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Accessible surface glycopeptide motifs on Spike glycoprotein of 2019-nCoV: implications on vaccination and antibody therapeutics

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Abstract

Corona viruses hijack human glycosylation enzymes to assembly sugar coat on Spike glycoproteins to evade antibody neutralization. The mechanism that human antibodies may uncover the antigenic viral peptide epitopes hidden by sugar coat are unknown. In this study, we analyzed the high-resolution Cryo-EM structure of Spike glycoproteins with the focus on calculating the accessible surface and studying the effect of glycosylation. The results showed that electron densities of glycans cover most of the SARS-CoV Spike protein RBD region except FSPDGKPCTPPALNCYWPLNDYGFYTTTGIGYQ. The glycosylated 2019-nCoV Spike protein by homology structure modeling showed a similar exposed sequence in RBD, YQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQ. Other surface-exposed domains included Central Helix, which is located between amino acids 967 and 1016 of SARS-CoV Spike protein, and amino acids 985 to 1034 of 2019-nCoV Spike protein. As the majority of antibody paratopes bind to peptide portion with or without sugar modification, we propose a snake-catcher model that a minimal length of peptide portion is first clamped by a paratope, and the binding is either strengthened by sugars close to peptide portion, or not interfered by sugar modification.

Key words: 2019-nCoV; corona virus; glycopeptide; N-linked glycans; antibody; cryo-EM structure; crystal structures; epitope prediction

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Introduction

Spike proteins are located on the surface of corona viruses and serve as entry proteins for infection (1). The Spike molecule forms trimers, which must be cleaved by cellular proteases so that the fusion peptide can facilitate the fusion of virus membrane with the infected cells. The proteases generate S1 and S2 subunits from Spike molecule, and the S1 subunit contains the critical receptor binding domain (RBD) to bind ACE2 of host cells. The receptor binding motif (RBM) of the receptor binding domain, rich in tyrosine, forms direct contacts with ACE2. The fusion of the virus with the host cells involves several other critical structures of the Spike protein, including Central Helix (CH) and heptad repeat 1 and 2 (HR1 and HR2) domains.

Spike glycoproteins are major targets for vaccine design and antibody-based therapies for corona viruses. Several antibodies targeting Spike proteins of SARS-CoV showed promising efficacy in preclinical trials (2-18). Besides the crucial RBD, structural studies suggest that other domains including fusion peptide, HR1 and Central Helix are also potential targets for antibody binding (19). In all corona viruses, Spike glycoproteins are densely glycosylated, with more than 20 predicted sites for N-glycosylation. The function of these glycans in immune escape of virus remain unknown.

In this study, we analyzed the cryo-EM structure of recombinant SARS-CoV Spike protein expressed by insect (Sf9) cells (19). We further used the homology-modeled structure of glycosylated 2019-CoV Spike protein, to identify surface-exposed epitopes for antibody recognition as well as vaccine design.

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Results

Predicted N-glycosylation sites for coronal viruses

A total of 22 N-glycosylation sites were found in Spike protein of 2019-nCoV. Among them 8 are located in N-terminal domain (NTD), 2 are located in receptor-binding domain (RBD), 3 are located in the rest of S1 subunit. 9 are located in the S2 subunit. The glycosylation pattern of Spike protein is highly conserved in SARS, MERS, and 2019-nCoV corona viruses. The NTD and HR2 domains are densely glycosylated. The fusion peptide (FP) domain is closely neighbored by a glycosylation site. In contrast, the receptor binding motif, the CH domain and the HR1 domain are free of glycosylation (Figure 1 and Supplemental Figure 1).

By Cryo-EM structure modeling (PDB: 5X58), 14 sites of N-glycosylation were observed. The GlcNAc (NAG) groups were identified at the reducing end of glycans, and the density map of extending glycan chains are still visible although the density is relatively weak (Figure 2A, B, and C). The RBD region of SARS-CoV Spike protein is covered by glycan density except FSPDGKPCTPPALNCYWPLNDYGFYTTTGIGYQ, which overlaps with an "Achilles heel" for antibody binding as pointed out by Berry et al (9).

The predicted glycosylation sites for Spike protein of 2019-nCoV include 22 N-glycosylation sites (displayed in yellow in Figure 2D). When trimer structures of S protein of SARS-CoV and 2019-nCoV are aligned (RMSD~1.32 for single chain), the structures are very similar except few loops, such as those at the N-terminal of NTD (Supplemental Figure 2). The predicted glycosylation sites are most conserved by sequence alignment and structure comparison. Fourteen of 22 sites are

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observed by Cryo-EM for SARS-CoV S protein, and most predicted sites of 2019-nCoV are located similarly to SARS-CoV (Figure 2E). The RBD domain are overall highly conserved with sequence identity (74.5%), structure (RMSD~1.14Å), and two identical glycosylation-sites near the N terminal (Figure 2F), while the sequence specificity of epitopes remains unique in some region (Tables 1&2). A similar surfaced exposed region, or "Achilles heel", YQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQ, was identified in RBD of 2019-nCoV. Interestingly, the "Achilles heel" for both SARS-CoV and 2019-nCoV is also free of glycosylation, while its neighbor fragments are covered or interacted by glycosylation. This region free of glycosylation is favorable for ACE2 and other protein binding (Figure 2G).

Accessible surface area (ASA) calculated according to electron density of glycans on Spike proteins of SARS-CoV and 2019-nCoV

The ASA profiling was used for mAb epitopes prediction (Supplemental Figure 3). Candidate epitopes were listed in Table 1 and Figure 3. In addition to RBD domains, multiple potential candidate epitopes from amino acid sequences at FP, HR1 and CH domains. Figure 4 shows the alignment of epitopes of Spike proteins of SARS-CoV and 2019-nCoV. Similar sites were found in RBD domains and CH domains of both viruses. However, unique sites were also found for each virus (Table 2 and Supplemental Figure 4). A unique epitope only existing in 2019-nCoV, but not in SARS-CoV, is the RARR (682-685) site for furin recognition (Supplemental Figure 5).

Discussion

Neutralizing antibodies toward Spike proteins are critical for protective immunity. Traggiai et al. reported Spike-specific monoclonal antibodies isolated from a patient who recovered from

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SARS-CoV infection, with in vitro neutralizing activity ranging from 10^{-8} M to 10^{-11} M (2). Several other groups reported monoclonal antibodies targeting Spike (3-15). Spike protein has also been the focus for vaccine development (20). High titers of IgG antibodies were reported to protect mice from SARS-CoV or MERS-CoV viral infection in mice vaccinated by DNA or subunit vaccines composed by Spike proteins (or RBD of Spike proteins) and adjuvants (21-29). TLR ligands, delta inulin, monophosphoryl lipid A were reported as effective adjuvants to be combined with subunit vaccines. To avoid the use of adjuvant, inactivated SARS-CoV viruses or recombinant adeno-associated virus encoding RBD of SARS-CoV spike protein have been studied, which induced potent protective antibody responses against infection (30-33). The safety and efficacy of antibody therapeutics and vaccines in human clinical trials remain to be studied, as well as the mechanism for specific vaccine component and formulation. For example, pulmonary pathology was reported when alum was used as adjuvant for Spike protein subunit vaccine (34). Antibody-induced lung injury was also reported in macaque model of SARS-CoV infection (35), which highlights the importance to avoid antibody-medicated inflammation.

RBD domain has been a main focus for antibody and vaccine studies. Three antibodies complexed with RBD of SARS-CoV has been co-crystalized, including 80R, m396, F26G19 (16-18). All three antibodies recognize non-continuous, conformational epitopes (Supplemental Table 1). Several mAb clones that recognize linear continuous peptide sequences have been reported (4D5, 17H9, F26G18, and 201), although co-crystal structures are not available yet.

In this study, we have identified the ASA profiling of RBD of 2019-nCoV, and found a vulnerable region, YQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQ. Previously, the

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structural counterpart of this region is termed as “the Achilles heel” of SARS-CoV (9). It is mostly overlapped with the interface between ACE2 and S protein (Figure 1G). For SARS-CoV, multiple mAbs targeting the “the Achilles heel” of SARS-CoV have been generated, including F26G18, 4D5, CR3006, m396, FM39, CR3014, F26G19 and 80R (Supplemental Table 1). Ongoing studies are being focused on the epitopes at “the Achilles heel” of 2019-nCoV for antibody and vaccine development.

In the past, it is well known that the predicted epitopes of protein antigens may be masked by glycosylation. Complex dataset and algorithm have been developed, which are based on training parameters related to interactions of glycans and surrounding amino acids, such as SEPPA 3.0 (36). However, no experimental data is available on the effect of glycosylation sites on epitope surface. With the recent breakthrough by high-resolution Cryo-EM, many glycoproteins can be solved and modeled with glycosylation sites. Here we directly exploit experiment data of SARS-CoV Spike protein from high resolution Cryo-EM, and screened epitopes for 2019-nCoV Spike protein by ASA profiling based on homology-modeled structure. By this approach, we have identified the “Achilles heel” of 2019-nCoV virus, as well as multiple other surface-exposed epitopes within and outside of RBD. For example, in NBD domain of SARS-CoV Spike protein, mAbs specific for linear epitopes have been reported (3, Supplemental Table 1). MAbs specific to other regions of S1 subunit and S2 subunits of SARS-CoV Spike protein were also reported (6). As summarized in Table 1, promising antibody binding sites within RBD and outside of RBD have been identified for 2019-nCoV, future studies will be focused on vaccination studies to validate their function as neutralizing epitopes with preventive and therapeutic effects in virus challenge experiments.

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Dense glycosylation of glycoproteins is a well-known strategy used by viruses to conceal surface peptide epitopes which elicit antibody responses, as exemplified by *Env* protein of HIV-1 virus. However, after decades of effort, monoclonal antibodies which bind to conformational epitopes on surface of the *Env* protein have been identified (36-38). Most of these antibodies bind to N-glycan portion neighboring the peptide epitopes, while some antibodies such as mAb 8ANC195 have evolved to recognize peptide epitope with no dependence on glycan binding (36). For antibodies specific to Spike glycoproteins, there is no data available whether their recognition is interfered by the glycosylation of Spike. We propose a "snake catcher" model that a minimum length of peptide portion, either linear continuous, or conformational, must first be first clamped by a paratope. This clamping effect may either be strengthened by sugars close to the peptide epitope, or not interfered by sugar modification. Clearly, the availability of surface-exposed glycopeptide motifs are critical for inducing antibody responses.

In summary, our study clearly identified list of linear surface exposed epitopes in Spike proteins of SARS-CoV and 2019-nCoV, and demonstrated the advantages to study glycosylation effect with real Cryo-EM data. These epitopes are critical for screening of monoclonal antibody therapeutics to treat 2019-nCoV viruses, as well as mechanistic studies on vaccine development.

Methods

Prediction of glycosylation sites

Spike proteins for 2019-nCoV (GenBank Accession Number: MN908947), SARS-CoV (AB263618), MERS (KM027290) were predicted by NetNGlyc.

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The sequence identity of the spike proteins between 2019-nCoV and SARS-CoV is as high as 84%, which is sufficient to build an accurate homolog model. The sequence of MN908947 was submitted and the structure model was built against all available homolog structures as templates by SWISS-MODEL (https://swissmodel.expasy.org). One stable conformation of trimer structure models for 2019-nCov is very close to Spike protein structure from SARS-CoV (PDB: 5X58), and their RMSD of single protein chain is about 1.32 Å after two structures are super-imposed and compared in PyMol (Figure 2D&E).

Calculation according to electron density of glycans on SARS-CoV Spike protein

Glycosylation sites were solved and determined from high-resolution Cryo-EM density map, while only N-Acetyl-D-glucosamine (NAG, GlcNAc) is determined to represent a whole glycan due to the glycan flexibility and disorder. The SARS spike protein structure (PDB:5X58), together with the NAG (GlcNAc) sites, were applied for molecular interface calculation with PISA (http://www.ccp4.ac.uk/pisa/). All the amino acids linking or interacting with NAG (GlcNAc) were selected and excluded in epitope prediction. Besides the interaction between NAG (GlcNAc at reducing end) and amino acids, the effects of larger structure of glycans extending from every NAG (GlcNAc) may also need to be considered, as shown as in Figure 2C, although their electron densities are weak.

Calculation according to homology-modeled structure of 2019-CoV protein

The same molecular interface calculation procedure described above was applied to calculate the ASA and screen the corresponding antigen epitopes, except the glycosylation effect could not be measured due to structure unavailable so far. As most glycosylation sites are conserved due to

high similarity of these two spike proteins, we could predict the glycosylation site effects in 2019-nCoV spike structure as well. When predicted epitopes collide with the amino acid residues interacting with NAG (GlcNAc), they were removed from the candidates by cross-reference of the SARS-CoV data.

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Conflict of interest disclosures

The authors declare no conflict of interest.

Author contributions

Dapeng Zhou and Wen Zhang designed this study. Dapeng Zhou, Ruibing Qi and Wen Zhang contributed to the collection, analysis and interpretation of data. Dapeng Zhou and Wen Zhang wrote the manuscript. All authors read and approved the final manuscript.

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Figure legends:

Figure 1. N-glycosylation sites of 2019-CoV. NTD, N-terminal domain; RBD, receptor binding domain; FP, fusion peptide; HR1, helix region 1; CH, central helix; HR2, helix region 2.

Figure 2. The spike structures of SARS and 2019-nCoV

A. The SARS-CoV spike protein structure (green, PDB:5X58) and its density map (yellow) with glycosylation (pink) from the solvent side view;

B. Bottom view with surface area of RBD (the "Achilles Heel", AH, blue) exposed in solvent;

C. The typical NAG and its density map, indicated with arrows, extending to outside solvent or neighbor amino acids;

D. The 2019-nCoV spike protein structure (cyan) with glycosylation amino acids (yellow) and RBD highlighted;

E. Structure comparison between The SARS-CoV (middle) and 2019-nCoV protein;

F. The comparison of RBD domains (dash line circled on 2019-nCoV S protein) between SARS-CoV S protein (RBD: Orange) and 2019-nCoV protein(RBD: deep blue) with AH surface map (blue); notes: the glycosylation sites from SARS-CoV and 2019-nCov S protein are surrounding the RBD domain;

G. AH fragment (sphere) of RBD domain (orange) in closeup view (dash line circled part); The interface (blue) between SARS -CoV S protein (wheat) and ACE2 (yellow) from the complex structure (PDB:6ACJ);notes: the interface is exactly located on the AH fragment of the complex structure (4.2 angstroms Cryo-EM structure).

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Figure 3. Surface-exposed amino acid sequences predicted by ASA profiling and glycosylation effect with Cryo-EM structure. Furin site (Red pentagram), N-glycosylation sites (*); epitopes for SARS-CoV (green) and 2019-nCoV (cyan).

Figure 4. Alignment of epitopes on the spike protein structure of SRAS and 2019-nCoV.

A. The comparison of the protein chain A between SARS-CoV trimer (in green, chain A specifically in sky blue) and 2019-nCov trimer (cyan), with glycosylation sites (pink at chain A, light pink from other Chains) and their interacting amino acids (yellow) for Chain A of SARS-CoV;

B. Four epitope pairs S1/n1, S2/n2, S3/n3, and S4/n4 compared between SARS-CoV (epitopes in red) and 2019-nCov S protein (epitopes in grey or light blue for site n3), and 2019-nCov S protein cartoon shown individually on right panel; the conserved fragments at FP (red), HR1 (yellow) and CH (orange) shown by small cartoon of SARS-CoV trimer (grey) in the middle. The epitopes pairs are listed in the Table 2.

C. Bottom solvent view of the RBD domain located at one side of trimer structure bottom;

D. Comparison of epitopes in RBD domains from SARS-CoV (epitopes in red) and 2019-nCov trimer (epitopes in light blue, RBD cartoon in cyan), together shown with AH (dark blue for whole AH, partially overlapping with AH/ah for epitopes predicted), glycosylation sites (pink) and their interacting amino acids (yellow).

E. The epitopes pairs I/i~IV/iv, AH/ah and g1/g2 are compared and listed in the Table 2.

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Table 1. Surface exposed amino acid sequences of SARS-CoV and 2019-nCoV

Sites		Epitope details	Nearby N-glycosite	Mab clone	Ref
		2019-nCoV			
L18-29	18 LTTRTQLPPAYT 29		17NLT		
G72-75	72 GTNG 75		74NGT		
L110-13	110 LDSK 113		122NAT		
Y144-48	144 YYHKN 148		149NKS		
W152-58	152 WMESEFR 158		149NKS		
A163-66	163 ANNC 166		165NCT		
E169-77	169 EYVSQPFLM 177				
G181-84	181 GKQG 184				
K206-15	206 KHTPINLVRD 215				
R246-56	246 RSYLTPGDSSS 256		234NIT		
L270-74	270 LQPRT 274		282NGT		
L303-06	303 LKSF 306				
P330-36	330 PNITNLC 336	RBD	331NIT		
A344-47	344 ATRF 347	RBD	343NAT		
P384-87	384 PTKL 387	RBD			
G413-16	413 GQTG 416	RBD			
S443-51	443 SKVG 446,448 NYNY 451	RBD		4D5	8
L455-463	455 LFRKSNLKP 463	RBD			
G476-490	476 GSTPC 480,482 GVEGFNCYF 490	RBD			
Q498-506	498 QPTNGVGYQ 506	RBD		201	3
L518-21	518 LHAP 521	RBD			
P527-33	527 PKKSTNL 533				
S555-62	555 SNKKFLPF 562				
Q580-83	580 QTLE 583				
N603-07	603 NTSNQ 607		603NTS,616NCT		
W633-36	633 WRVY 636		657NNS		
E654-62	654 EHVNNSYEC 662				
Y674-87	674 YQTQTNSPRRARSV 687				
Y707-71	707 YSNN 710		709NNS		
S746-51	746 STECSN 751				
D808-14	808 DPSKPSK 814		801NFS	5H10	6
T827-83	827 TLAD 830				
I834-54	834 IKQYG 838,840 CLGDIAARDLICAQK 854	CR			
T866-69	866 TDEM 869	CR			
Q920-23	920 QKLI 923	HR1			
D936-44	936 DSLSSTASA 944	HR1			
K986-91	986 KVEAEV 991	CH			
A1070-76	1070 AQEKNFT 1076		1074NFT		
T1100-03	1100 THWF 1103		1098NGT		
Q1113-18	1113 QIITTD 1118				
C1126-29	1126 CDVV 1129		1134NNT		
V1133-37	1133 VNNTV 1137		1134NNT		
		SARS-CoV			
R18-31	18 RCTTFDDVQAPNYT 31		29NYT		
K142-15	142 KPMG 145,146 QTHT 150		158NCT	68	3
S165-17	165 SDAFSL 170		158NCT		
E174-77	174 EKSG 177				
V205-08	205 VVRD 208				
L257-26	257 LKPT 260		269NGT		
I319-23	319 ITNLC 323	RBD	318NIT		
A331-34	331 ATKF 334	RBD	330NAT		
R342-47	342 RKKISN 347	RBD	357NST		
T425-28	425 TRNI 428	RBD			
P462-76	462 PDGKPCTPPALNCYW 476	RBD		17H9, F26G18,80R	8,18
Y484-92	484 YTTTGIGYQ 492	RBD		F26G19, m396, 80R,201	3,16,17,18
P513-22	513 PKLSTDLIKN 522				
N589-94	589 NASSEV 594		589NAS		
I610-14	610 IHADQ 614		602NCT	F26G8	9
Y622-27	622 YSTGNN 627				
E640-48	640 EHVDTSYEC 648				
H661-73	**661 HT 662,672 KS 673**				
P789-97	789 PDPLKPTKR 797		783NFS	5H10	6
Q917-26	917 QESLTTTSTA 926	HR1			
N935-39	935 NQNAQ 939	HR1			
K968-73	968 KVEAEV 973	CH			
C1064-69	1064 CHEGKA 1069		1056NFT		
G1081-84	1081 GTSW 1084		1080NGT		
Q1095-00	1095 QIITTD 1100				

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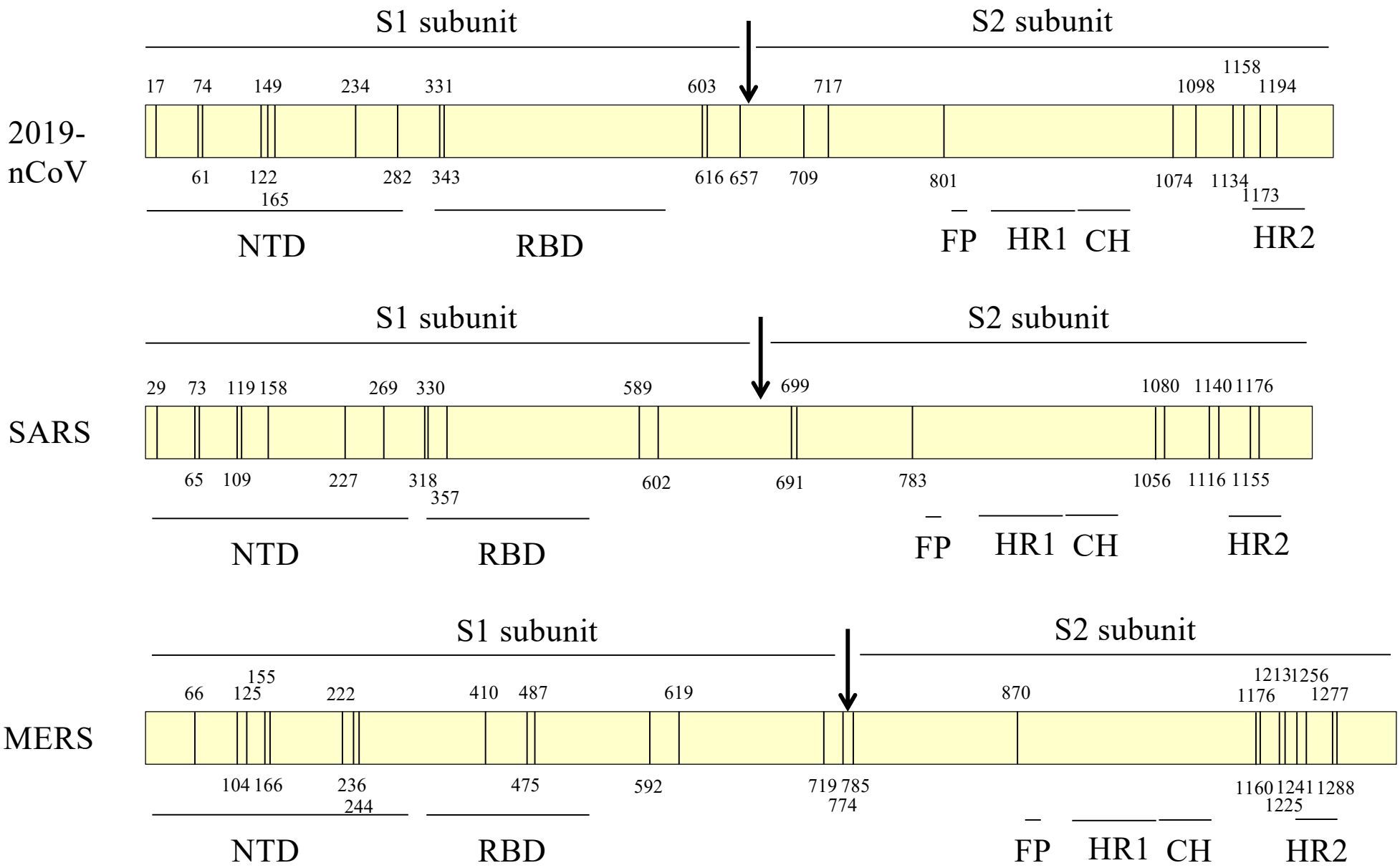
Zhou et al., Figure 1



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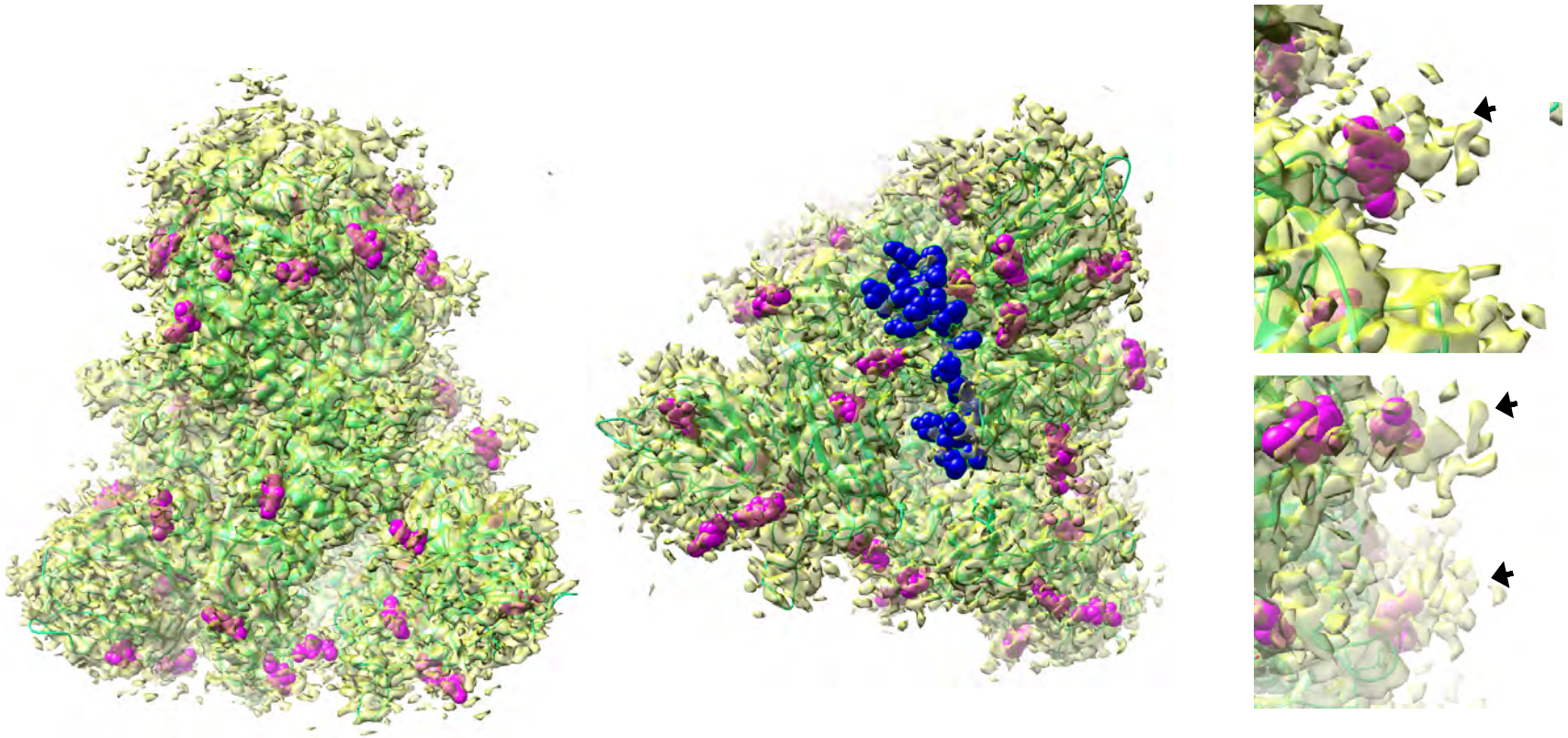
Zhou et al., Figure 2

A|B|C



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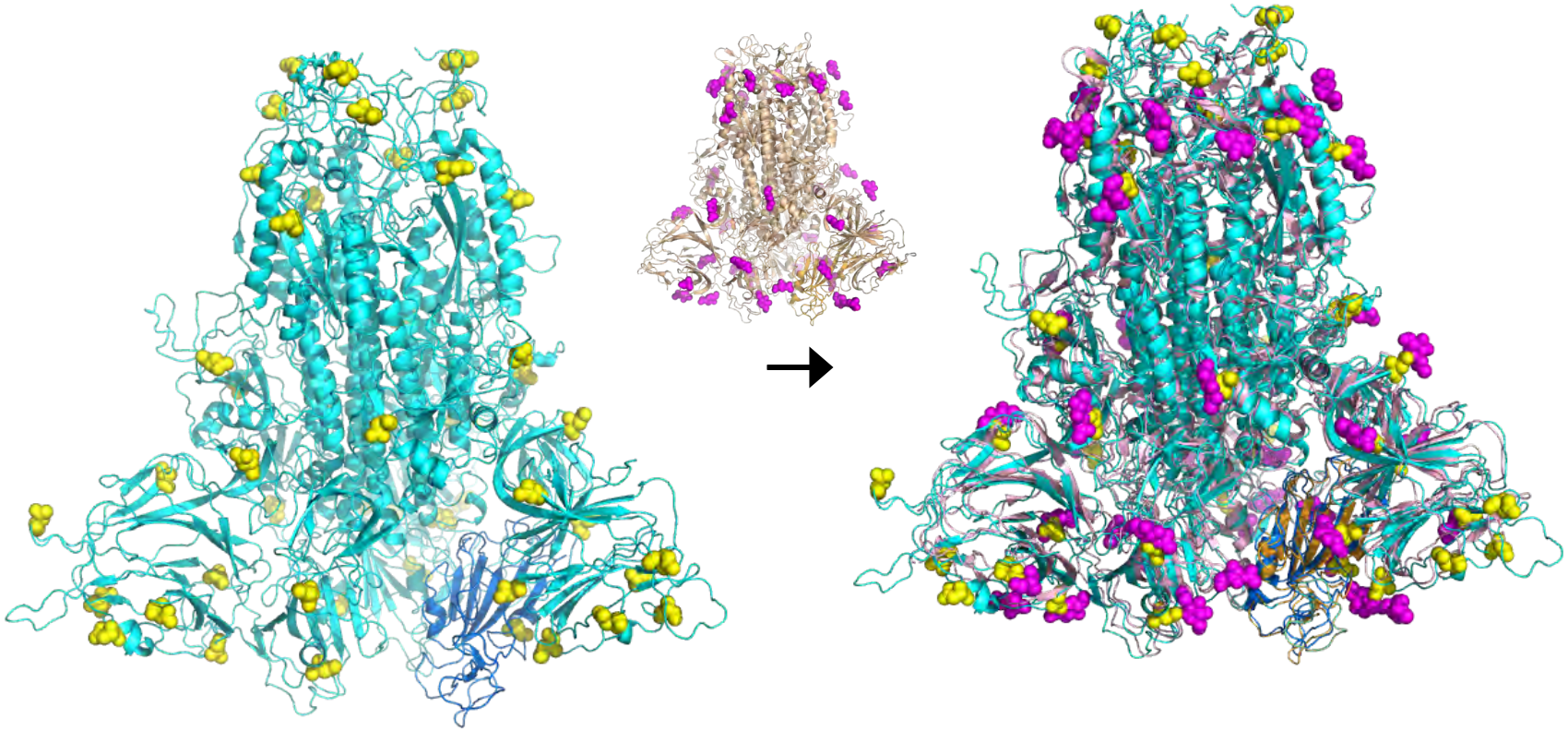
Zhou et al., Figure 2

D | E



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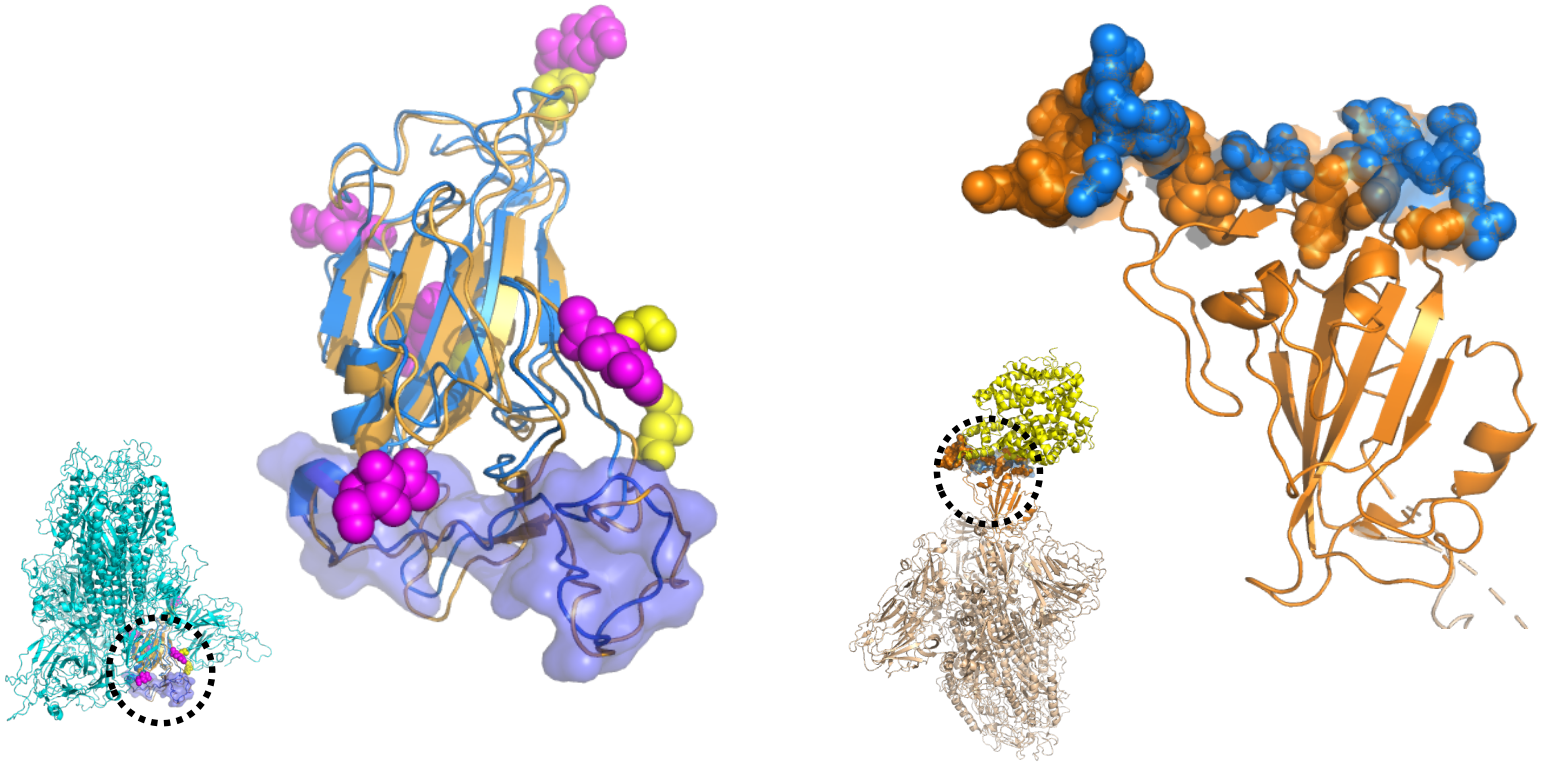
Zhou et al., Figure 2

F | G



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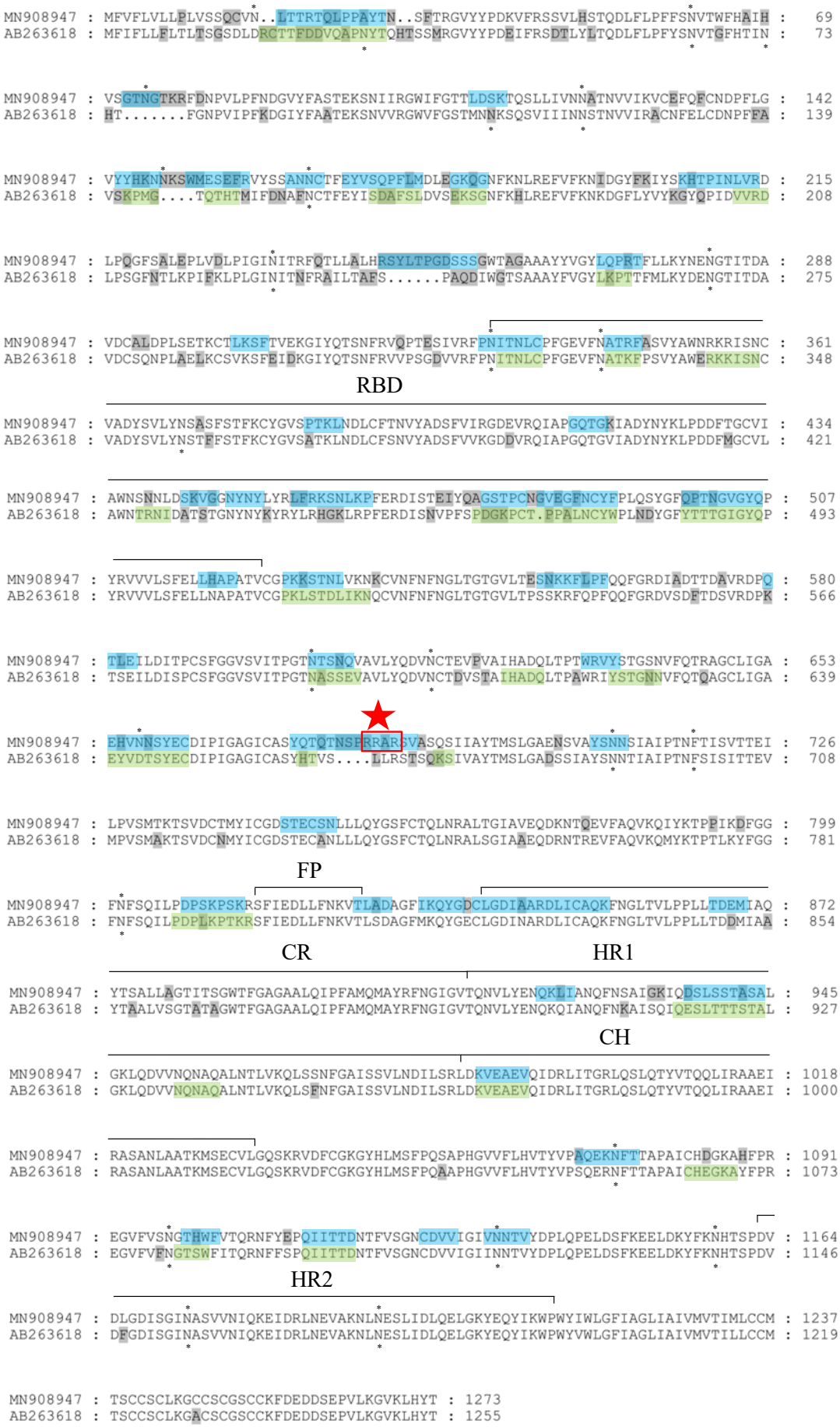

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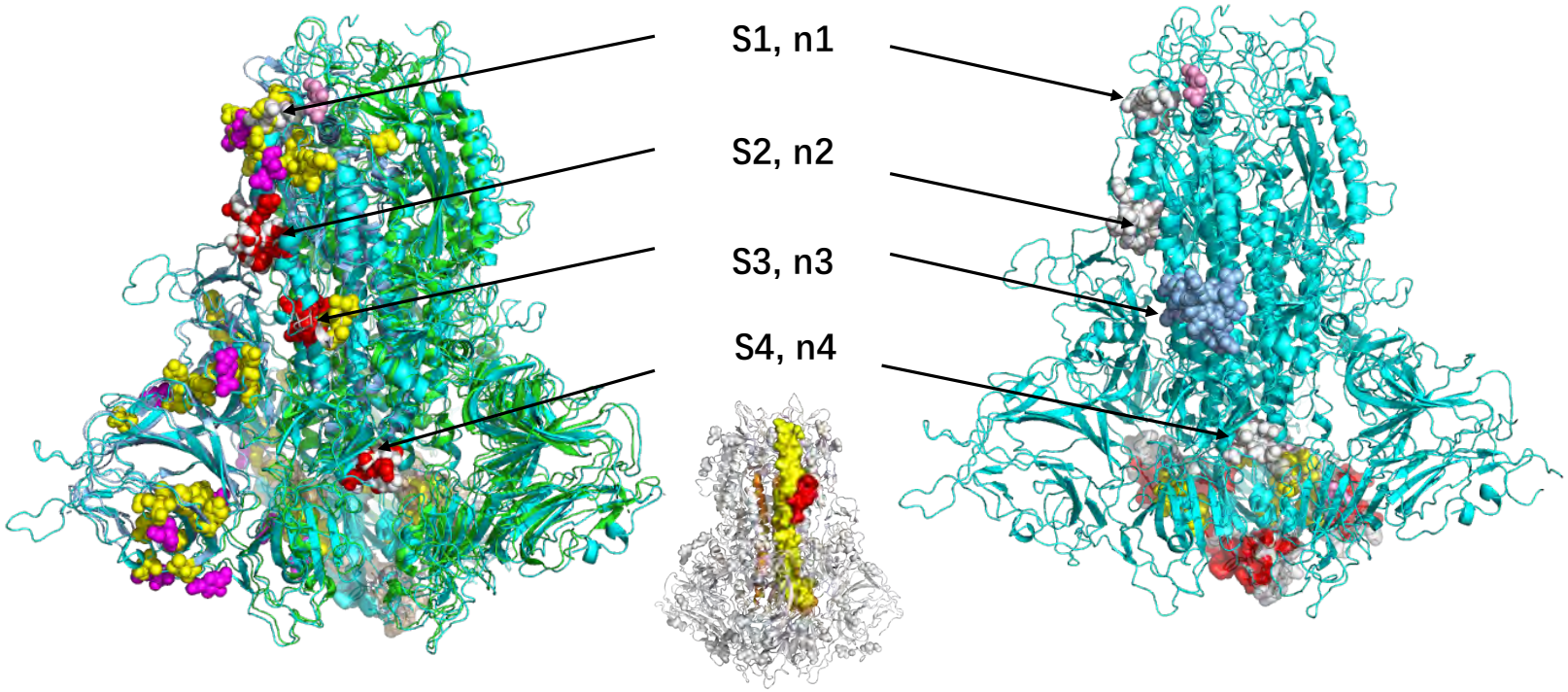
Zhou et al., Figure 4

A|B



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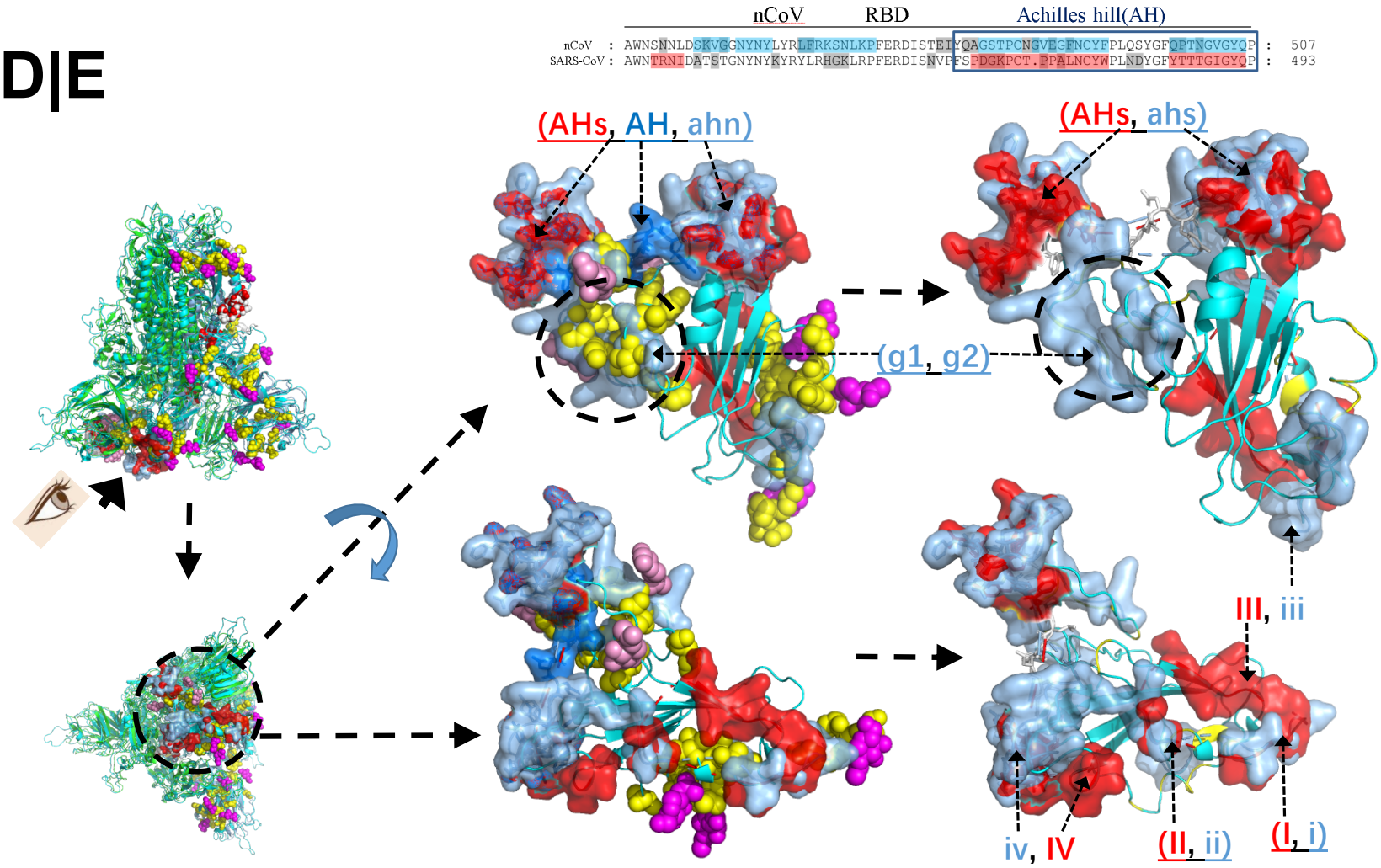
Zhou et al., Figure 4

C|D|E



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Supplemental Online Materials

Supplemental Table 1: List of monoclonal antibodies for Spike protein of SARS-CoV

Supplemental Figure 1: Scheme of Spike proteins of 2019-nCoV, SARS-CoV and MERS-CoV.

Supplemental Figure 2: Structure-based alignment of 2019-nCoV and SARS-CoV Spike proteins. The sequences are directly extracted from PDB 5X58 and 2019-nCoV homology model, and the sequence alignment was based on above two structures by ENDscript and ESPRIPT with default settings (http://espript.ibcp.fr/ESPript/ENDscript/index.php).

Supplemental Figure 3: Accessible surface area profiling of Spike proteins of 2019-nCoV and SARS-CoV. A) The epitopes predicted on the S protein structure for SARS-CoV, Epi (yellow) denotes the epitopes screened by simple ASA profiling (the same for nCoV), and EpiS (red) denotes the epitopes were calculated by excluding the glycosylation sites and the glyco-interacting amino acids; B) The epitopes predicted for nCoV. The values of Y axis means nm^2 of ASA.

Supplemental Figure 4: Connecting region (CR) of 2019-nCoV and SARS-CoV Spike proteins.

Supplemental Figure 5: Furin recognition site of 2019-nCoV Spike protein.

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Supplemental Table 1: List of monoclonal antibodies for Spike protein of SARS-CoV

Antibody binding site	Nearby N-glycosylation	Clone	Ref
N-terminal domain (NBD)			
130 FELCDNPFFAVSKPMGTQTHT 150	158NCT	68	3
236 TAFSPAQDIWGTSAAAYF 253	227NIT	114E3,115H2,116F8,112F9,120D9	14
Receptor binding domain (RBD)			
W423,N424		S-9-11,N-176-15	7
435 NYNYKYRYLRHGKLRPF 451		4D5	8
442 YLRHGKLRPFERDISNVPFSPDGK 465		17H9	
460 FSPDGKPCTPPALNCYW 476		F26G18	9
Unknown sites in SARS RBD		F26G9,F26G10,F26G19	
F360,Y442, L472, D480, T487	357NST	CR3006	10
Unknown sites in SARS RBD		CR3013 CR3014	
L472,N479,D480		80R	11
N479		CS5、CS84、FM6	
R395, R426, F483, Y484,I489, Y491, Q492		m396	
T332,N479, D463	330NAT	FM39	12
P462,N479		CR3014	13
Unknown sites outside of SARS RBD		CR3022	
T359,G391,D392,N424,R426,N427,T486,488 GIGYQ 492	357NST	F26G19	16
T359,T363,K365,390 KGD 392,V394,R395,R426,S432, Y436,G482,484 YTTTGIGYQ 492	357NST	m396	17
R426,S432,T433,Y436,N437,K439,Y440,Y442, 470 PALNCYWPLND 480,484 YTTTGI 489, Y491,Q492		80R	18
490 GYQPYRVVVLSFELLNAPATV 510		201	3
S1 (non-RBD) and S2 subunits			
536 GVLTPSSKRFQPFQQFG 552		114G5	14
612 ADQLTPAWR 620	602NCT	F26G8	9
549 QQFGRDVSDF 558		101F10,103F2,104D4,111A7, 121B8	14
731 CANLLLQYGSFCTQL 745		65B3,63B10	6
791 PLKPTKRSFIEDLLF 805	783NFS	5H10*	6
814 GFMKQYGECL 823		102D7	14
1125 PELDSFKEELDKYFKNH 1141	1116NNT	119F6	14

*Generated by immunizing mice with recombinant Spike protein produced in *Escherichia coli.*

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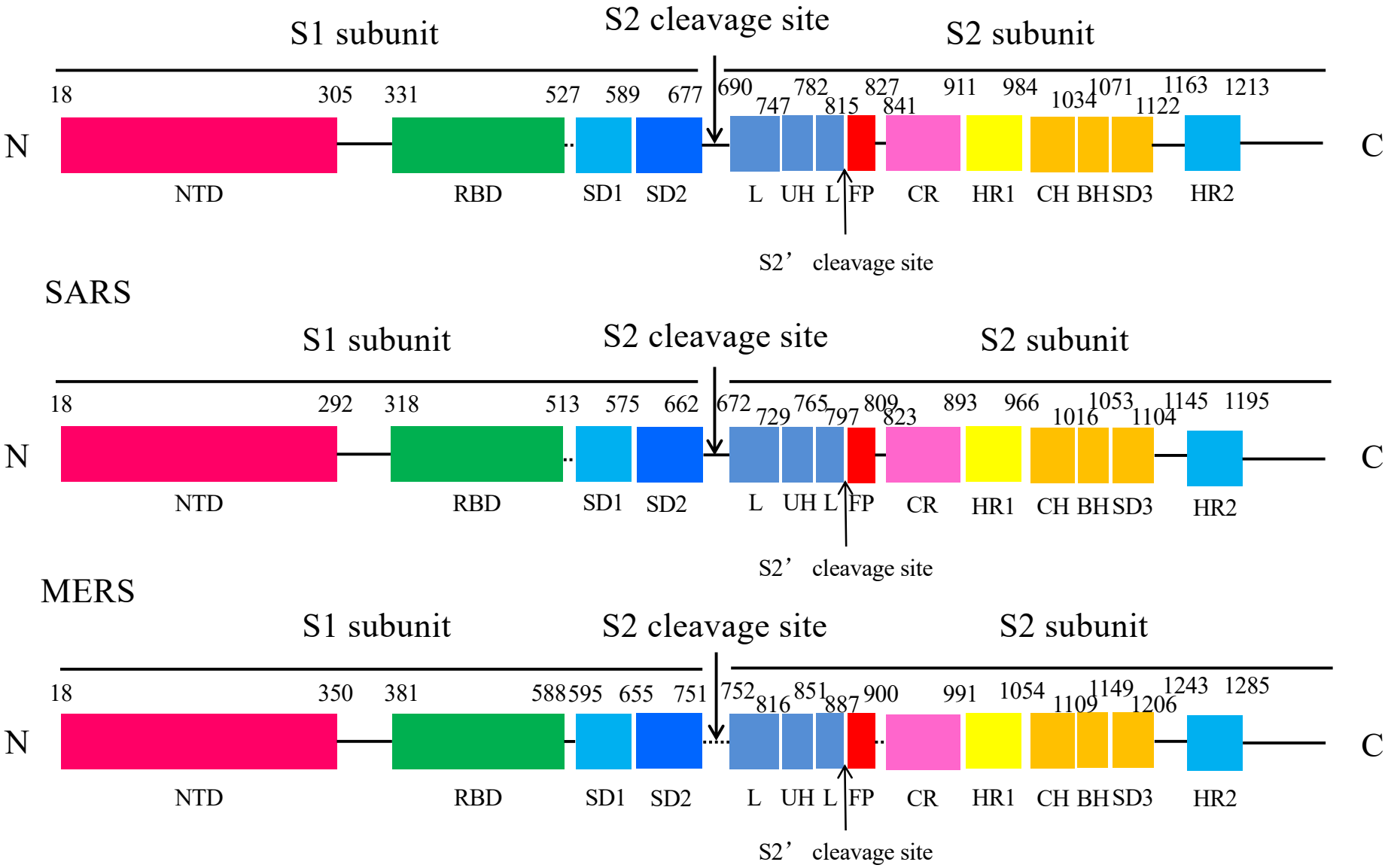
Zhou et al., Figure S1



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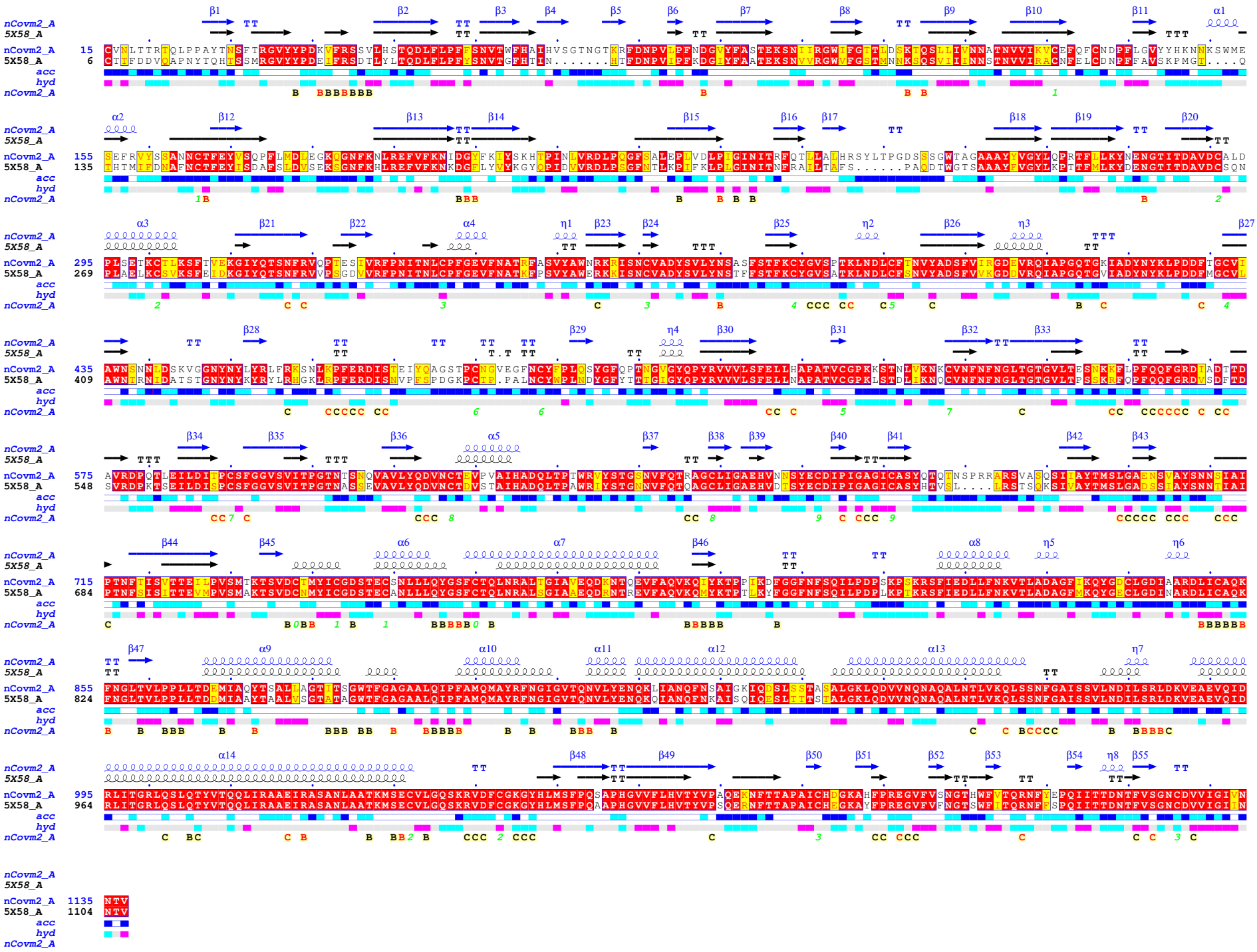

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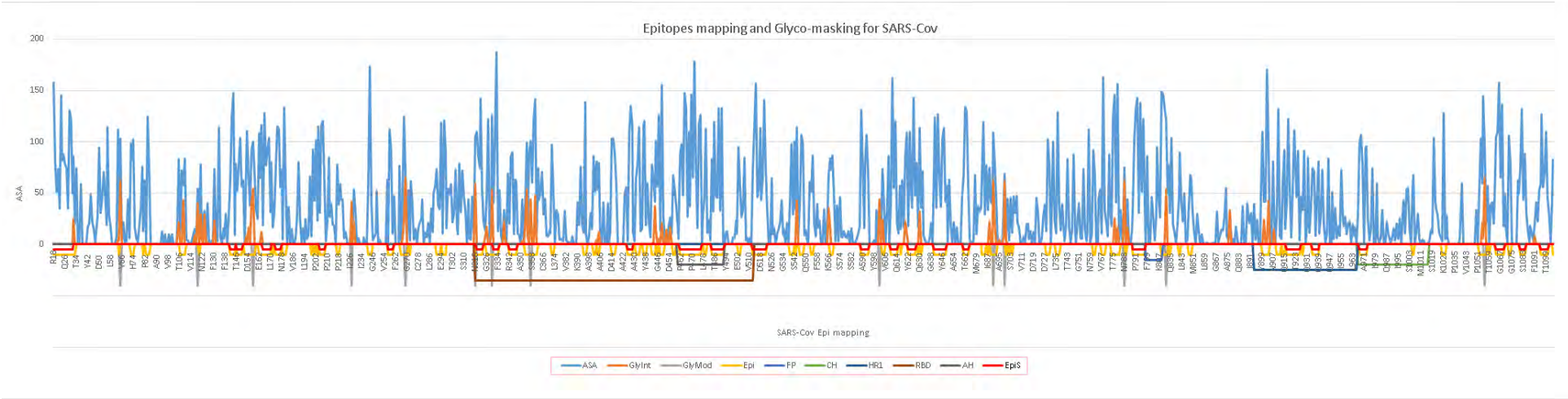
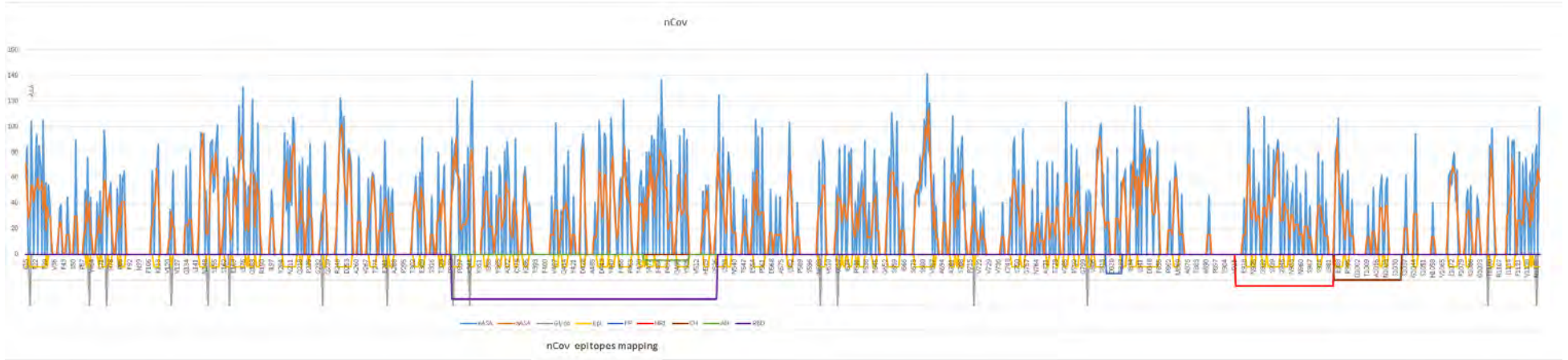
Zhou et al., Figure S3

SARS RBD



2019-nCoV



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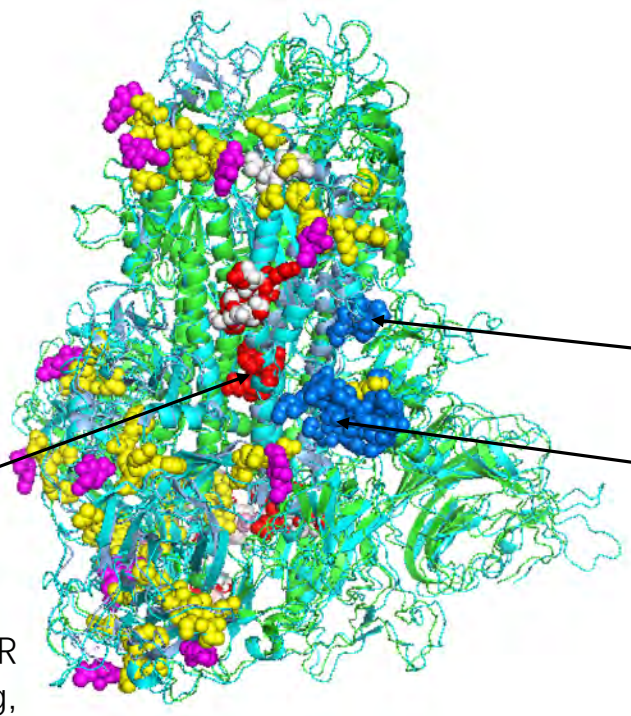
Zhou et al., Figure S4



HR1(S3)
N935-39:
NQNAQ

The fragment of CR for SARS is missing, thus the buried HR1(S3) fragment is exposed

CR nCoV(close epitope)
T866-69:
TDEM

CR nCoV(n3)
I834-54:
IKQYGCLGDIAARDLICAQK

CR(n3) is exposed, and located exactly outside of S3

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Zhou et al., Figure S5