772 and 4,435 movies for the ADI-
530 85647 or ADI-85666 complex, respectively, were collected in counting mode at 30 e-/px/s for a
531 total dose of 52.44 $e^{-}/Å^{2}/s$. Images were collected at a magnification of 81000, corresponding to a
532 calibrated pixel size of 0.825 Å/pixel. Defocus values ranged between -1 and -2.5 µm.
533
534 **Cryo-EM data processing**
535 The data were processed (**Supplementary Figs. 5 and 6**) using cryoSPARC v4.3.1[54].
536 Briefly, the movies were aligned and corrected for both global and local motion using Patch
537 Motion Correction within cryoSPARC. Then, contrast transfer function estimation was done using

538 Patch CTF and particles were picked using template picker. The picked particles were extracted
539 with a box size 400 pixels and 4x binned for 2D classification. Two rounds of 2D classification
540 were performed and the good particles were selected for the generation of the initial model at 12
541 Å/pixel with 3 classes. Non-uniform refinement was performed in C1 symmetry for each
542 individual class. The particles from the best class were re-extracted with a box size of 400 pixels
543 and binned 2x and then subject to 3D classification into 3 classes with a target resolution of 4Å.
544 The particles from the top class were combined and used for non-uniform refinement and local
545 CTF refinement with the best volume obtained from the 3D classification. The particle stack from
546 the local CTF refinement was then used for new round of non-uniform refinement, and then a new
547 round of 3D classification was performed with 3 classes at a target resolution of 3Å. The 3 classes
548 were subject to a new round of non-uniform refinement. Local CTF refinement was then performed
549 the final particle stack and then subjected to a new round of non-uniform refinement with C3
550 symmetry imposed. A new round of local CTF refinement followed by non-uniform refinement in
551 C3 symmetry was performed and then subject to global CTF refinement follow by new round of
552 non-uniform refinement in C3 symmetry. The particles were re-extracted at full box size of 400
553 pixels without binning and a new round of non-uniform refinement and local CTF refinement was
554 performed. This particle stack was used for a last non-uniform refinement with C3 symmetry and
555 yielded a reconstruction at 3Å nominal resolution. The two half-maps were used for sharpening in
556 DeepEMhancer[55]. The reported resolution is based on the gold-standard Fourier shell correlation
557 of 0.143 criterion.
558
559 **Model building and refinement**
560 The DeepEMhancer sharpened maps were used for model building with ModelAngelo[56]
561 and then manually built using COOT[57]. N-linked glycans were built manually in COOT using the
562 glyco extension and their stereochemistry and fit to the map validated with Privateer[58]. The models
563 were then refined in Phenix[59] using real-space refinement and validated with MolProbity[60].
564
565 **Computational sequencing analyses**
566 Computational sequencing analyses were performed exactly as described in Maurer et al.[33].
567 Briefly, barcodes and amino acid variants were linked from PacBio sequencing using *alignparse*[61].
568 Variants with insertions were removed from analysis. Sequences with ≥3 PacBio CCSs per

569 barcode were retained for analysis. After amplification of the barcodes, paired ends were merged
570 with PEAR[62], merged reads were filtered for the correct lengths, and sequences with ambiguous
571 base calls were removed. Unique barcode-UMI combinations were used for counting. The
572 functional scores were calculated using *dms_variants* (https://jbloomlab.github.io/dms_variants/)
573 comparing the change in variant frequency from the plasmid libraries to after the low MOI (0.01)
574 passage. Relative escape values are derived from differential selection
575 (https://jbloomlab.github.io/dms_tools2/diffsel.html) using a pseudocount of 10. For analysis, we
576 filtered for variants that were positively selected in both independent replicates and set other
577 variants to 0. Relative escape values are normalized to the most selected variant in each selection.
578 These relative escape values are summed by position to obtain a "summed relative escape" value.
579
580 **Data and code availability**
581 Raw sequencing data have been deposited to NCBI in BioProjects PRJNA1130551 and
582 PRJNA1056469. All code used for the analyses here and the resulting output files used to generate
583 the figures can be found at https://github.com/dpmaurer/flu_ab_escape. Atomic models have been
584 deposited to the PDB under accession codes 9BDF and 9BDG and the corresponding maps have
585 been deposited to the EMDB under accession codes EMD-44451 and EMD-44452. Aligned
586 micrographs for these datasets can be found in EMPIAR under accession codes EMPIAR-12059
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588
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AUTHOR INFORMATION

Author Contributions D.P.M. and M.V. produced recombinant proteins, performed ELISAs, and performed virus neutralization assays. D.P.M. performed deep mutational scanning assays and analyses. H.L.D., P.K., and J.C.G. sorted human B cells and performed *in vitro* affinity maturation. A.S.F.R. and G.B. determined cryo-EM structures. D.P.M. and A.G.S. wrote the original drafts of the paper. All authors reviewed and commented on the paper.

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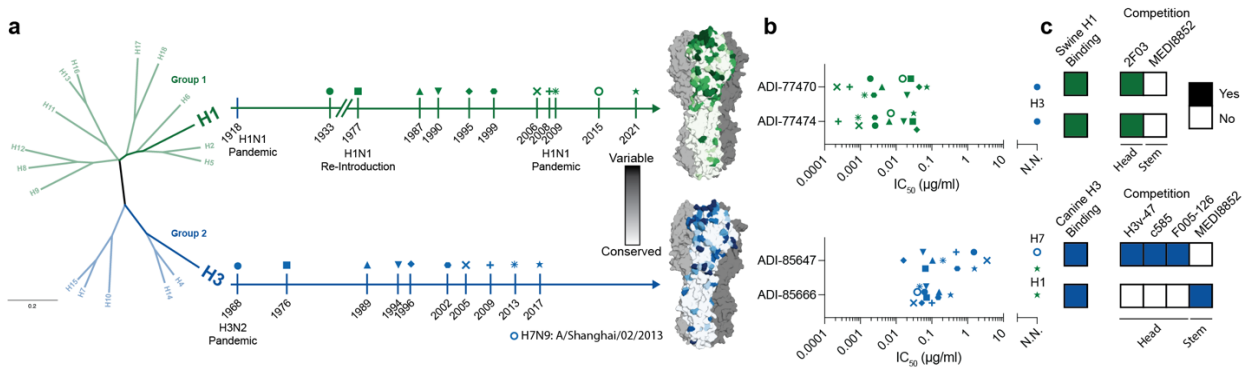


Fig. 1: Broad subtype neutralization by human antibodies targeting the HA head. (**A**) Diversity of influenza A HA. Phylogenetic tree of influenza A virus HAs (left). Timeline of influenza H1N1 and H3N2 circulation; symbols represent strains used in this study (middle). Pairwise identity of HAs from viruses used in this study mapped onto structures of HA (PDB: 3LZG and 4FNK) (right). (**B**) Neutralization potencies determined by microneutralization for down-selected antibodies against a panel of influenza A viruses. Strains are indicated as in **A**. H1N1 strains: A/WSN/1933, A/USSR/90/1977, A/Memphis/4/1987, A/Massachusetts/1/1990, A/Beijing/262/1995, A/New Caledonia/20/1999, A/Solomon Islands/3/2006, A/New York/08-1326/2008, A/California/07/2009, A/Michigan/45/2015, A/Sydney/5/2021. H3N2 strains: A/Aichi/2/1968, A/Bilthoven/1761/1976, A/Beijing/353/1989, A/Johannesburg/33/1994, A/Brisbane/8/1996, A/Fujian/411/2002, A/Wisconsin/67/2005, A/Perth/16/2009, A/Switzerland/9715293/2013, A/Kansas/14/2017. (**C**) ELISA binding reactivity at 10 or 1 µg/ml to A/Swine/Henan/SN13/2018[30] or A/Canine/Hainan/79/2019[31] (left) and BLI-based antibody competition with the indicated antibodies (right).

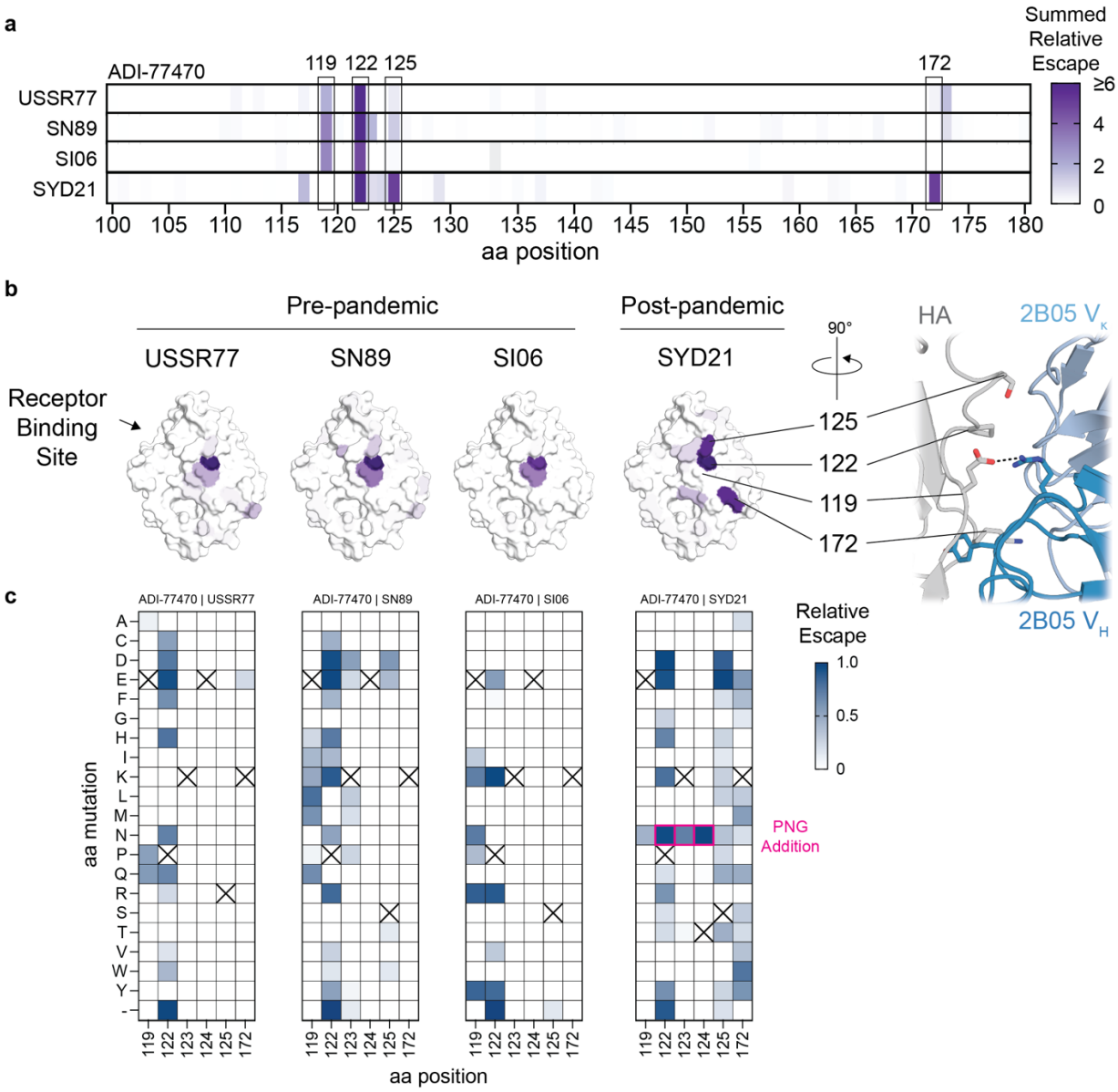
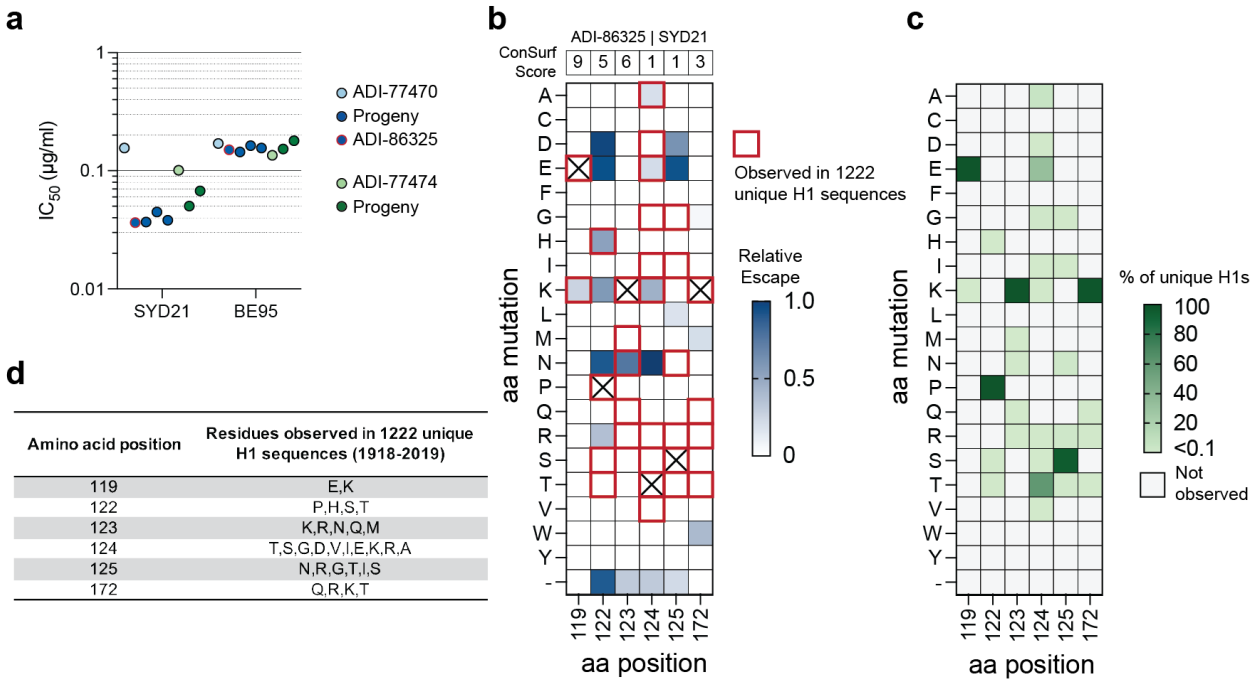


Fig. 2: Escape from a broad H1 neutralizing antibody in multiple H1N1 viruses. (**A**) Relative escape values summed by amino acid position in the HA head (H3 numbering) for ADI-77470 against USSR77 (A/USSR/90/1977), SN89 (A/Siena/10/1989), SI06 (A/Solomon Islands/3/2006), and SYD21 (A/Sydney/5/2021). (**B**) Summed relative escape values shown in **A** mapped onto the structure of the SI06 head (PDB: 4HKX) (left) with specific residues highlighted on the structure of the lateral patch-targeting antibody 045-09 2B05 bound to A/California/04/2009 (PDB: 7MEM) (right). (**C**) Relative escape values from ADI-77470 for individual amino acid mutations for each of the strains in **A** for a subset of positions. Highlighted asparagine mutations in SYD21 add potential N-linked glycosylation (PNG) sites.



d

Amino acid position	Residues observed in 1222 unique H1 sequences (1918-2019)
119	E,K
122	P,H,S,T
123	K,R,N,Q,M
124	T,S,G,D,V,I,E,K,R,A
125	N,R,G,T,I,S
172	Q,R,K,T

Fig. 3: Naturally occurring escape mutations from an engineered lateral patch antibody are rare. (**A**) Neutralization potencies for in vitro affinity-matured antibodies against SYD21 (A/Sydney/5/2021) and BE95 (A/Beijing/262/1995). (**B**) Relative escape values from ADI-86325 for individual amino acid mutations in SYD21 for the positions that can cause escape. ConSurf[36] values from a previous analysis[35] are shown at the top. Amino acids boxed in red indicate these were observed in the ConSurf analysis of 1222 H1 sequences that are representative of sequence clusters[35]. (**C**) Frequency of observed mutations in the same set of 1222 unique H1 sequences. (**D**) Table of observed residues at each position.

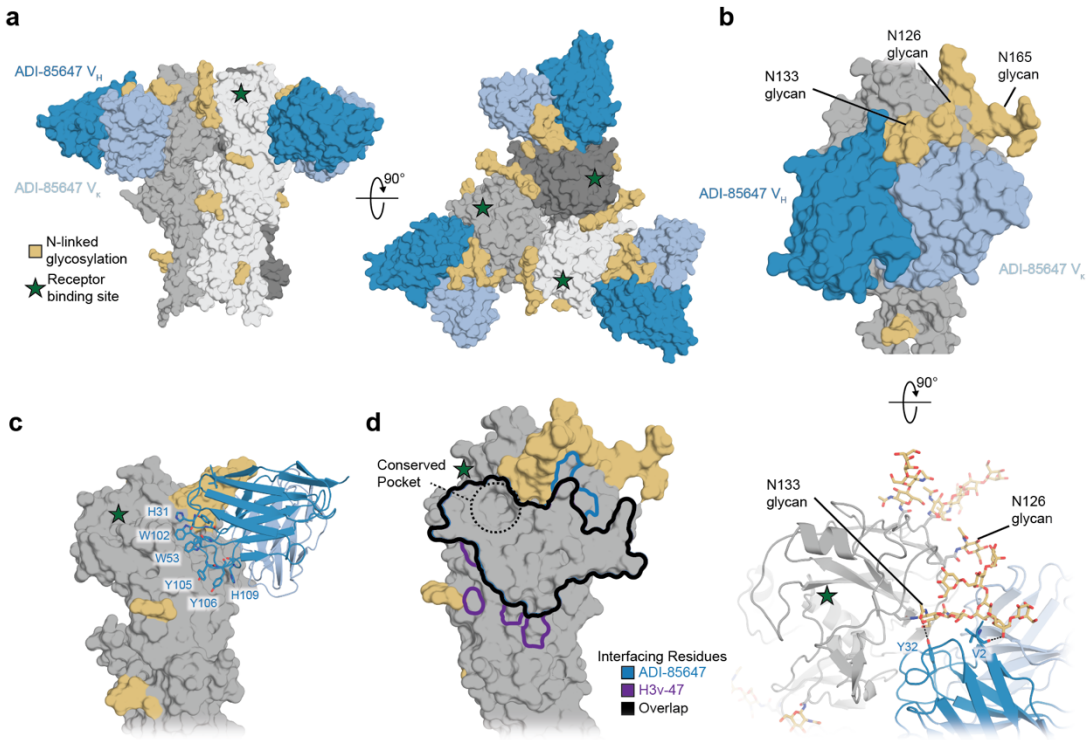


Fig. 4: A broad H3 antibody targeting the side of the HA head. (**A**) Cryo-EM structure of ADI-85647 bound to DAR21 (A/Darwin/6/2021) HA. (**B**) A glycan at N133 sits within a cleft formed between the V_H and V_κ of ADI-85647 (top). Hydrogen bonds formed between the N133 glycan and the V_H of ADI-85647 (bottom). (**C**) Several aromatic residues in the heavy chain interact with DAR21 HA. (**D**) The epitopes of ADI-85647 and H3v-47 are highlighted, showing the high degree of overlap between the two epitopes. PISA[53] was used to determine interfacing residues.

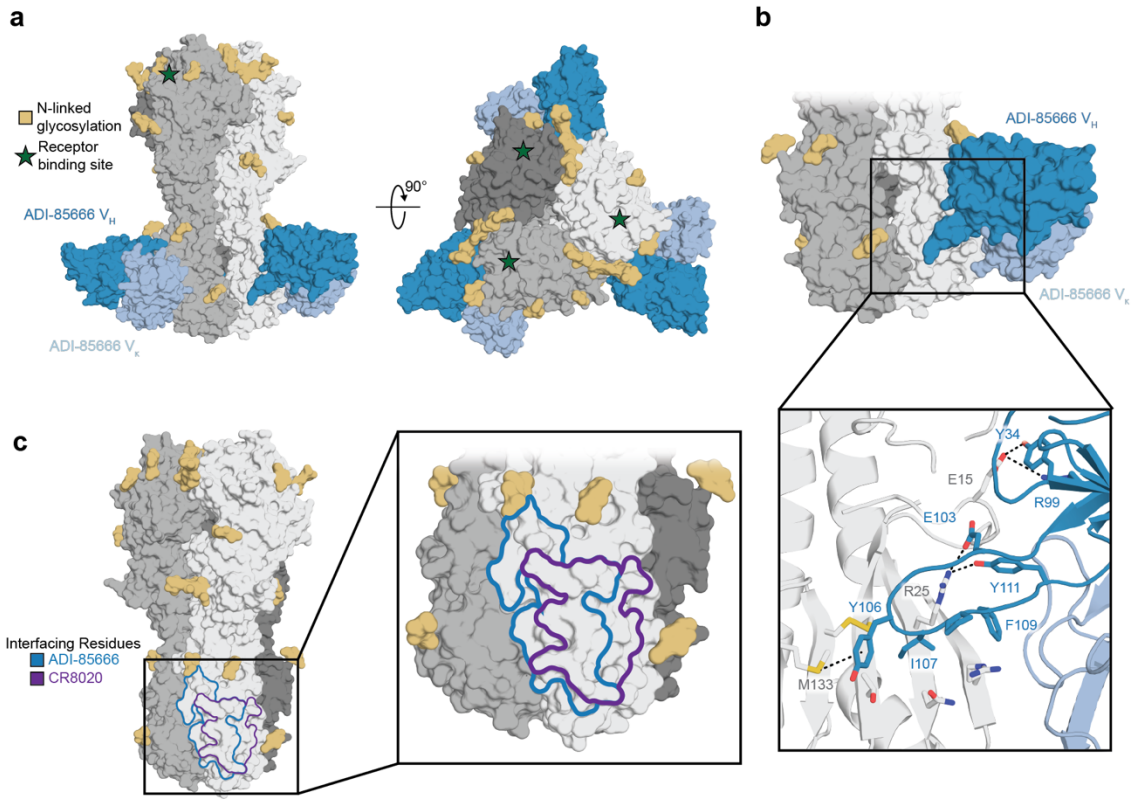


Fig. 5: A group 2 neutralizing antibody targets a unique epitope in the stem. (**A**) Cryo-EM structure of ADI-85666 bound to DAR21 (A/Darwin/6/2021) HA. (**B**) Close up of the interactions between the CDRH3 of ADI-85666 and DAR21 HA. (**C**) The epitopes of ADI-85666 and CR8020 are highlighted showing overlapping, but distinct epitopes. PISA[53] was used to determine interface residues.

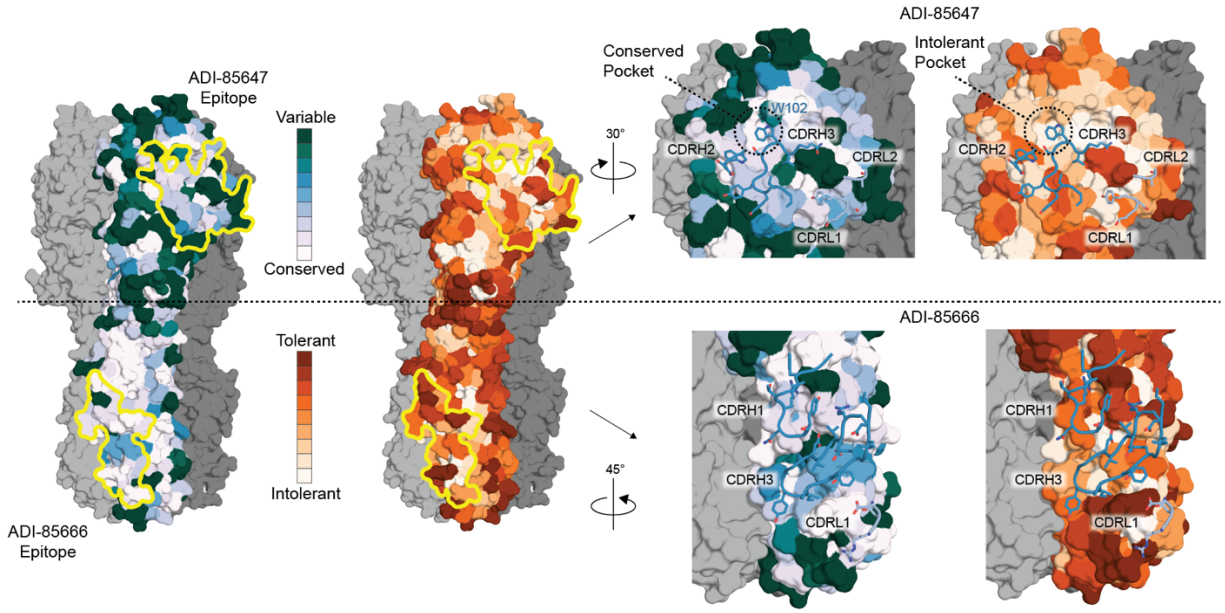


Fig. 6: Conservation of epitopes targeted by broad H3 antibodies. ConSurf[36] scores from 3408 unique H3 HA sequences and mutational tolerance values (0.3-2.7)[28] are mapped onto the cryo-EM structure of A/Darwin/6/2021 HA. Footprints of ADI-85647 and ADI-85666 are outlined (left). CDRs from ADI-85647 (top, right) and ADI-85666 (bottom, right) are shown in the context of the ConSurf scores and mutational tolerance values. Color spectrums were obtained from colorbrewer2.org.