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2 **A glycan gate controls opening of the SARS-CoV-2 spike protein**

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20

21 **Abstract**

22 SARS-CoV-2 infection is controlled by the opening of the spike protein receptor binding domain

23 (RBD), which transitions from a glycan-shielded “down” to an exposed “up” state in order to

24 bind the human ACE2 receptor and infect cells. While snapshots of the “up” and “down” states  
25 have been obtained by cryoEM and cryoET, details of the RBD opening transition evade  
26 experimental characterization. Here, over 130  $\mu$ s of weighted ensemble (WE) simulations of the  
27 fully glycosylated spike ectodomain allow us to characterize more than 300 continuous,  
28 kinetically unbiased RBD opening pathways. Together with ManifoldEM analysis of cryo-EM  
29 data and biolayer interferometry experiments, we reveal a gating role for the N-glycan at position  
30 N343, which facilitates RBD opening. Residues D405, R408, and D427 also participate. The  
31 atomic-level characterization of the glycosylated spike activation mechanism provided herein  
32 achieves a new high-water mark for ensemble pathway simulations and offers a foundation for  
33 understanding the fundamental mechanisms of SARS-CoV-2 viral entry and infection.

34

## 35 **Main Text**

### 36 **Introduction**

37 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped RNA virus and  
38 the causative agent of COVID-19, a disease that has caused significant morbidity and mortality  
39 worldwide.<sup>1,2</sup> The main infection machinery of the virus is the spike (S) protein, which sits on  
40 the outside of the virus, is the first point of contact that the virion makes with the host cell, and is  
41 a major viral antigen.<sup>3</sup> A significant number of cryoEM structures of the spike protein have been  
42 recently reported, collectively informing on structural states of the spike protein. The vast  
43 majority of resolved structures fall into either “down” or “up” states, as defined by the position  
44 of the receptor binding domain (RBD), which modulates interaction with the ACE2 receptor for  
45 cell entry.<sup>4,5,6</sup>

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47 The RBDs must transition from a “down” to an “up” state for the receptor binding motif to be  
48 accessible for ACE2 binding (**Fig. 1**), and therefore the activation mechanism is essential for cell  
49 entry. Mothes et al.<sup>7</sup> used single-molecule fluorescence (Förster) resonance energy transfer  
50 (smFRET) imaging to characterize spike dynamics in real-time. Their work showed that the  
51 spike dynamically visits four distinct conformational states, the population of which are  
52 modulated by the presence of the human ACE2 receptor and antibodies. However, smFRET, as  
53 well as conventional structural biology techniques, are unable to inform on the atomic-level  
54 mechanisms underpinning such dynamical transitions. Recently, all-atom molecular dynamics  
55 (MD) simulations of the spike protein with experimentally accurate glycosylation together with  
56 corroborating experiments indicated the extensive shielding by spike glycans, as well as a  
57 mechanical role for glycans at positions N165 and N234 in supporting the RBD in the “open”  
58 conformation.<sup>8</sup> Conventional MD simulations as performed in Casalino et al.<sup>8</sup> also revealed  
59 microsecond-timescale dynamics to better characterize the spike dynamics but were limited to  
60 sampling configurations that were similar in energy to the cryoEM structures. Several enhanced  
61 sampling MD simulations have been performed to study this pathway, however, these  
62 simulations lacked glycosylation for the spike protein<sup>9</sup> or involve the addition of an external  
63 force<sup>10</sup> or did not provide mechanistic detail.<sup>11</sup>

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66 In this study, we characterized the spike RBD opening pathway for the fully glycosylated SARS-  
67 CoV-2 spike protein, in order to gain a detailed understanding of the activation mechanism. We  
68 used the weighted ensemble (WE) path sampling strategy<sup>12,13</sup> (**Supplementary Fig. 1**) to enable  
69 the simulation of atomistic pathways for the spike opening process. As a path sampling strategy,  
70 WE focuses computing power on the functional transitions between stable states rather than the

71 stable states themselves.<sup>14</sup> This is achieved by running multiple trajectories in parallel and  
72 periodically replicating trajectories that have transitioned from previously visited to newly  
73 visited regions of configurational space,<sup>15</sup> thus minimizing the time spent waiting in the initial  
74 stable state for “lucky” transitions over the free energy barrier. Given that these transitions are  
75 much faster than the waiting times,<sup>16,17</sup> the WE strategy can be orders of magnitude more  
76 efficient than conventional MD simulations in generating pathways for rare events such as  
77 protein folding and protein binding.<sup>18,19</sup> This efficiency is even higher for slower processes,  
78 increasing *exponentially* with the effective free energy barrier.<sup>20</sup> Not only are dynamics carried  
79 out without any biasing force or modifications to the free energy landscape, but suitable  
80 assignment of statistical weights to trajectories provides an *unbiased* characterization of the  
81 system’s time-dependent ensemble properties.<sup>13</sup> The WE strategy therefore generates continuous  
82 pathways with unbiased dynamics, yielding the most direct, atomistic views for analyzing the  
83 mechanism of functional transitions, including elucidation of transient states that are too fleeting  
84 to be captured by laboratory experiments. Furthermore, while the strategy requires a progress  
85 coordinate toward the target state, the definition of this target state need not be fixed in advance  
86 when applied under equilibrium conditions,<sup>21</sup> enabling us to refine the definition of the target  
87 “open” state of the spike protein based on the probability distribution of protein conformations  
88 sampled by the simulation.

89

90 Our work characterizes a series of transition pathways of the spike opening, in agreement with  
91 conformations detected in cryoEM dataset by ManifoldEM,<sup>22</sup> and identifies key residues,  
92 including a glycan at position N343, that participate in the opening mechanism. Our simulation

93 findings are corroborated by biolayer interferometry experiments, which demonstrate a reduction  
94 in the ability of the spike to interact with ACE2 after mutation of these key residues.

95

## 96 **Results and Discussion**

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### 98 **Weighted ensemble simulations of spike opening**

99 As mentioned above, simulations of the spike opening process require an enhanced sampling  
100 strategy as the process occurs beyond the microsecond timescale (*i.e.* seconds timescale<sup>7</sup>). We  
101 therefore used the weighted ensemble (WE) path sampling strategy, which enabled the  
102 generation of continuous, atomistic pathways for the spike opening process with unbiased  
103 dynamics (**Fig. 2A-E, Supplementary Video 1**); these pathways were hundreds of ns long,  
104 excluding the waiting times in the initial “down” state. The protein model was based on the head  
105 region (residues 16 to 1140) of the glycosylated SARS-CoV-2 spike from Casalino et al.<sup>8</sup> (**Fig.**  
106 **1**), which in turn was built on the cryoEM structure of the three-RBD-down spike (PDB ID:  
107 6VXX<sup>5</sup>). The entire simulation system, including explicit water and salt ions, reaches almost  
108 half a million atoms. We focused sampling along a two-dimensional progress coordinate to track  
109 RBD opening: the difference in the center of mass of the spike core to the RBD and the root  
110 mean squared deviation (RMSD) of the RBD (**Fig. 2F-G**). On the SDSC Comet and TACC  
111 Longhorn supercomputers, 100 GPUs ran the WE simulations in parallel for over a month,  
112 generating over 130  $\mu$ s of glycosylated spike trajectories and more than 200 TB of trajectory  
113 data. We simulated a total of 310 independent pathways, including 204 pathways from the RBD-  
114 down conformation (PDB ID: 6VXX<sup>5</sup>) to the RBD-up conformation (PDB ID: 6VSB<sup>4</sup>) and 106  
115 pathways from the RBD-down to the RBD-open state, in which the RBD twists open beyond the

116 6VSB<sup>4</sup> cryoEM structure. Remarkably, the RBD-open state that we sampled includes  
117 conformations that align closely with the ACE2-bound spike cryoEM structure (PDB ID: 7A95<sup>6</sup>)  
118 even though this structure was not a target state of our progress coordinate (**Fig. 2F-G**,  
119 **Supplementary Video 1, Supplementary Figs. 2 and 3**). This result underscores the value of  
120 using (i) equilibrium WE simulations that do not require a fixed definition of the target state and  
121 (ii) a two-dimensional progress coordinate that allows the simulations to sample unexpected  
122 conformational space along multiple degrees of freedom. The ACE2-bound spike conformation  
123 has also been sampled by the Folding@home distributed computing project,<sup>11</sup> and RBD rotation  
124 has been detected in cryoEM experiments.<sup>6</sup>

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#### 126 **Comparison to spike conformations detected by ManifoldEM**

127 To validate our simulated RBD-down to RBD-up pathway, the ManifoldEM framework<sup>22</sup> was  
128 applied using the cryo-EM dataset of PDB 6VSB from McLellan and colleagues.<sup>4</sup> The  
129 ManifoldEM method allows characterization of conformational variations as obtained from a  
130 single-particle cryo-EM ensemble of a molecule in thermal equilibrium. Two conformational  
131 coordinates (CCs) (i.e., collective motion coordinates) were discovered from this dataset, and  
132 observed from several exemplary projection directions (PDs) showing a (1) RBD-down to RBD-  
133 up pathway and (2) RBD outward opening pathway (**Supplementary Fig. 4 Supplementary**  
134 **Videos 2 and 3**).

135

136 These projections were next aligned to corresponding 2D projections of Coulomb potential maps  
137 generated with frames from the WE simulation (**Supplementary Fig. 5, Supplementary Videos**  
138 **2 and 3**). Overall, there was very good agreement between the ManifoldEM conformational

139 coordinates and WE trajectory, aside from two discrepancies. First, the CC2 observed in the  
140 ManifoldEM included concerted opening of all three RBDs, while the WE focused sampling on  
141 the opening of a single RBD (**Supplementary Video 2**). Second, the WE trajectory ultimately  
142 opens to an RBD — core distance 11 Å greater than the most open conformation in the  
143 ManifoldEM. This is likely because the simulations sample the S1 subunit *en route* to the post-  
144 fusion conformation, whereas the experimental dataset does not.

145

### 146 **The N343 glycan gates RBD opening**

147 In the “down” state, the RBD of the SARS-CoV-2 spike is shielded by glycans at positions  
148 N165, N234, and N343.<sup>24</sup> While glycan shielding had been investigated for the RBD-down and  
149 RBD-up states,<sup>8</sup> our WE simulations allowed characterization of shielding *during* the opening  
150 process, revealing an abrupt decrease in glycan shielding when the RBD transitions from the  
151 “down” to the “up” state. The glycans at position N165 and N234 consistently shield the receptor  
152 binding motif, while shielding by the N343 glycan decreases with RBD opening.

153 (**Supplementary Fig. 6**). Beyond shielding, a structural role for glycans at positions N165 and  
154 N234 has been recently reported, stabilizing the RBD in the “up” conformation through a “load-  
155 and-lock” mechanism.<sup>8</sup>

156

157 Our WE simulations reveal an even more specific, critical role of a glycan in the opening  
158 mechanism of the spike: the N343 glycan acts as a “glycan gate” pushing the RBD from the  
159 “down” to the “up” conformation by intercalating between residues F490, Y489, F456, and R457  
160 of the ACE2 binding motif in a “hand-jive” motion (**Fig. 2A-E, Fig. 3, Supplementary Video**  
161 **4**). Therefore, the N343 glycan plays an active role in initiating the transition, distinct from the

162 stabilizing roles of glycans N165 and N234. This gating mechanism was initially visualized in  
163 several successful pathways of spike opening and then confirmed through analysis of all 310  
164 successful pathways in which the N343 glycan was found to form contacts (within 3.5 Å) with  
165 each of the aforementioned residues in every successful pathway (**Supplementary Fig. 7**). The  
166 same mechanistic behavior of the N343 glycan was observed in two fully independent WE  
167 simulations, suggesting the result is robust despite potentially incomplete sampling that can  
168 challenge WE and other enhanced sampling simulation methods.<sup>15</sup>

169  
170 To test the role of the N343 glycan as a key-gating residue, we performed biolayer  
171 interferometry (BLI) experiments. BLI experiments assess the binding level of the spike receptor  
172 binding motif (RBM, residues 438 to 508) to ACE2, acting as a proxy for the relative proportion  
173 of RBDs in the “up” position for each spike variant. No residues directly involved in the binding  
174 were mutated (i.e., at the RBM-ACE2 interface), to ensure controlled detection of the impact of  
175 RBD opening in response to mutations. Although previous results have shown reduced binding  
176 levels for N165A and N234A variants in the SARS-CoV-2 S-2P protein,<sup>8</sup> the N343A variant  
177 displayed an even greater decrease in ACE2 binding, reducing spike binding level by ~56 %  
178 (**Fig. 4, Supplementary Table 1**). As a negative control, the S383C/D985C variant,<sup>25</sup> which is  
179 expected to be locked by disulfides into the three-RBD-down conformation, showed no  
180 association with the ACE2 receptor. These results support the hypothesis that the RBD-up  
181 conformation is significantly affected by glycosylation at position N343.

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#### 184 **Atomic details of the opening mechanism**

185 The RBD-down state features a hydrogen bond between T415 of the RBD (chain A) and K986 of  
186 chain C, a salt bridge between R457 of RBD<sub>A</sub> and D364 of RBD<sub>B</sub>, and a salt bridge between



187 K462 of RBD<sub>A</sub> and D198 of NTD<sub>C</sub> (**Fig. 5A-C,E Supplementary Fig. 8**). The hydrogen bond  
188 T415<sub>A</sub> - K986<sub>C</sub> spends an average of 12% of the successful pathways to the “up” state before  
189 K986<sub>C</sub> makes a short lived (2% average duration to “up” state) salt bridge with RBD<sub>A</sub> D427.  
190 (**Fig. 5B,E Supplementary Fig. 8**) Next, K986<sub>C</sub> forms salt bridges with E990<sub>C</sub> and E748<sub>C</sub> as the  
191 RBD<sub>A</sub> continues to open. These contacts are formed in all 310 successful pathways  
192 (**Supplementary Fig. 8**). Mutation of K986 to proline has been used to stabilize the pre-fusion  
193 spike,<sup>23,26</sup> including in vaccine development,<sup>27</sup> and these simulations provide molecular context  
194 to an additional role of this residue in RBD opening.  
195  
196 Subsequently, at an average of 16% of the way through the successful pathways to the “up” state,  
197 the R457<sub>A</sub> - D364<sub>B</sub> salt bridge is broken, prompting the RBD<sub>A</sub> to twist upward, away from RBD<sub>B</sub>  
198 towards RBD<sub>C</sub> and forming a salt bridge between R408 of RBD<sub>A</sub> and D405 of RBD<sub>C</sub> (**Figs. 5C**  
199 **and 5E, Supplementary Fig. 8**). This salt bridge persists for 20% of the successful trajectories  
200 to the “up” state and is present in all 310 successful pathways.  
201  
202 A salt bridge between R466 of RBD<sub>A</sub> and E132 from NTD<sub>B</sub> is present in 189 out of 204  
203 successful pathways to the “up” state, and all 106 pathways to the “open” state. This contact is  
204 most prevalent during the transition between the “up” and “open” state. Finally, the salt bridge  
205 between D428 of RBD<sub>A</sub> and R454 of RBD<sub>C</sub> is present only in all 106 pathways from the “up” to  
206 the “open” state and is the last salt bridge between the RBD and the spike in the “open” state  
207 before the S1 subunit begins to peel off (**Fig. 5D and 5E, Supplementary Fig. 8**), at which point  
208 the last remaining contact to the RBD<sub>A</sub> is the glycan at position N165 of NTB<sub>B</sub>.  
209

210 Additional BLI experiments of the key identified spike residues R408A, D405A, and D427A  
211 corroborate the pathways observed in our simulations. Each of these reduces the binding  
212 interactions of the spike with ACE2 by ~13%, ~27%, and ~52%, respectively (**Fig. 4,**  
213 **Supplementary Table 1**). We also note that identified residues D198, N343, D364, D405, R408,  
214 T415, D427, D428, R454, R457, R466, E748, K986 and E990 are conserved between SARS-  
215 CoV and SARS-CoV-2 spikes, supporting their significance in coordinating the primary spike  
216 function of RBD opening. The emerging mutant SARS-CoV-2 strains B.1: D614G; B.1.1.7:  
217 H69-V70 deletion, Y144-Y145 deletions, N501Y, A570D, D614G, P681H, T716I, S982A,  
218 D1118H; B.1.351: L18F, D80A, D215G, R246I, K417N, E484K, N501Y, D614G, A701V; P1:  
219 L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, T1027I and  
220 CAL.20C: L452R, D614G<sup>28</sup> do not contain mutants in the residues we identified here to facilitate  
221 RBD opening. Analysis of neighboring residues and glycans to those mutated in the emerging  
222 strains along the opening pathway is detailed in **Supplementary Table 2**, and distances between  
223 each residue and glycan to RBD<sub>A</sub> is summarized in **Supplementary Video 5**.

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## 226 **Conclusions**

227 We report extensive weighted ensemble molecular dynamics simulations of the glycosylated  
228 SARS-CoV-2 spike head characterizing the transition from the “down” to “up” conformation of  
229 the RBD. Over 130 microseconds of simulation provide more than 300 independent RBD  
230 opening transition pathways. The simulated opening pathways align very well to conformations  
231 detected from cryoEM with the ManifoldEM method. Analysis of these pathways from  
232 independent WE simulations indicates a clear gating role for the glycan at N343, which lifts and  
233 stabilizes the RBD throughout the opening transition. We also characterize an “open” state of the

234 spike RBD, in which the N165 glycan of chain B is the last remaining contact with the RBD *en*  
235 *route* to further opening of S1. Biolayer interferometry experiments of residues identified as key  
236 in the opening transitions, including N343, D405, R408, and D427, broadly supported our  
237 computational findings. Notably, a 56% decrease in ACE2 binding of the N343A mutant,  
238 compared to a 40% decrease in N234A mutant, and 10% decrease in the N165A mutant reported  
239 previously<sup>8</sup> evidenced the key role of N343 in gating and assisting the RBD opening process,  
240 highlighting the importance of sampling functional transitions to fully understand mechanistic  
241 detail. None of the individual mutations fully abolished ACE2 binding, indicating the virus has  
242 evolved a mechanism involving multiple residues to coordinate spike opening. Our work  
243 indicates a critical gating role of the N343 glycan in spike opening and provides new insights to  
244 mechanisms of viral infection for this important pathogen.

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357

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375

### 376 **Author contributions**

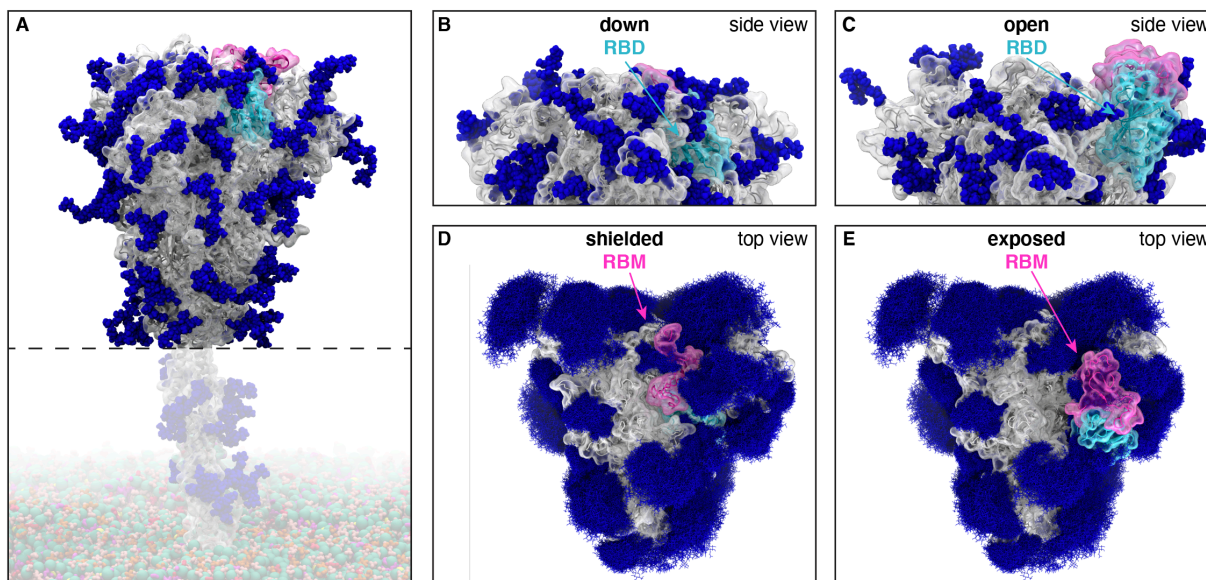
377 T.S. and S.A. contributed equally to this work. R.E.A. and L.T.C. oversaw the project. T.S. and  
378 L.C. prepared the simulation model. T.S. and S.A. performed WE simulations and A.B. provided  
379 WESTPA scripts. S.A., A.B., T.S., and L.T.C. carried out WE analysis. T.S., and F.L.K.  
380 performed simulation analyses. L.C., T.S., and F.L.K. created figures and movies. J.S.M.  
381 designed and oversaw biolayer interferometry experiments. R.S.M. and J.A.G. performed  
382 biolayer interferometry experiments and wrote the corresponding parts in the Results and  
383 Methods sections. J.F. and A.O. directed the ManifoldEM study, E.S., F.A., S.M. and G.M.  
384 performed the ManifoldEM study. E.S. and F.A. analyzed the results and compared them with  
385 WE simulations. E.S. and J.F. described the ManifoldEM methods and results. T.S., S.A.,  
386 L.T.C., and R.E.A. wrote the manuscript with contributions from all authors.

387

### 388 **Competing interests statement**

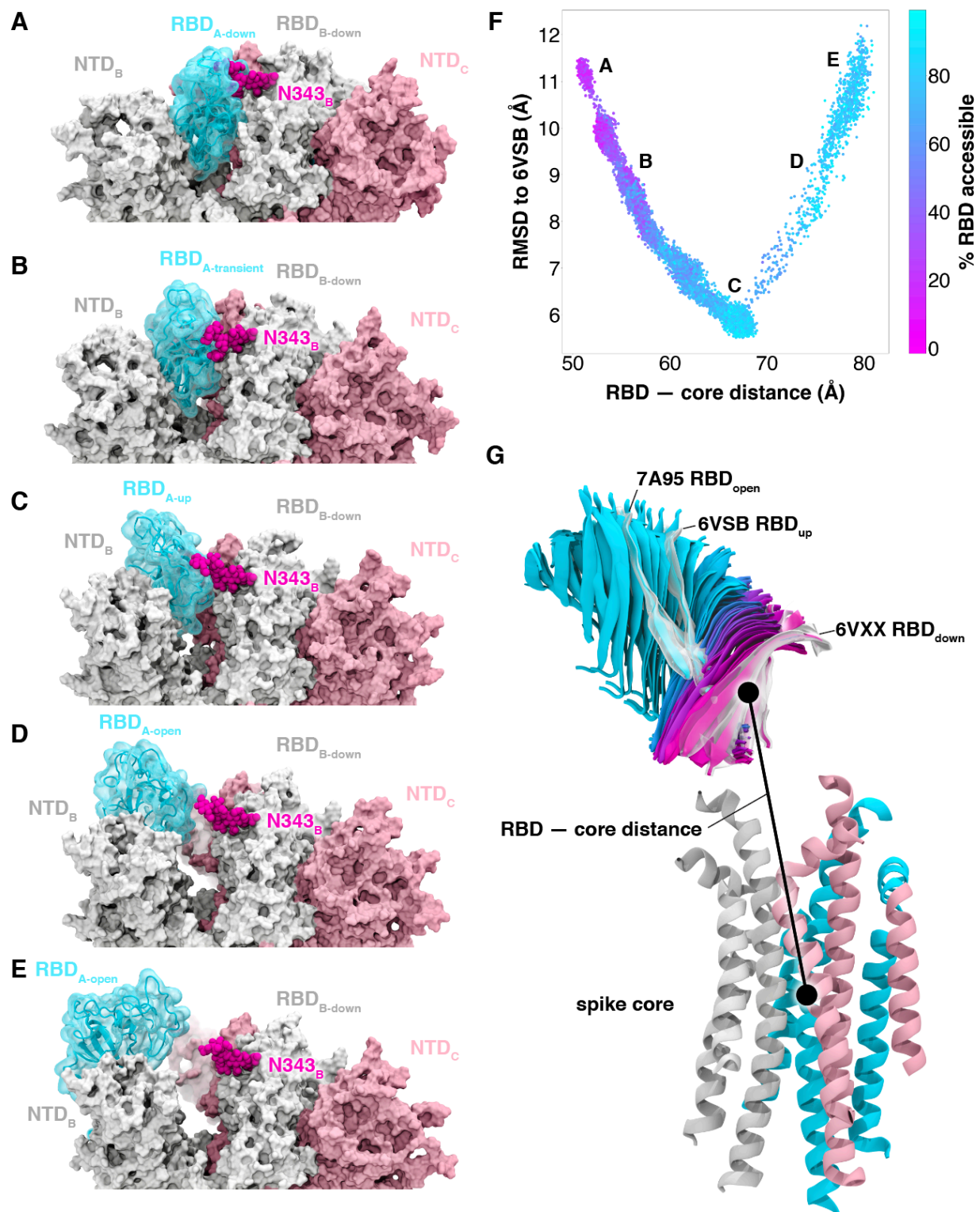
389 The authors declare no competing financial interests.

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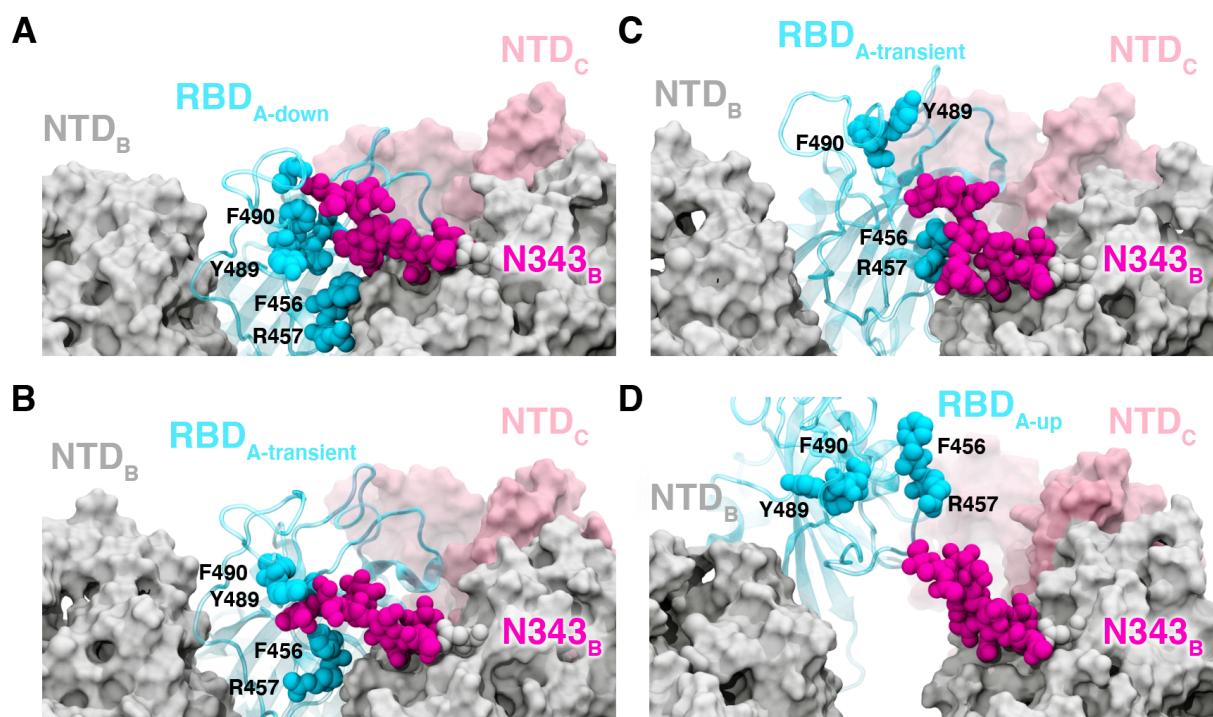
392 **Figure 1** Glycosylated spike RBD “down” and “open” conformations. (A) The SARS-CoV-2  
393 spike head (gray) with glycans (dark blue) as simulated, with the stalk domain and membrane  
394 (not simulated here, but shown in transparent for completeness). RBD shown in cyan, receptor  
395 binding motif (RBM) in pink. Side view of the “down” (shielded, B) and “open” (exposed, C)  
396 RBD. Top view of the closed (shielded, D) and “open” (exposed, E) RBM. Composite image of  
397 glycans (dark blue lines) shows many overlapping snapshots of the glycans over the microsecond  
398 simulations.



399

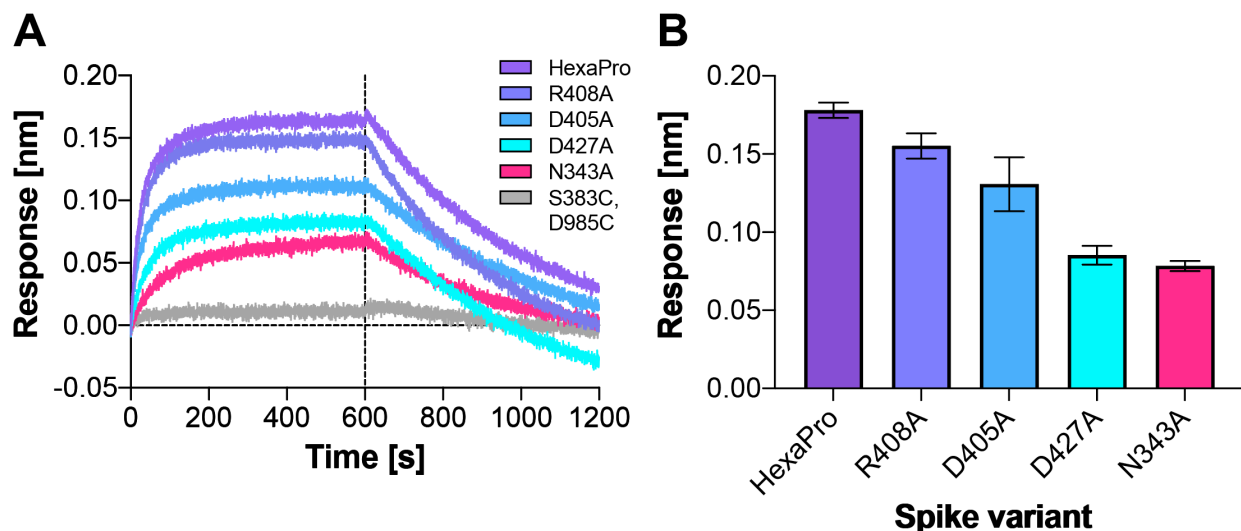
400 **Figure 2** Atomically detailed pathways of spike opening. (A-E) Snapshot configurations along  
 401 the opening pathway with chain A shown in cyan, chain B in gray, and chain C in pink and the

402 glycan at position N343 is shown in magenta. (F) Scatter plot of data from the 310 continuous  
403 pathways with the C $\alpha$  RMSD of the RBD from the 6VSB “up” state plotted against the RBD —  
404 core distance. Data points are colored based on % RBD solvent accessible surface area compared  
405 to the RBD “down” state 6VXX. Location of snapshots shown in A-E are labeled. (G) Primary  
406 regions of spike defined for tracking progress of the opening transition. The spike core is  
407 composed of three central helices per trimer, colored according to chain as in (A-E). The RBD  
408 contains a structured pair of antiparallel beta sheets and an overlay of snapshots from a  
409 continuous WE simulation are shown colored along a spectrum resembling the palette in (F).  
410 Overlaid cryoEM structures are highlighted and labeled including the initial RBD “down”  
411 state, 6VXX, the target RBD “up” state and the ACE2 bound “open” state, 7A95.

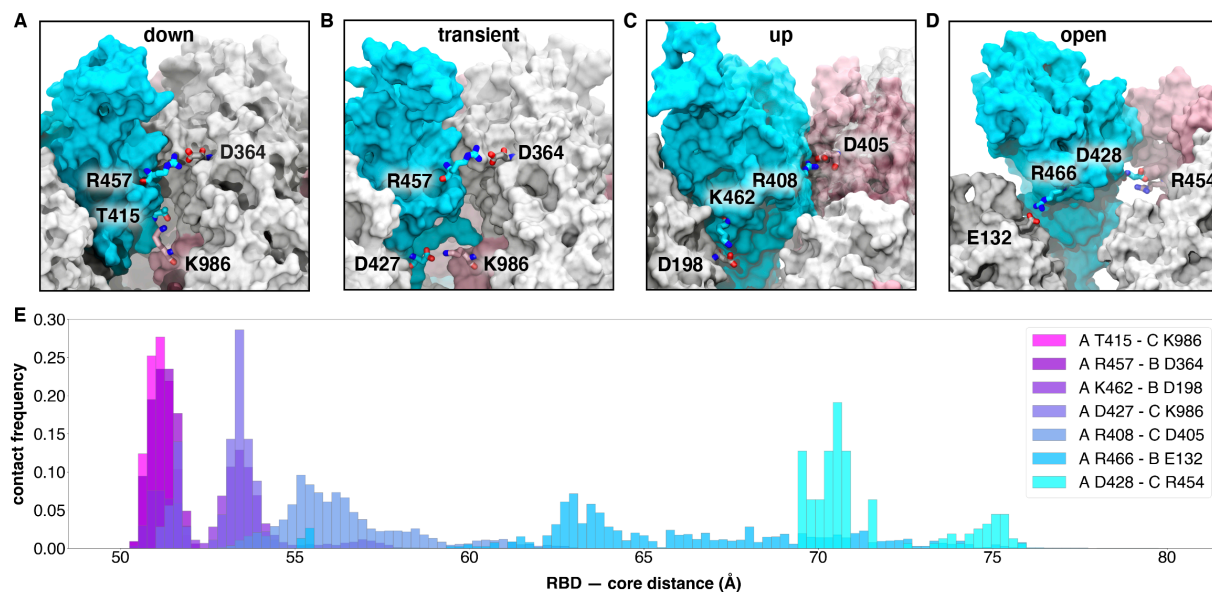


412  
413 **Figure 3** Glycan gating by N343. (A-D) Snapshot configurations along the opening pathway  
414 with chain A shown in cyan, chain B in gray, chain C in pink, and the glycan at position N343 is  
415 shown in magenta. (A) RBD A in the “down” conformation is shielded by the glycan at position  
416 N343 of the adjacent RBD B. (B-D) The N343 glycan intercalates between and underneath the  
417 residues F490, Y489, F456, F457 to push the RBD up and open (D).

418



419  
420 **Figure 4** ACE2 binding is reduced by mutation of N343 glycosylation site and key salt bridge  
421 residues. (A) Biolayer interferometry sensorgrams of HexaPro spike variants binding to ACE2.  
422 For clarity, only the traces from the first replicate are shown. (B) Graph of binding response for  
423 BLI data collected in triplicate with error bars representing the standard deviation from the mean.



424  
425 **Figure 5** Salt bridges and hydrogen bonds along the opening pathway. (A-D) salt bridge or  
426 hydrogen bond contacts made between RBD A, shown in blue, with RBD B, shown in gray, or  
427 RBD C shown in pink within the “down”, transient, “up”, and “open” conformations,  
428 respectively. (E) Histogram showing the frequency at which residues from (A-D) are within 3.5  
429 Å of each other relative to RBD — core distance. Frequencies are normalized to 1.  
430

431 **Data availability**

432 Data supporting the findings of this study are included in the Article and its Supplementary  
433 Information files. We endorse the community principles around open sharing of COVID19  
434 simulation data.<sup>29</sup> All simulation input files and data are available at the NSF MolSSI COVID19  
435 Molecular Structure and Therapeutics Hub at <https://covid.molssi.org> and the Amaro Lab  
436 website <http://amarolab.ucsd.edu>.

437

438 **Code availability**

439 This study utilized the standard builds of the simulation software WESTPA 2020.02  
440 (<https://github.com/westpa/westpa>) and AMBER 18 (<https://ambermd.org>) according to best  
441 practices for running WE simulations<sup>30</sup> with no special modifications.

442

443 **Supplementary information**

444 Sztain\_Ahn\_Supplementary\_Information.pdf

445 Supplementary\_Table2.xlsx

446 Supplementary\_Video1.mp4

447 Supplementary\_Video2.mp4

448 Supplementary\_Video3.mp4

449 Supplementary\_Video4.mp4

450 Supplementary\_Video5.mp4

451 **Source data**

452 Source\_data\_fig2.zip

453 Source\_data\_fig4.zip

454 Source\_data\_fig5.zip

455