LETTER

Pre-fusion structure of a human coronavirus spike protein

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HKU1 is a human betacoronavirus that causes mild yet prevalent respiratory disease[1], and is related to the zoonotic SARS[2] and MERS[3] betacoronaviruses, which have high fatality rates and pandemic potential. Cell tropism and host range is determined in part by the coronavirus spike (S) protein[4], which binds cellular receptors and mediates membrane fusion. As the largest known class I fusion protein, its size and extensive glycosylation have hindered structural studies of the full ectodomain, thus preventing a molecular understanding of its function and limiting development of effective interventions. Here we present the 4.0 Å resolution structure of the trimeric HKU1 S protein determined using single-particle cryo-electron microscopy. In the pre-fusion conformation, the receptor-binding subunits, S1, rest above the fusion-mediating subunits, S2, preventing their conformational rearrangement. Surprisingly, the S1 C-terminal domains are interdigitated and form extensive quaternary interactions that occlude surfaces known in other coronaviruses to bind protein receptors. These features, along with the location of the two protease sites known to be important for coronavirus entry, provide a structural basis to support a model of membrane fusion mediated by progressive S protein destabilization through receptor binding and proteolytic cleavage. These studies should also serve as a foundation for the structure-based design of betacoronavirus vaccine immunogens.

Betacoronavirus S proteins are processed into S1 and S2 subunits by host proteases[5]. Like other class I viral fusion proteins, the two subunits trimerize and fold into a metastable pre-fusion conformation. The S1 subunit is responsible for receptor binding, while the S2 subunit mediates membrane fusion. Coronaviruses typically possess two domains within S1 capable of binding to host receptors: an amino (N)-terminal domain (NTD) and a carboxy (C)-terminal domain (CTD), with the latter recognizing protein receptors for SARS-CoV and MERS-CoV[6,7]. Although these individual domains have been structurally characterized, the organization of the complete spike has not yet been determined, preventing a mechanistic understanding of S protein function.

Here, we present the structure of the HKU1 S protein ectodomain determined using cryo-electron microscopy (cryo-EM) to 4.0 Å resolution (Fig. 1a and Extended Data Figs 1 and 2 and Extended Data Table 1). The protein construct contains a C-terminal T4 fibritin trimerization motif and a mutated S1/S2 furin-cleavage site (Extended Data Fig. 3). The S1 subunit adopts an extended conformation with short linkers between domains and sub-domains (Fig. 1b). The S1 NTD (amino acids 14–297) has strong structural and sequence homology to the bovine coronavirus (BCoV) S1 NTD (Extended Data Fig. 4), which recognizes acetylated sialic acids on glycosylated cell-surface receptors[8]. The glycan-binding site in the BCoV S1 NTD is conserved in the HKU1 S1 NTD and is located at the apex of the trimer, oriented towards target cells. Indeed, HKU1 S1 was recently shown to bind O-acetylated sialic

acids on host cells, and these glycans were required for efficient infection of primary human airway epithelial cultures[9].

The HKU1 S1 CTD (amino acids 325–605) consists of a structurally conserved core connected to a large, variable loop (HKU1 S amino acids 428–587)[10] that is partially disordered (Extended Data Figs 5 and 6). The CTD is located at the trimer apex close to the threefold axis, and the core interacts with the other two S1 CTD cores and with one NTD from an adjacent protomer. The domain swapping between protomers results in a woven appearance when viewed looking down towards the viral membrane (Fig. 2a). Structural alignment of the SARS-CoV and MERS-CoV CTD–receptor complexes[11,12] with the HKU1 pre-fusion S protein reveals that the protein-receptor-binding surface of the S1 CTD is buried in the HKU1 S protein trimer and is therefore incapable of making equivalent interactions without some initial breathing and transient exposure of these domains (Fig. 2b).
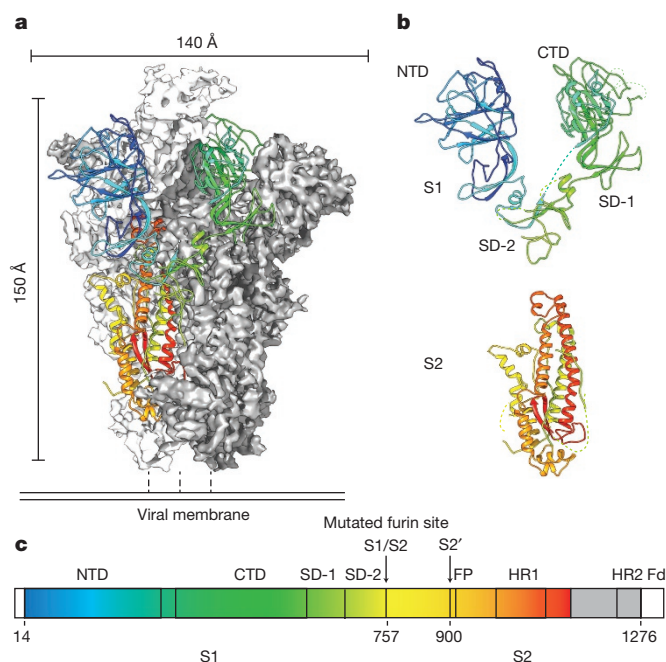

Figure 1 | Structure of the HKU1 pre-fusion spike ectodomain.
a, A single protomer of the trimeric S protein is shown in cartoon representation coloured as a rainbow from the N to C terminus (blue to red) with the reconstructed EM density of remaining protomers shown in white and grey. **b**, The S1 subunit is composed of the NTD and CTD as well as two sub-domains (SD-1 and SD-2). The S2 subunit contains the coronavirus fusion machinery and is primarily α-helical. **c**, Domain architecture of the HKU1 S protein coloured as in **a**.

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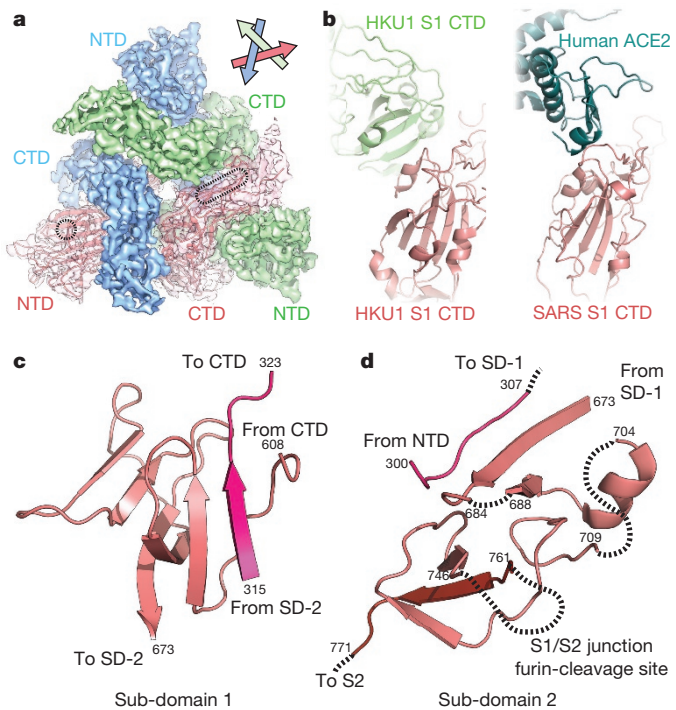*These authors contributed equally to this work.

Figure 2 | Architecture of the HKU1 S1 subunit. a, EM density corresponding to each S1 protomer is shown. The putative glycan-binding and protein-receptor-binding sites are indicated with dashed shapes on the NTD and CTD, respectively. **b**, The HKU1 S1 CTD forms quaternary interactions with an adjacent CTD using a surface similar to that used by SARS CTD to bind its receptor, ACE2 (ref. 11). **c**, Sub-domain 1 is composed of amino acid residues before and after the S1 CTD. **d**, Sub-domain 2 is composed of S1 sequence C-terminal to the CTD, a short peptide following the NTD, and the N-terminal strand of S2, which follows the S1/S2 furin-cleavage site.
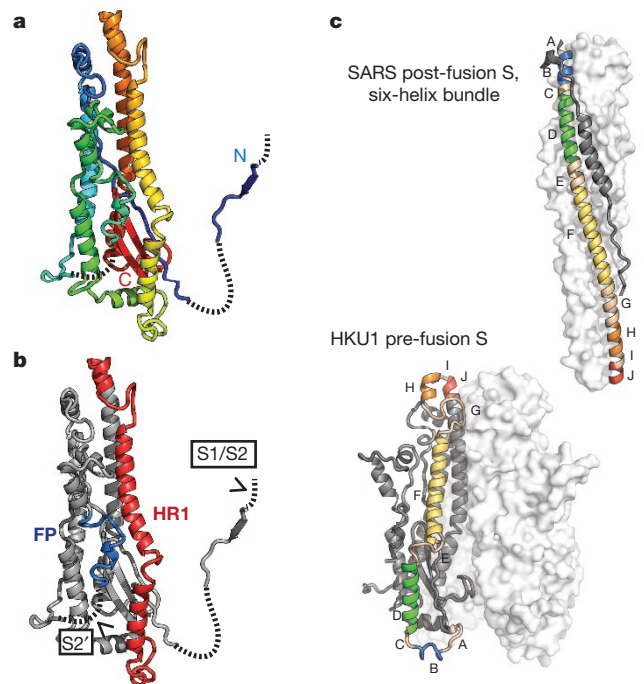

Figure 3 | HKU1 S2 subunit fusion machinery. a, The HKU1 S2 subunit is coloured like a rainbow from the N-terminal β-strand (blue), which participates in S1 sub-domain 2, to the C terminus (red) before HR2. **b**, The HKU1 S2 structure contains the fusion peptide (FP) and a heptad repeat (HR1). Protease-recognition sites are indicated within disordered regions of the protein (dashed lines). **c**, A comparison of coronavirus S2 HR1 in the pre- and post-fusion[22] conformations. Five HR1 α-helices are labelled and coloured like a rainbow from blue to red, N to C terminus, respectively. The structures are oriented to position similar portions of the central helix (red).

Although a protein receptor has not yet been identified for HKU1, antibodies against the CTD, but not those against the NTD, blocked HKU1 infection of cells[13]. These data suggest that the S1 CTD is the primary HKU1 receptor-binding site[13], whereas the NTD mediates initial attachment via glycan binding.

HKU1 S1 also contains two sub-domains (which we term SD-1 and SD-2) that lack significant homology to previously determined structures (Fig. 2c, d). These sub-domains are primarily composed of S1 amino acid sequences following the CTD. However, stretches of amino acids preceding the CTD as well as S2 residues adjacent to the S1/S2 cleavage site also contribute to the sub-domains. This complex folding of elements dispersed throughout the primary sequence may allow receptor-induced conformational changes in the CTD to be transmitted to other parts of the structure.

In contrast to other viral fusion proteins such as influenza haemagglutinin (HA)[14] or HIV-1 envelope (Env)[15,16], the HKU1 S1 subunits are rotated about the trimeric threefold axis with respect to the S2 subunits, causing the S1 subunit from one protomer to sit above the S2 subunit of an adjacent protomer (Extended Data Fig. 7). Similar to HA and Env, a region in the HKU1 S1 CTD (amino acids 371–380) caps the S2 central helix, thereby preventing the fusion machinery from springing into action.

Processing of coronavirus S proteins by host proteases plays a critical role in the entry process[5]. HKU1 S is cleaved by furin into S1 and S2 subunits during protein biosynthesis. Though mutated in the protein construct used here and disordered in the density map, the HKU1 S furin-cleavage site at the S1/S2 junction lies in a loop of SD-2 (Fig. 3 and Extended Data Fig. 6). Furin cleavage would leave a single S2 β-strand participating in the SD-2 β-sheets (Fig. 2d). Coronavirus S proteins also have a secondary cleavage site, termed S2′ (Arg900)[5],

adjacent to the viral fusion peptide (amino acids 901–918)[17] (Fig. 3b and Extended Data Fig. 6). This is similar to the multiple endoproteolytic cleavage events that occur in the fusion proteins of respiratory syncytial virus (RSV) and Ebola virus[18,19]. Protease cleavage at S2′ likely follows S1/S2 cleavage and may not occur until host-receptor engagement at the plasma membrane or viral endocytosis[5].

As in all class I viral fusion proteins, the coronavirus S2 subunit contains the four elements required for membrane fusion: a fusion peptide or loop, two heptad repeats (HR1 and HR2), and a transmembrane domain[14,20,21]. Refolding of HR1 into a long α-helix thrusts the fusion peptide into the host-cell membrane, and as the two heptad repeats interact to form a coiled-coil, the host and viral membranes are brought together. The fusion peptide, conserved among coronavirus S proteins[17] (Extended Data Fig. 6), is located on the exterior of the HKU1 S protein and is adjacent to the putative S2′ cleavage site, which remains uncleaved in our structure. The fusion peptide forms a short helix and a loop, with most of the hydrophobic amino acids buried in an interface with other elements of S2. Unlike influenza HA where the C terminus of the fusion peptide is only 14 amino acids away from the N terminus of HR1, the fusion peptide of HKU1 S is 60 amino acids away from HR1. This span of protein contains four short α-helices and several longer regions lacking regular secondary structure. This intervening sequence is also buried beneath SD-2 and the S2′ cleavage site, suggesting that cleavage may affect the proclivity of S2 for undergoing the transition to the post-fusion conformation.

Coronavirus S protein heptad repeats are unusually large with HR1 encompassing more than 90 amino acids[20]. In the cryo-EM structure, HR2 is located at the base of the HKU1 S protein near the viral membrane, but is poorly ordered, precluding unambiguous assignment of the residues. However, HR1 is well ordered and arranged along the length of the S2 subunit, forming four short helices and part of the
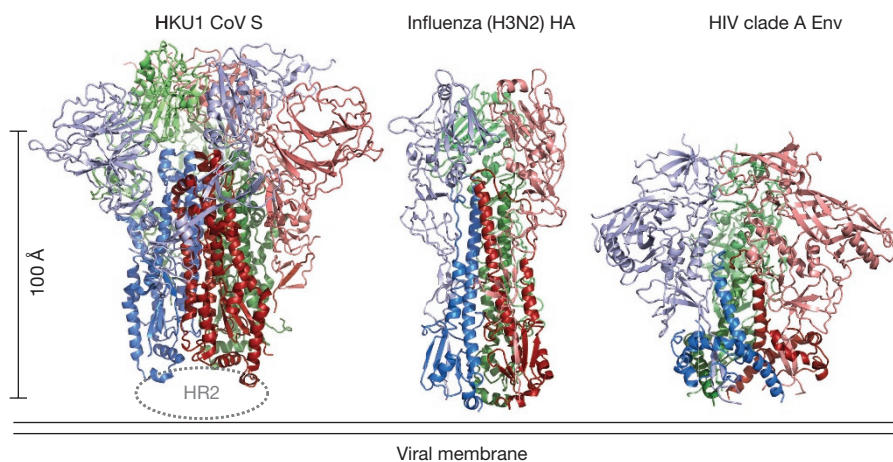
Figure 4 | Comparison of structurally related class I viral fusion proteins. The fusion proteins from coronaviruses, influenza virus and HIV-1 are cleaved into receptor-binding subunits (pink, light green, light blue) and the viral fusion machinery (dark red, dark green, blue)[14–16,28]. Comparison to other class I fusion proteins can be found in Extended Data Fig. 8.

central three-helix bundle. This arrangement of HR1 is similar to that of influenza HA, although in HA the HR1 is organized as two helices connected by a long loop[14]. Conversion of influenza HA to the post-fusion conformation requires these protein elements to transition into a single long α-helix[21]. The post-fusion six-helix bundle structures of SARS-CoV and MERS-CoV S2 heptad repeats[22,23] reveal that coronavirus S proteins also undergo a similar transition (Fig. 3c). However, the S protein must carry out five such loop-to-helix transitions, highlighting the complexity of S proteins relative to other class I fusion proteins. In addition, the membrane distal regions of the pre-fusion S2 central three-helix bundle (S2 amino acids 1070–1076), which is the C-terminal portion of HR1, are splayed outwards from the threefold axis (Extended Data Fig. 7). In the available coronavirus post-fusion HR1–HR2 structures, this portion of HR1 forms a tight three-helix bundle[22,23]. Formation of this three-helix bundle may be prevented by interactions between the C-terminal end of the S2 HR1 and the S1 CTD, and thus disruption of these interactions through receptor-induced conformational changes would provide an additional means by which receptor binding in S1 can initiate S2-mediated membrane fusion. Indeed, protease cleavage and an acidic pH are thought to be insufficient to trigger the transition to the post-fusion conformation without additional destabilization provided by receptor binding[24–26].

The formation of anti-parallel six-helix bundles composed of HR1 and HR2 in the post-fusion conformation is a unifying feature of class I viral fusion proteins. However, the pre-fusion conformations of this protein family are incredibly diverse in size and topology (Extended Data Fig. 8). The HKU1 S protein structure presented here most closely resembles influenza virus HA and HIV-1 Env (Fig. 4), which also have receptor-binding subunits that cap the central helix of the fusion subunit[14,15,27,28]. However, some core elements of the fusion machinery are conserved amongst all class I fusion proteins, including paramyxovirus F proteins.

The HCoV-HKU1 S protein trimer in a pre-fusion conformation is, to our knowledge, the largest class I viral fusion glycoprotein structure determined to date (Fig. 4 and Extended Data Figs 8 and 9). Since betacoronavirus S proteins are similar in size and have a conserved domain organization, our findings should be generally applicable to other betacoronaviruses, including SARS-CoV and MERS-CoV (Extended Data Fig. 6). Our studies provide a structural basis for S protein function wherein the pre-fusion S protein is progressively matured and destabilized by receptor binding and protease cleavage. Following dissociation of the S1 subunits, HR1 would transition to a long α-helix, and the fusion peptide would be released from the side of the S2 subunit and inserted into host membranes. The structure and mechanistic insights presented here should enable engineering of pre-fusion

stabilized coronavirus S proteins as vaccine immunogens against current and emerging betacoronaviruses, similar to recent efforts for other viral fusion proteins[29,30]. This work also acts as a springboard for future studies to define mechanisms of antibody recognition and neutralization, which will lead to an improved understanding of coronavirus immunity.

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Author Contributions H.M.Y. and K.S.C. designed the HKU1 S expression constructs. N.W. expressed and purified the proteins. C.A.C., H.L.T., and J.P. prepared samples for cryo-EM, collected images, and processed the data. R.N.K. and C.A.C built and refined the model. B.S.G., J.S.M., and A.B.W. conceived of the project. R.N.K, C.A.C., N.W., B.S.G, J.S.M and A.B.W analysed the results and wrote the manuscript, with all authors editing and approving the final manuscript.

METHODS

Data reporting. No statistical methods were used to predetermine sample size. The investigators were not blinded to allocation during experiments and outcome assessment.

Protein expression and purification. A mammalian-codon-optimized gene encoding HKU1 S (isolate N5, NCBI accession Q0ZME7) residues 1–1276 with a C-terminal T4 fibritin trimerization domain, a HRV3C cleavage site, and a 6xHis-tag was synthesized and subcloned into the eukaryotic expression vector pVRC8400. The S1/S2 furin-recognition site 752-RRKRR-756 was mutated to GGSGS to generate the uncleaved construct used for cryoEM studies. Three hours after this plasmid was transfected into FreeStyle 293-F cells (Invitrogen), kifunensine was added to a final concentration of 5 μM. FreeStyle 293-F cells are a high-transfection-efficiency cell line adapted for suspension culture derived from low passage clonal cultures and after purchase were not further authenticated. Cells were not confirmed to be free of mycoplasma, but were only used for protein expression. Cultures were harvested after six days, and protein was purified from the medium using Ni-NTA Superflow resin (Qiagen). The buffer was then exchanged using a HiPrep 26/10 desalting column (GE Healthcare Biosciences) from a high-imidazole elution buffer to a low pH buffer (20 mM Bis-Tris pH 6.5, 150 mM NaCl). Afterward, endoglycosidase H (EndoH) (10% w/w) and HRV3C protease (1% w/w) were added to the protein and the reaction was incubated overnight at 4 °C. The digested protein was further purified using a Superose 6 16/70 column (GE Healthcare Biosciences).

The furin-cleaved HKU1 S construct analysed by negative-stain EM was similar to the one described above except that it encoded residues 1–1249 and contained the wild-type RRKRR furin-recognition site. Expression and purification were also similar, except that a plasmid expressing furin was co-transfected into the FreeStyle 293-F cells to ensure complete processing of the protein.

Sample preparation for negative-stain electron microscopy. HKU1 S proteins were placed directly onto 400 copper mesh grids and then stained with 1% uranyl formate. Tris-buffered saline (TBS) was used as buffer if dilution was necessary.

Negative-stain electron microscopy data collection. Grids were loaded into a Tecnai T12 Spirit operating at 120 keV and imaged using a Tietz TemCam-F416 CMOS at 52,000 × magnification at ~1.5 μm under focus. Micrographs were collected using Leginon[31] and processed within Appion[32]. Particles were picked using a difference-of-Gaussians approach[33] and aligned using reference-free 2D classification employing iterative multivariate statistical analysis/multi-reference alignment (MRA/MSA) using a binning factor of 2 to remove amorphous particles[34]. Particles in classes that did not represent views of HKU1 S proteins were discarded. ISAC[35] was used to generate a template stack from which initial 3D models were generated using the EMAN2 (ref. 36) procedure initialmodel.py. 3D models were refined using EMAN1 (ref. 37).

Sample preparation for cryo-electron microscopy. Sample solution (3 μl) was applied to the carbon face of a CF-2/2-4C C-Flat grid (Electron Microscopy Sciences, Protochips) that had been plasma cleaned for five seconds using a mixture of Ar/O$_2$ (Gatan Solarus 950 Plasma system). The grid was then manually blotted and immediately plunged into liquid ethane using a manual freeze plunger.

Cryo-electron microscopy data collection. Movies were collected via the Leginon interface on a FEI Titan Krios operating at 300 keV mounted with a Gatan K2 direct-electron detector[31]. Each movie was collected in counting mode at 22,500 × nominal magnification resulting in a calibrated pixel size of 1.31 Å/pix at the object level. A dose rate of ~10 e$^-$/((cam pix) × s) was used; exposure time was 200 ms per frame. The data collection resulted in a total of 1,049 movies containing 50 frames each. Total dose per movie was 57 e$^-$/Å^2. Data were collected at 1.0 to 3.5 μm under focus.

Cryo-electron microscopy data processing. Frames in each movie were aligned[38], and CTF estimation was carried out using CTFFIND3 (ref. 39). Particles were picked from a subset of the data employing a difference-of-Gaussians approach[33] and aligned using reference-free 2D classification employing iterative MRA/MSA using a binning factor of two[34]. The resulting 2,188 particles were used to generate an initial 25 Å lowpass-filtered 3D reconstruction using EMAN2. SPIDER refproj. spi[40] with a delta theta angle of 15 degrees was used to generate 83 projection images of the initial 3D reconstruction. These projection images were used as templates for picking particles from the entire cryo data set. Particles from the entire data set were aligned and classified with the same methods used for the subset of particles stated above. After 2D classification, unbinned selected particles were symmetrically refined in RELION version 1.3 (refs 41, 42) against the initial 3D reconstruction filtered to 60 Å resolution. This refinement was followed by particle polishing and refinement of the resulting realigned, B-factor-weighted and signal-integrated particles using RELION version 1.4b1. The resolution of the final map was 4.04 Å at an FSC cutoff of 0.143. A mask was generated in RELION using a threshold that accounted for the entire structure. From this threshold, the mask was further dilated by 3 voxels and a Gaussian fall-off was generated over an additional 6 voxels. The mask effect on FSC was taken into consideration. Phases were randomized in the unfiltered half-set maps for initial FSC lower than 0.8 and a new FSC between these phase-randomized maps was generated and used to correct for mask effects in the final FSC-based resolution estimate. The reported resolution of 4.04 Å is the RELION CorrelationCorrected value

The map was B-factor sharpened employing FSC-weighting. The B-factor was estimated in RELION based on the resolution range from 10 Å to 2.62 Å (B-factor = −117 Å^2). The detector MTF file was provided to RELION.

Model building and refinement. An initial model of the S1 NTD was generated using the Modeller[43] homology modelling tool in UCSF Chimera[44] with the BCoV NTD (PDB 4H14)[8] as a template. The NTD homology model was docked into the HKU1 S protein EM density and refined with Rosetta density-guided iterative local refinement[45] while imposing C3 symmetry. Rosetta output models were clustered based on pairwise r.m.s.d. using a cluster radius of 2.15 Å. The lowest energy model from the largest cluster was selected for additional refinement. This model and the conserved CTD core from SARS-CoV (PDB 2AJF)[11] were used as starting structures for model building and refinement. These starting models and the remaining HKU1 protein sequence were modelled manually using COOT[46] and refined using RosettaRelax[47]. Structures were evaluated using EMRinger[48] and Molprobity[49]. Figures were produced in the PyMol[50] or UCSF Chimera[44] software packages.

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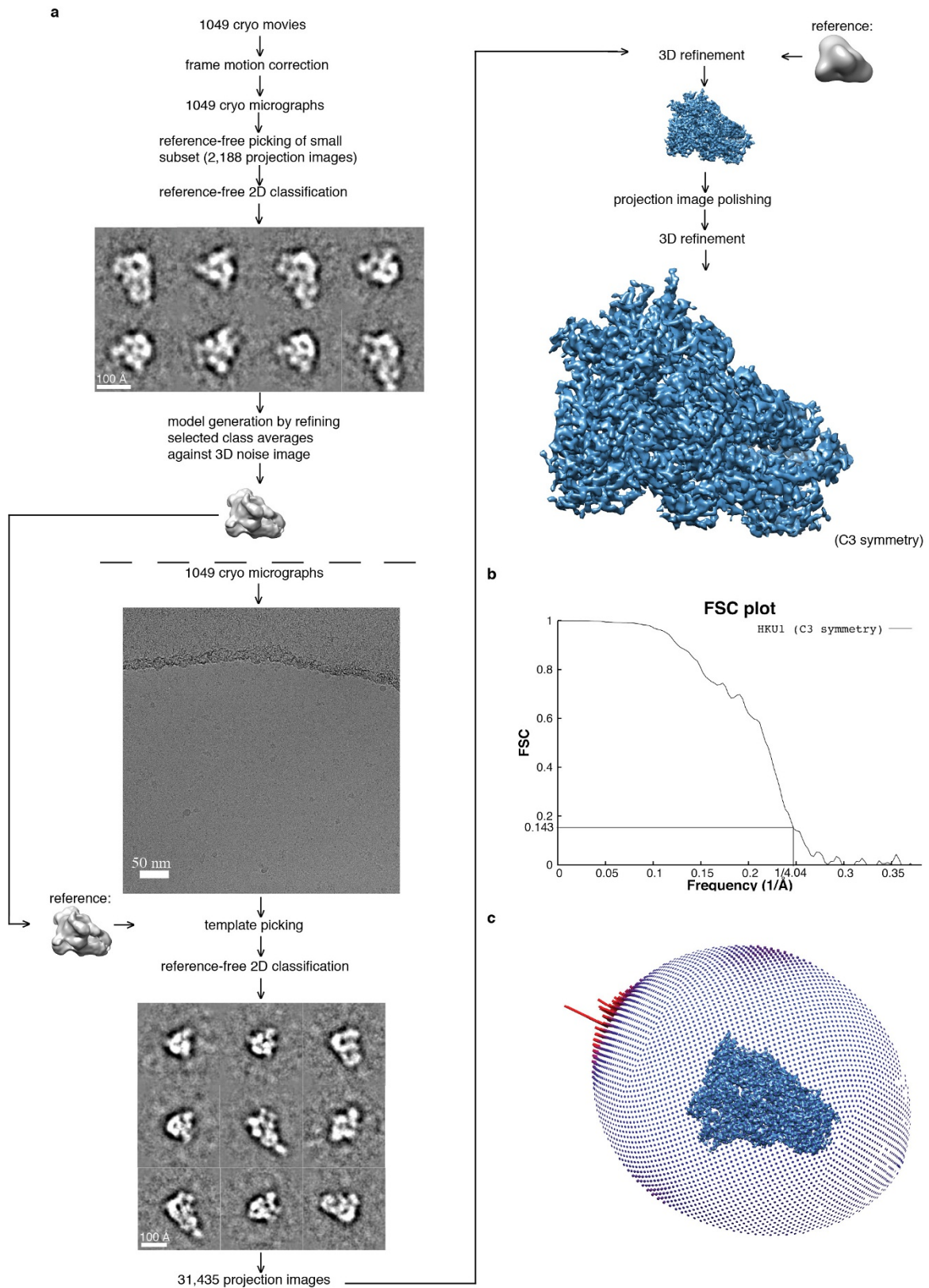
Extended Data Figure 1 | Data processing flowchart. a, Processing resulting in density map of pre-fusion HKU1 spike glycoprotein at 4.04 Å resolution. **b**, FSC plot illustrating correlation between two volumes refined independently from two distinct half sets of raw data. A final resolution of 4.04 Å is indicated in the plot. **c**, Angular distribution of raw data within the data set. A slight, but within normal range, over-representation of top views was observed (tall red bars).

a

🟩	3.74Å
🟨	4.16Å
🟧	4.58Å
🟥	5.00Å
⬜	>5.00Å

b
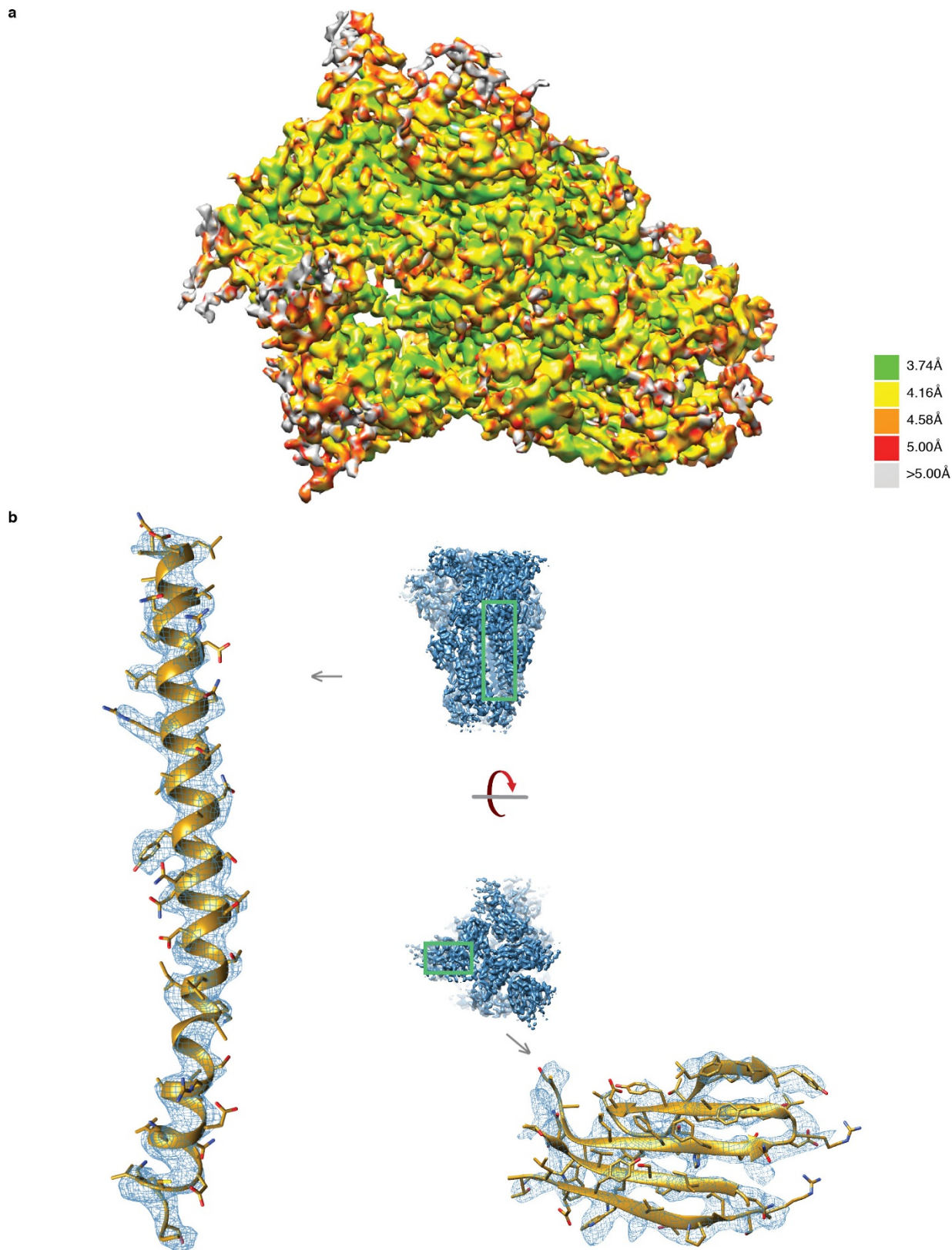
Extended Data Figure 2 | Resolution of the pre-fusion HKU1 S density map. a, Local resolution within the EM density map. Local resolution was calculated using ResMap[51] discretizing every 0.25 Å over a range from 2 × voxel size (2.62 Å) to 4 × voxel size (5.24 Å). Resolution significance criterion was set to 0.05. The resolution ranges from 3.74 Å in stable internal secondary structures to greater than 5.00 Å in flexible peripheral loops. **b**, Close-ups of secondary-structure densities. To the left is displayed the central α-helix of an S2 monomer and to the right is a β-sheet from the NTD domain in an S1 monomer.

	a	b	c
Wild-type S1/S2 Cleavage Site	+	–	–
Foldon trimerization domain	+	+	–
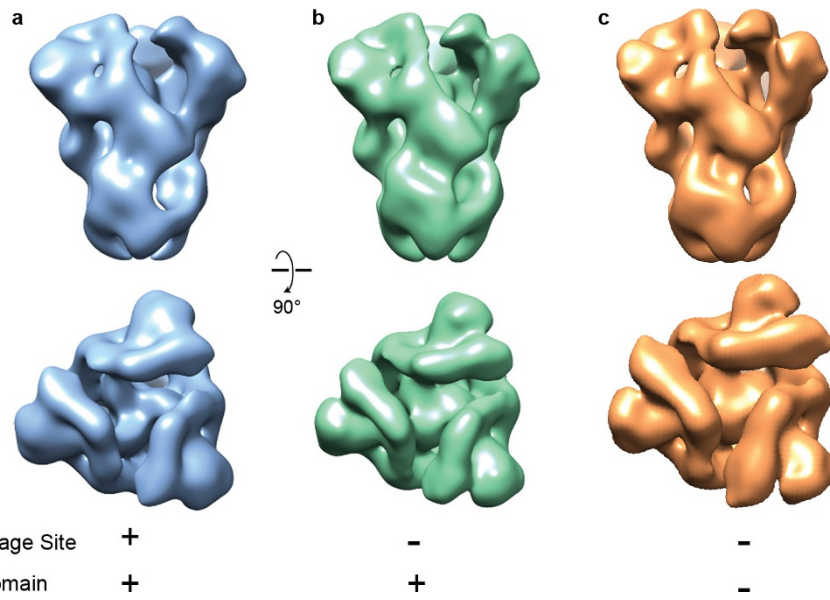
Extended Data Figure 3 | Cleavage at the S1/S2 junction does not induce large conformational changes in HKU1 spike. a, HKU1 spike 1–1249 with an attached foldon domain and wild-type furin-cleavage site was reconstructed using negative-stain electron microscopy. **b**, HKU1 spike 1–1276 with an attached foldon and a mutated furin-cleavage site reconstructed using negative-stain electron microscopy. **c**, HKU1 spike 1–1249 without foldon and with mutated furin-cleavage site. Side and top views are shown.
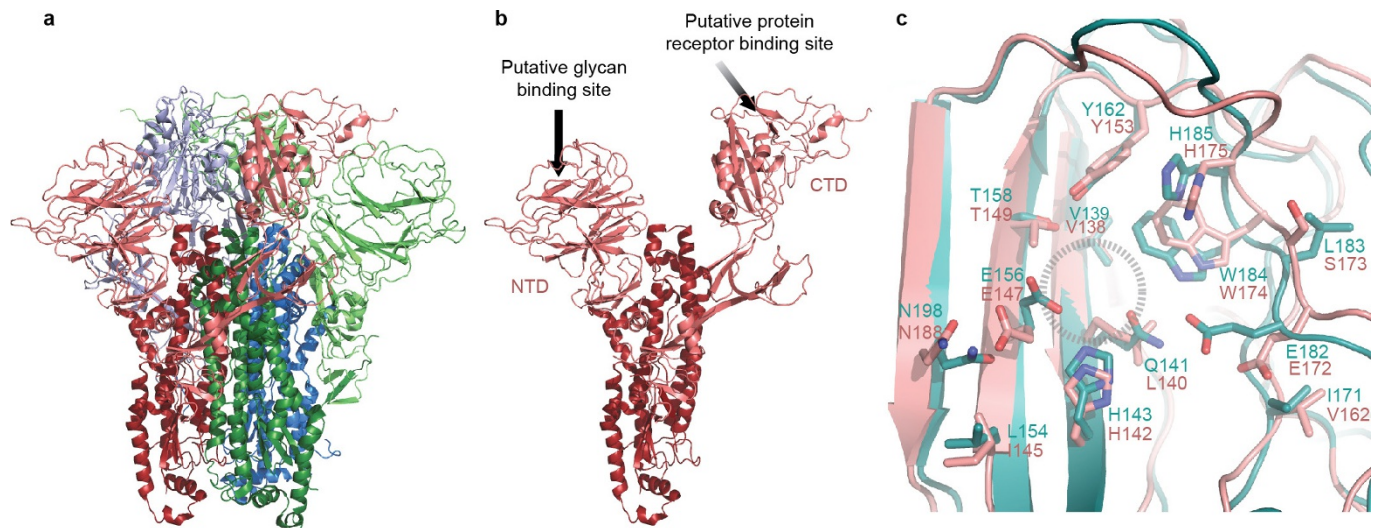
Extended Data Figure 4 | Putative glycan binding site of the HKU1 S1 NTD. a, HKU1 trimeric S and **b**, an isolated monomer. Putative host glycan-binding and protein-receptor-binding sites are indicated. **c**, The bovine coronavirus (BCoV) S1 NTD structure from Peng *et al.*[8] (teal) is superposed onto the HKU1 S NTD (pink). Residue side-chains involved in the putative glycan-binding site (dashed circle) are shown as sticks, with oxygen atoms coloured red and nitrogen atoms coloured blue. Note that N198 (BCoV) and N188 (HKU1) are predicted *N*-linked glycosylation sites.
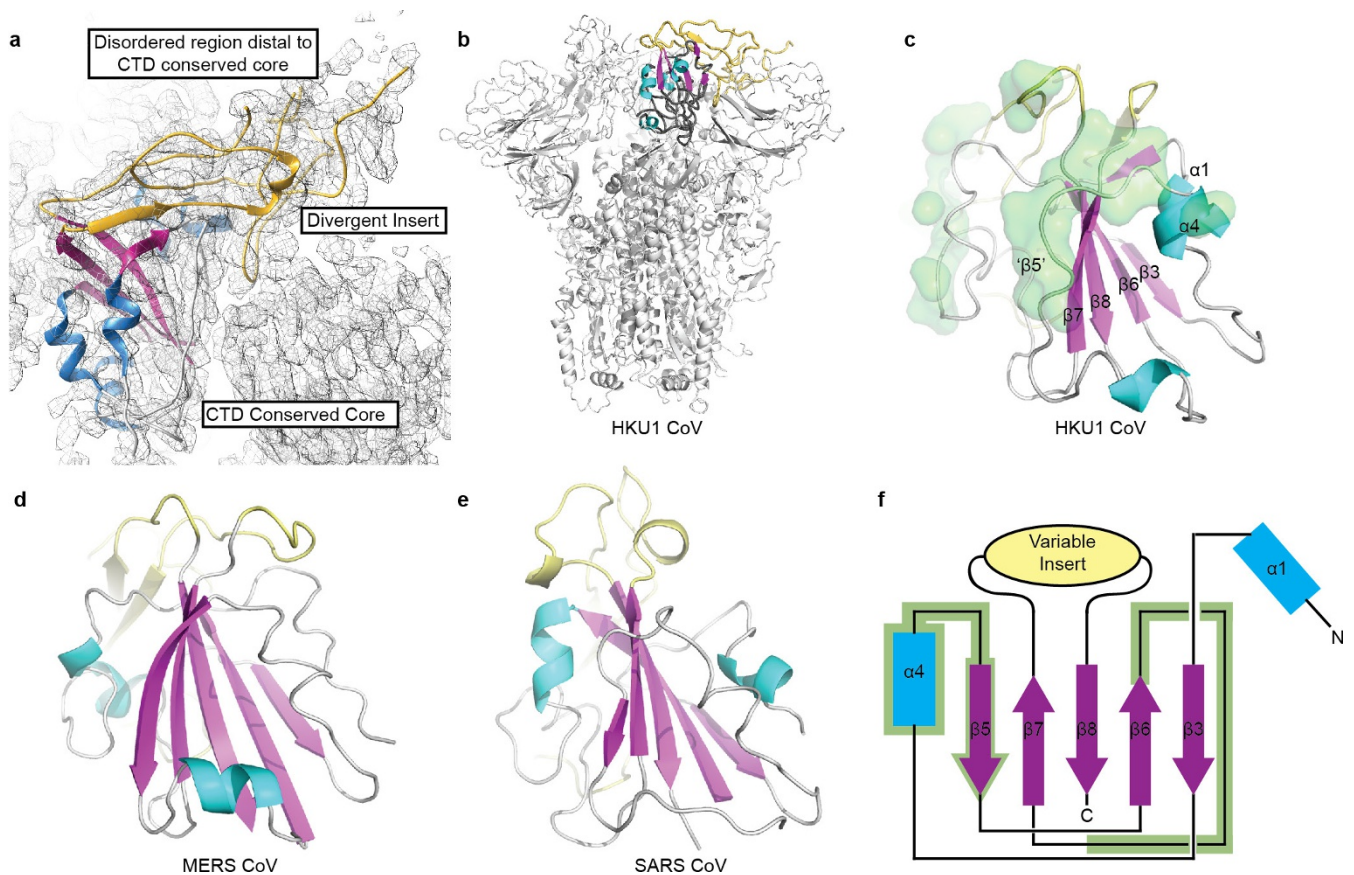
Extended Data Figure 5 | Betacoronavirus S proteins possess a conserved structural core in their C-terminal domains. a, The structurally divergent loop of the S1 CTD is poorly ordered distal to the core CTD domain. The conserved S1 CTD cores[10] of **b,** HKU1-CoV highlighted in the trimeric pre-fusion S, **c,** HKU1-CoV as an isolated domain, **d,** MERS-CoV[12] and **e,** SARS-CoV[11] are coloured according to secondary structure (β-sheets: pink, α-helices: blue, lacking regular secondary structure: grey) and the insert which differs amongst coronaviruses is coloured yellow. Atoms participating in quaternary interactions with other HKU1 S protomer CTDs are shown in green surface in **c. f,** The positions of these interacting atoms are mapped on to the conserved core topology. The sheet and helix nomenclature is taken from reference 10.

```
                          NTD
HKU1   ----MFLIIFILPTTLAV--IGD----FNCTNSF--INDYNKTIPRISEDVVDVSLGLGTYYVLNRVYLNTTLLFTGYFPKSGANFRDLALKGSIY--------LSTLWYKPPFLSDFNN    100
SARS   ----MFIFLLFLTLTS----G--SDLDRCTTFD------DVQAPNYT--QHTSSMRGVYYPDEIFRSDTLYLTQDLFLPFYSNVTGFHT-------------INHTFGN-PVIPFKD       85
MERS   MIHSVFLLMFLLTPTES YVDVGPDSVKSACIEVDIQQTFFDKTWPR-P---IDVSKADGIIYPQGRTYSNITITYQG-LFPYQGDHGDMVYYSAGHATGTTPQKLFVANYSQ-DVKQFAN   114
       :*:::::*  *   *     *     *          *          .. *    *  * *          *   *     . ..   *      *        *     *

HKU1   GIFSKVKNTKLYVNN---------TLYSEFSTIVIGSVFVNTSYTIV--VQPHNGILEITACQYTMCEYPHTVCKSKGSIRNE------------SWHIDSSE-PLCLFKKNFTYNVSAD   196
SARS   GIYFAATEK---------------SNVVRGWVFGSTMNNKSQSVI--IINNSTNVVIRACNFELCDNPFFAVSKPMGTQTHTMIFDNAFNCTFEYISDAF--SLDVSEKSGNFKHL--    182
MERS   GFVVRIGAAANSTGTVIISPSTSATIRKIYPAFMLGSSVGNFSDGKMGRFFNHTLVLLPDGCGTL--LRAFYCILEPRSGNHCP--AGNSYTSFATYHTPATDCSDGNYNRNASLNSF-   228
       *:                       .    ::**.  * *   : .  .*       .          : :             :              ::.   :

HKU1   WLYFHFYQERGVFYAYY-----------------------ADV-GMPTTFLFSLYLGTILSHYYVMPLTCNAISSNTD---NETLEYWVTPLSRRQYLLNFDEHGVITNAVDCSSSF    286
SARS   -REFVFKNKDGFLYVYKGYQPID----VVRDLPSGFNTLKP-----------IFKLPLGINITNFRAI---LTAFSPAQDIWGTSAAAYFVGYLKPTTFMLKYDENGTITDAVDCSQNP    282
MERS   -KEYFNLRNCTFMYTYNITEDEILEWFGITQTAQGVHLFSSRYVDLYGGNMFQFATLPVYDTIKYYSIIPHSIRSIQSDRKAW----AAFYVYKLQPLTFLLDFSVDGYIRRAIDCGFND   343
         :    .:   .:*.*                           :  .* .:.:  .      ::.        .    ::*  *.  ::*.::..  * *  *:**.  .

                                 CTD
HKU1   LSEIQCKTQSFAPNTGVYDLSGFTVKPVATVYRRIPNLP DCDIDNWLNNVSVPSPLNWERRIFSNCNFNLSTLLRLVHVDSFSCNNLDKSIFGSCFNSITVDKFAIPNRRRDDLQLGSS   406
SARS   LAELKCSVKSFEIDKGIYQTSNFRVVPSGDVVRFPNITNLCPFGEVFNATKFPSVYAWERKKISNCVADYSVLYNSTFFSTFKCYGVSATKLNDLCFSNVYADSFVVKGDDVRQIAPGQT   402
MERS   LSQLHCSYESFDVESGVYSVSSFEAKPSGSVVEQAEG-VECDFSPLLS-GTPPQVYNFKRLVFTNCNYNLTKLLSLFSVNDFTCSQISPAAIASNCYSSLILDYFSYPLSMKSDLGVSSA   461
       *:::::*. :**  *:*:*. * *   . *. .     *:  :. .*.   ::* ::** :: *    .. *.* :: :  *   *:::.: * *           ::   ..:

                    Variable Loop
HKU1   GFLQSSNYKIDISSSSCQLYYSL PLVNVTINNFNPSSWNRRYGFGSFNLSSYDVVYSDHCFSVNS------------DFCPCADPSVVNSCAKSKPPSAICPAGTKYRHCDLDTTLYVK   513
SARS   GVIADYNYKLPDDFMGCVLAWNTRNIDATSTGN----YNYKY-----------RYLRHG--KLRPFERDISNVPFSPDGKPCTPPA---------LNCYWPLNDYG-----------    482
MERS   GPISQFNYKQSFSNPTCLILATVPHNLTTITKP------LKY-----------SYINKCSRLLSDDRTEVPQLVNANQYSPCVSIV----------PSTVWEDGDYYRKQL--SPLEGG   551
       *   : .*** . *:  .*.      .*.          :*             *  :        :   **.          :   **.          .

HKU1   NWCRCSCLPDPISTYSPNTCPQKKVVVGIGEHCPGLGINEEKCGTQLNHSSCFCSPDAFLGWSFDSCISNNRCNIFSNFIFNGINSGTTCSNDLL--YSNTEISTGVCVNYDLYGITGQG   631
SARS   -FYTTTG------IG----YQPYRVVV-----------------------------------------LSFELL----NAPATVCGP-----KLSTDLIKNQCVNFNFNGLTGTG       536
MERS   GWLVASG-----ST----VAMTEQLQ-----------------------------------------MGFGITVQYGTDTNSVCPKLEFANDTKIASQLGNCVEYSLYGVSGRG        615
        :  :         :      .:                                                 *         .:*             **::.: *::*  *

HKU1   IFKEVSAAYYNNWQNLLYDSNGNIIGF-----KDFLTNKTYTILPCYSGRVSAAFY--QNSSSPALLYRNLKCSYVLNNISFISQ-----------PFYFDSYLGCVLNAVNLTSYSVS   732
SARS   VLTPSSKRF-QPFQQFGRD-----VSDFTDSVRDPKTSEILDISPCAFGGVSVITPGTNASSEVAVLYQDVNCTDVSTAIHADQLT--PAWRIYSTGNNVFQTQAGCLIGAEHVD--TSY   646
MERS   VFQNCTAVG-VRQQRFVYDAYQNLVGYYSD------DGNYYCLRACVSVPVSVIYD--KETKTHATLFGSVACEHISSTMSQYSRSTRSMLKRRDSTYGPLQTPVGCVLGLVNSS-LFVE   725
       ::   .           *.:  .         :      :    *   **.     .:.  *   .*:*  *  :. *:.     :           .:   **::  .

                   S1/S2     S2
HKU1   SCDLRMGSGFCIDYALPSSRRRRGISSPYR--FVTFEPFNVSFVNDSVETVGGLFEIQIPTNFTIAGHEEFIQTSSPKVTIDCSAFVCSNYAACHDLLSEYGTFCDNINSILNEVNDLL   850
SARS   ECDIPIGAGICASYHTVSLLRSTSQKSIVA---------YTMSLGA-DSSIAYSNNTIAIPTNFSISITTEVMPVSMAKTSVDCNMYICGDSTECANLLLQYGSFCTQLNRALSGIAAEQ   756
MERS   DCKLPLGQSLCALPDTPSTLTPRSVRSVPGEMRLASI-AFNHPIQV--DQFNSSYFKLSIPTNFSFGVTQEYIQTTIQKVTVDCKQYICNGFQKCEQLLREYGQFCSKINQALHGANLRQ   842
       .*.:  :* .:*  *       *       *        :.  :  .    .   :  * *****::.  * .:  *.::*. .::*     * :**.:** ** ::*  *

                        S2'  FP
HKU1   DITQLQVANALMQGVTLSSNLNTNLHSDVDNIDFKSLLGCLGSQCGSSR SLLEDLLFNKVKLSDVGF VEAYNNCT--GGSEIRDLLCVQSFNGIKVLPPILSETQISGYTTAATVAAMF   968
SARS   DRNTREVFAQVKQMY-----KTPTLKYFGG-FNFSQILPDPL---KPTKRSFIEDLLFNKVTLADAGF MKQYGECL--GDINARDLICAQKFNGLTVLPPLLTDDMIAAYTAALVSGTAT   865
MERS   DDSVRNLFASVKSSQ-----SSPIIPGFGGDENLTLLEPVSISTGSRSAR SAIEDLLFDKVTIADPGYMQGYDDCMQQGPASARDLICAQYVAGYKVLPPLMDVNMEAAYTSSLLGSIAG   957
       *  .    ::   :       .  :        ::.*  *::   :  : ** *****:**.::* *::: * :*    * . ***:*.* .* .****::   :.**::.  .

            HR1
HKU1   PPWSA----AAGVPFSLNVQYRINGLGVTMDVLNKNQKLIANAFNKALLSIQNGFTATNSALAKIQSVVNANAQALNSLLQQLFNKFGAISSSLQEILSRLDNLEAQVQIDRLINGRLTA   1084
SARS   AGWTFGAGAALQIPFAMQMAYRFNGIGVTQNVLYENQKQIANQFNKAISQIQESLTTTSTALGKLQDVVNQNAQALNTLVKQLSSNFGAISSVLNDILSRLDKVEAEVQIDRLITGRLQS   985
MERS   GGWTAGLSSFAAIPFAQSIFYRLNGVGITQQVLSENQKLIANKFNQALGAMQTGFTTTNEAFRKVQDAVNNNAQALSKLASELSNTFGAISASIGDIIQRLDVLEQDAQIDRLINGRLTT   1077
        .      ::*::.:* :    * :**** :* ** ** :*** ***:. :*  :: ::.:**.:: :*.*. *. ***::* .* ****::  :.**:: *** *.*****.**:*

HKU1   LNAYVSQQLSDITLIKAGASRAIEKVNECVKSQSPRINFCGNGNHILSLVQNAPYGLYFIHFSYKPTSFKTVLVSPGLCLSGDR---GIAPKQGYFIKQ-----NDSWMFTGSSYYYPEPI   1197
SARS   LQTYVTQQLIRAAEIRASANLAATKMSECVLGQSKRVDFCGKGYHLMSFPQAAPHGVVFLHVTYVPSQERNFTTAPAICHEGKA---YFPREGVFVFN-----GTSWFITQRNFFSPQII   1097
MERS   LNAFVAQQLVRSESAALSAQLAKDKVNECVKAQSKRSGFCGQGTHIVSFVVNAPNGLYFMHVGYYPSNHIEVVSAYGLCDAANPTNCIAPVNGYFIKTNNTRIVDEWSYTGSSFYSPEPI   1197
       *::::*:***    .. *  * *:*** .**   ...***:* *::*:     * :*::*. *  *.  .   .   **    .      * :* *:         .*   .:: *: *

                                                    HR2                                           TM
HKU1   SDKNVVFMNSCSVNFTKAPFIYL--NNSIPNLSDFEAELSLWFKNHTSIAPNLTFSHNIATFLDLYYEMNVIQESIKSLNSSFINLKEIGTYEMYVKWPWYIWLLIVILFIIFLMILFF   1315
SARS   TTDNTFVSGNCDVVIGIINNTVYDPLQPELDSFKEELDKYFKNHTSPDVDLGDISGINATFLDLYYEMNVIQESIKSLNSSFINLKEIGTYEMYVKWPWYIWLLIVILFIIFLMILFF   1215
MERS   TSLNTKYVAPQVT-YQNISTNLPPPLLGNSTGIDFQDELDEFFKNVSTSIPNFGSLTQINTTLLDLTYEMLSLQQVVKALNESYIDLKELGNYTYYNKWPWYIWLGFIAGLVALALCVFF   1316
        : *  .   .       . :*            *::*. ::*** . : *:   . *: *:*** :***   :*::*:.*:*: ** *:* *.** ***** ::  *: :: :: : ::

HKU1   ICCCTGCGSACFSK--CHNCCDEYGGHNDFVIK-ASHDD-- 1351
SARS   LCCMTSCCSCLKGACSCGSCCKFD-EDDSEPVLKGVKLHYT 1255
MERS   ILCCTGCGTNCMGKLKCNRCCDRYEEYDLEPHKVHVH---- 1353
       : *  *.: .   *     *  **:  :     :
```

Extended Data Figure 6 | Sequence alignment of human betacoronavirus S proteins. Sequence alignment of S proteins from HKU1, SARS-CoV and MERS-CoV using Clustal Omega[52]. Protein features described in the text are indicated: N-terminal domain (NTD), C-terminal domain (CTD) which contains the large variable loop, the S1/S2 and S2′ cleavage sites, fusion peptide (FP), heptad repeats 1 and 2 (HR1, HR2) and transmembrane helix (TM).

Extended Data Figure 7 | S1 sits atop an adjacent protomer's S2. a, The HKU1 S1 subunits are rotated about the trimeric threefold axis relative to their corresponding S2 subunits such that the S1 CTD from one protomer caps the S2 central helix from an adjacent protomer (CTD$_1$, blue, caps S2$_2$, red). The third protomer of the trimer has been omitted for clarity. **b**, HKU1 S1 CTD (blue) uses a short helix to cap the central helix and HR1 (red). **c**, The influenza haemagglutinin HA2 central helix (red) is also capped by a helix in HA1 (blue)[14,28]. **d**, The S2 N-terminal β-strand is connected to the remainder of the S2 subunit via a loop and an α-helix (dotted lines). These regions of the EM density are of insufficient quality to confidently build this protein region but enable interpretation of connectivity. **e**, In the pre-fusion HKU1 S protein, the tops of the central S2 helices (blue, red, green) are splayed outwards from the threefold axis and capped by the S1 CTDs (white). The S1 NTD, SD-1 and SD-2 have been omitted for clarity. **f**, In the post-fusion six-helix-bundle structure of SARS S[22], the corresponding helical regions from (e) form a well-packed three-helix bundle.
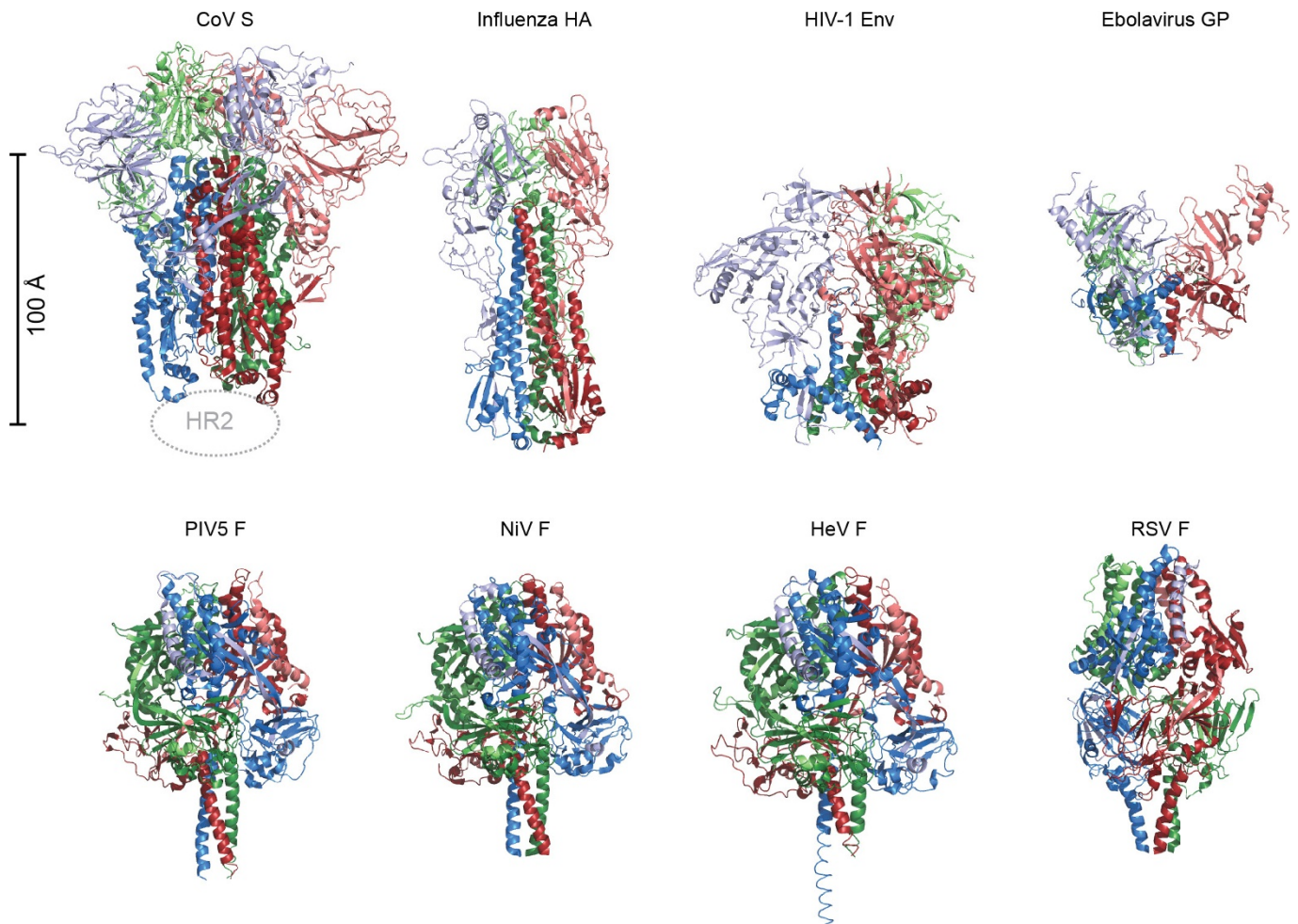
Extended Data Figure 8 | Class I viral fusion proteins. All class I fusion proteins require proteolytic cleavage adjacent to the fusion peptide or loop, and the metastable pre-fusion state is triggered by a series of events that involve pH change or receptor binding. The post-fusion conformations all contain anti-parallel six-helix bundles composed of the HR1 and HR2 from the membrane-proximal subunit. However, there is a great diversity in pre-fusion conformations as shown here. Members of this class that also participate in receptor binding[14–16,28,53] (top row), including S glycoproteins of coronaviruses, are organized such that their receptor binding subunits sit atop the fusion machinery, and need to be shed in order for membrane fusion to proceed. Paramyxovirus F proteins[54–57] (bottom row) have a different architecture than the capped fusion proteins on the top row. The F proteins all have disulfide bonds between the membrane proximal and membrane distal subunits, and the two subunits remain interconnected throughout the rearrangement process.
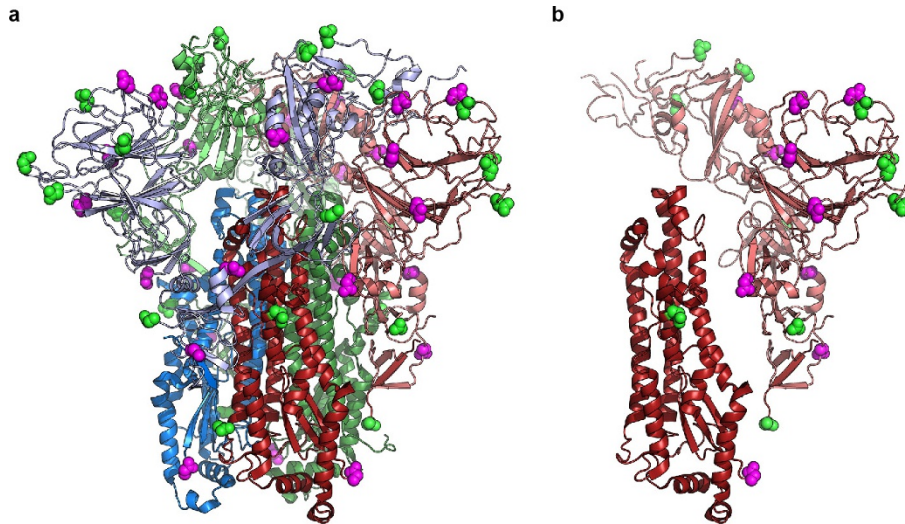
Extended Data Figure 9 | HKU1 S glycosylation. a, Sites of *N*-linked glycosylation on the HKU1 S trimer and **b**, a single monomer. Of the 30 potential *N*-linked glycosylation sites in a single protomer, the asparagine residues are observed for 21 sites and of these a small portion of density in the EM map is observed for 10 sites corresponding to the EndoH-trimmed sugars. Asparagines where glycan density is observed are shown as magenta spheres. Asparagines lacking glycan density are shown in green.

Extended Data Table 1 | CryoEM data collection, processing and refinement metrics

Data collection/processing

Microscope	Titan Krios
Voltage (keV)	300
Defocus range (µm)	1.0 to 3.5
Movies	1,049
Frames per movie	50
Exposure time per frame (ms)	200
Magnification	22,500x
Dose rate (e⁻/pixel/s)	10
Total dose per movie (e-/Å^2)	57
Particles	31,435
Map Resolution (Å)	4.04

Model Refinement

Chimera CC[44]	0.87
EMRinger Score[48]	2.7
MolProbity[49]	1.6
Clashscore[49]	3.0
Ramachandran (%)[49]	
Favored	92.1
Allowed	7.0
Outliers	0.9

CC = cross correlation