SUPPLEMENTARY INFORMATION

Megabodies expand the nanobody toolkit for protein structure determination by single-particle cryo-EM

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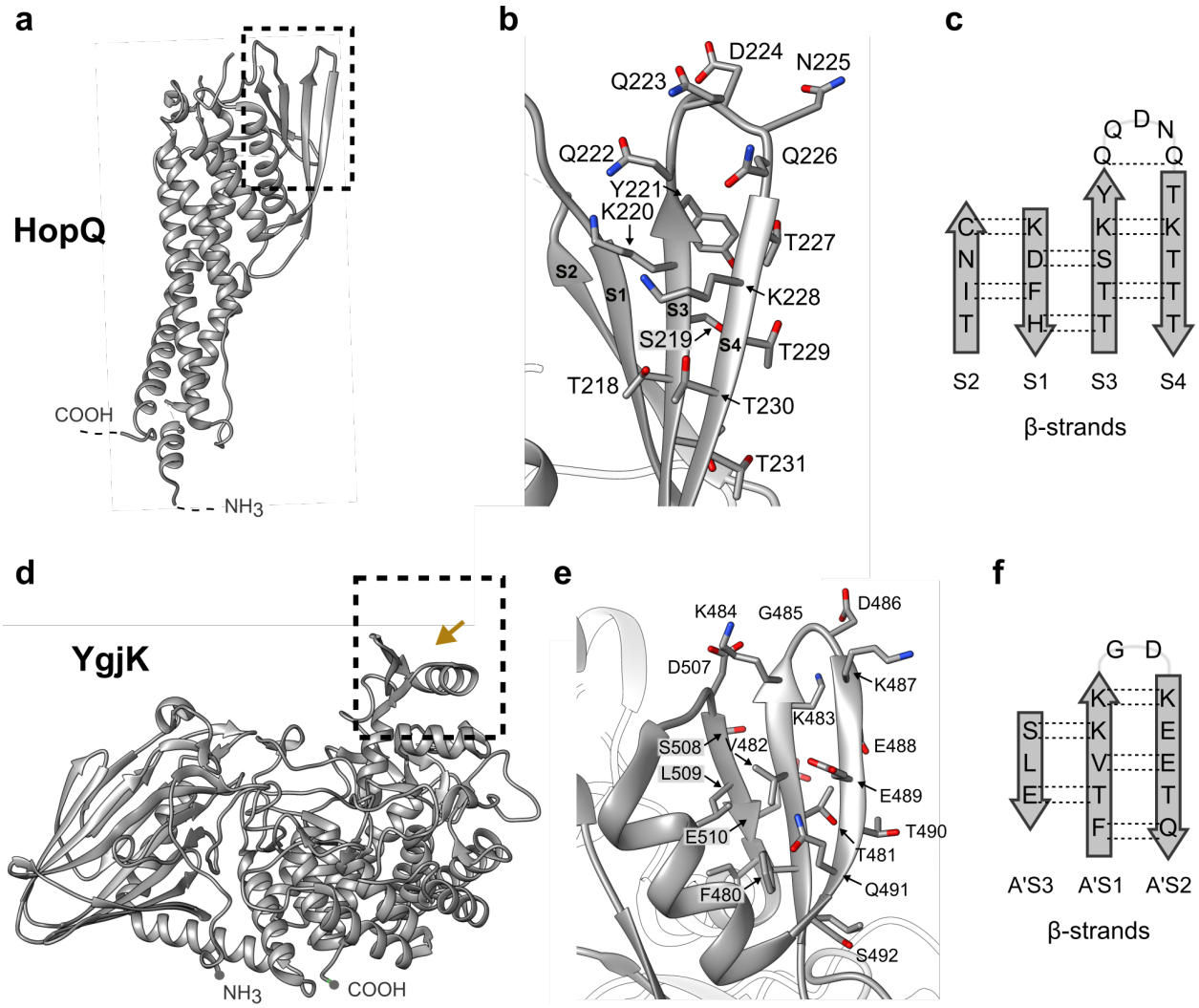
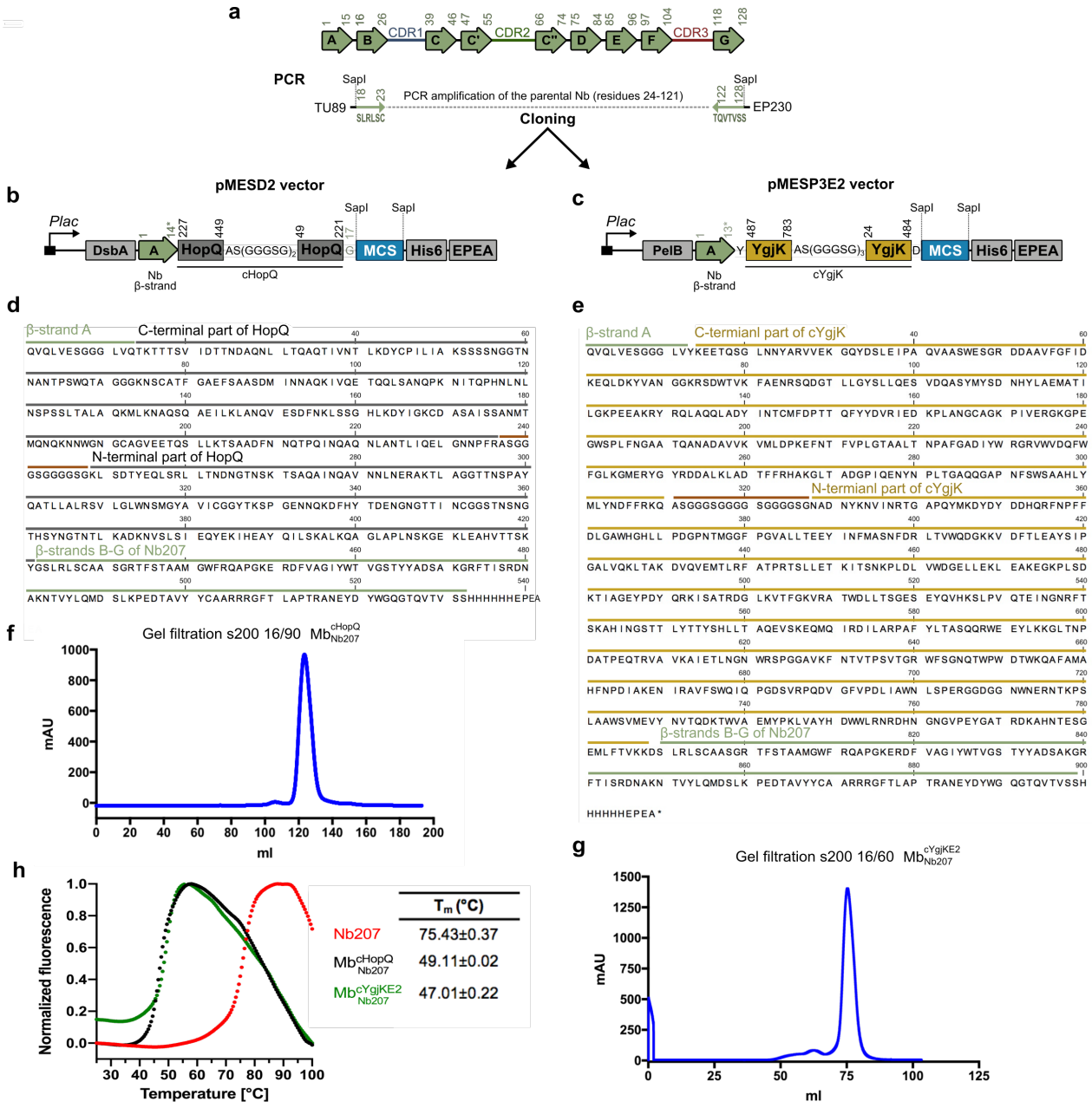
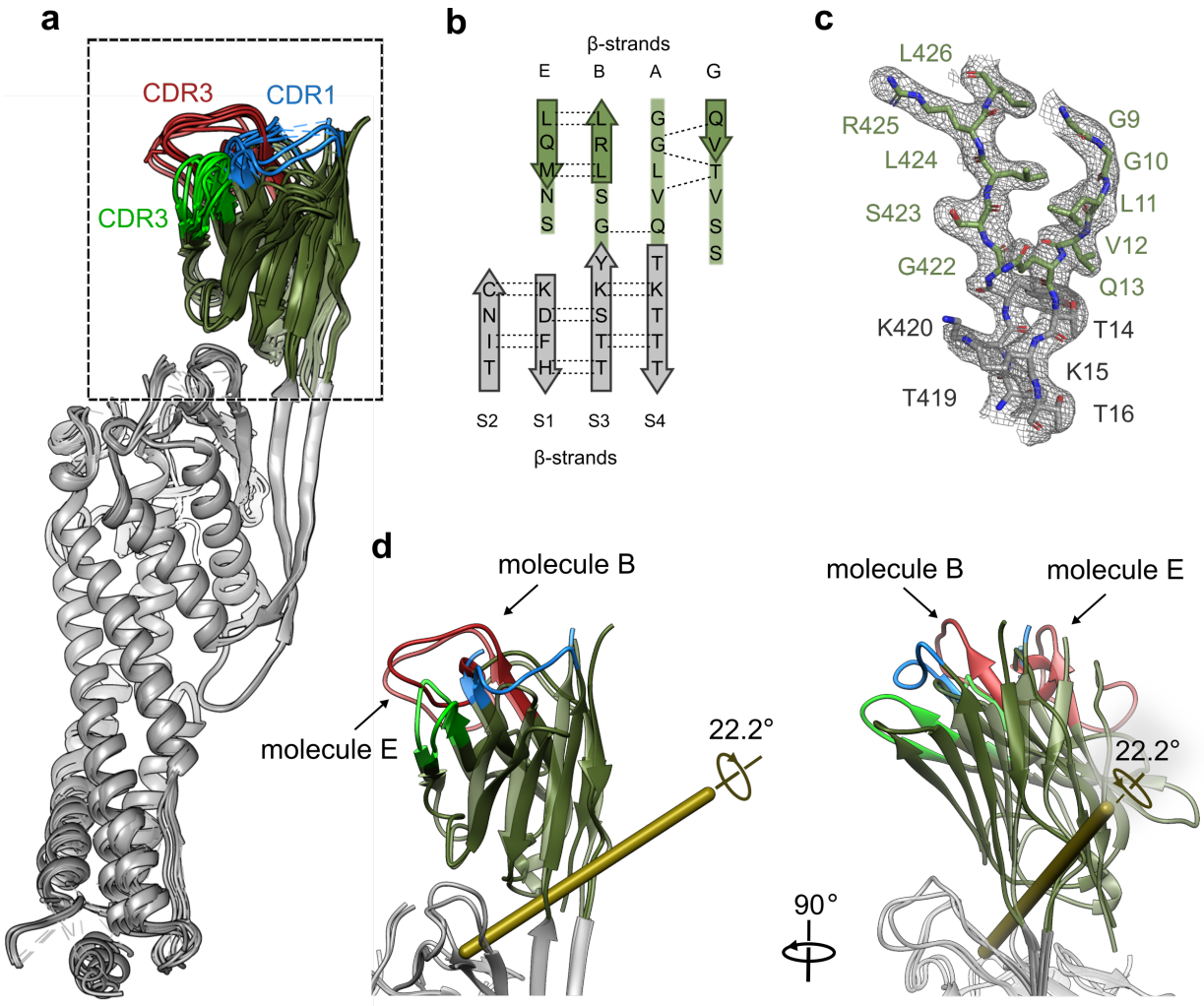
Keywords: megabody, nanobody, single-particle cryo-EM



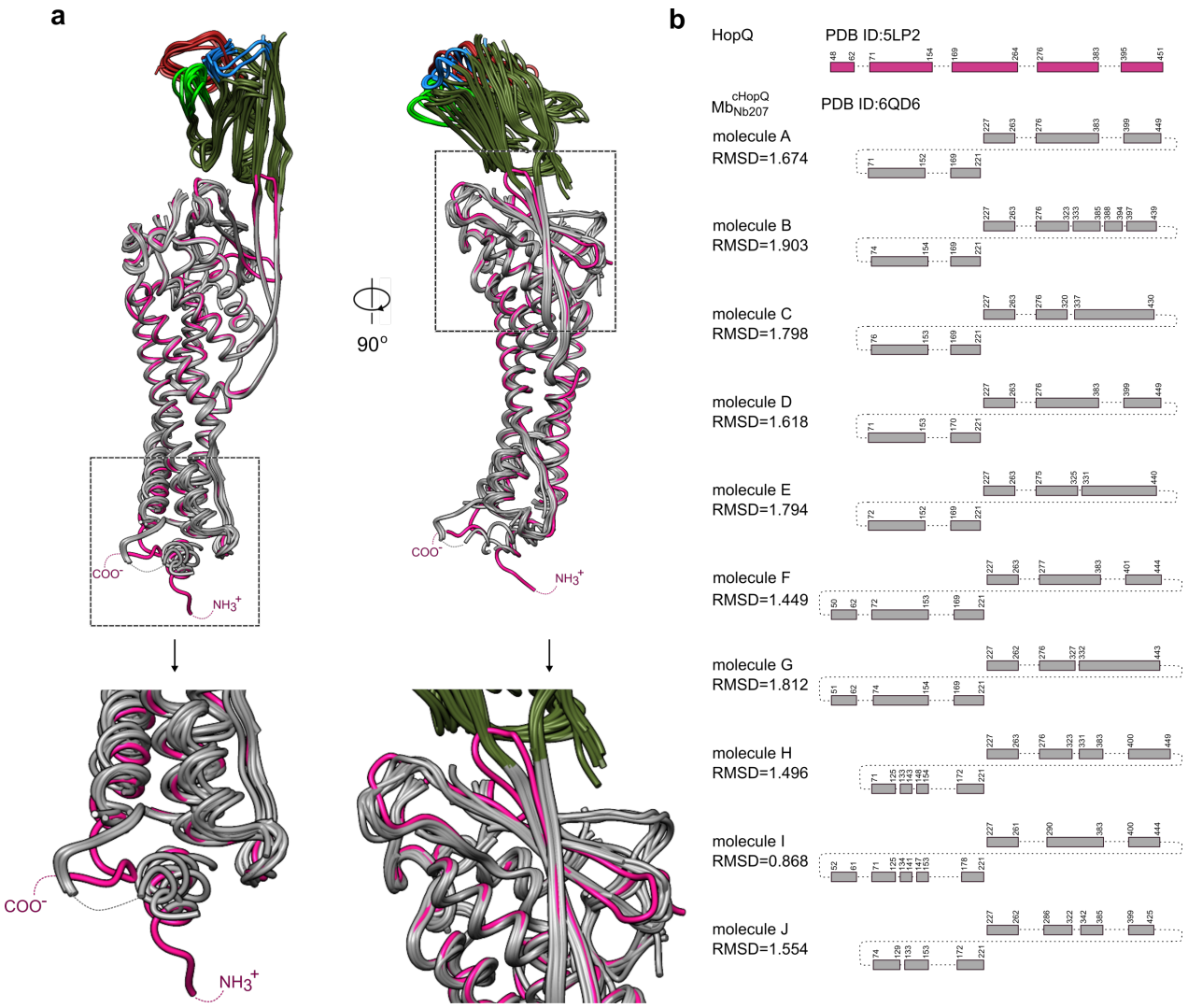
Supplementary Figure 1. Structures of the scaffold proteins HopQ and YgjK. a-c, Cartoon representation of the extracellular adhesin domain of *H. pylori* crystal structure (HopQ, PDB ID: 5LP2). **a**, The flexible N- and C-terminal regions are invisible in the electron density and are indicated by dashed lines. The boxed region is enlarged in (**b**). Residues are numbered according to UniProtKB B5Z8H1. **c**, Secondary structure of the solvent-exposed S3-S4 β-turn. Hydrogen bonds between the backbone atoms are indicated by dotted lines. **d-f,** Cartoon representation of the *E. coli* K12 Glucosidase crystal structure (YgjK, PDB ID: 3W7T). **d**, N- and C-termini are indicated by dots. The boxed region is enlarged in (**e**). Residues are numbered according to UniProtKB P42592. **f**, Secondary structure of the solvent-exposed A'S1-A'S2 β-turn.



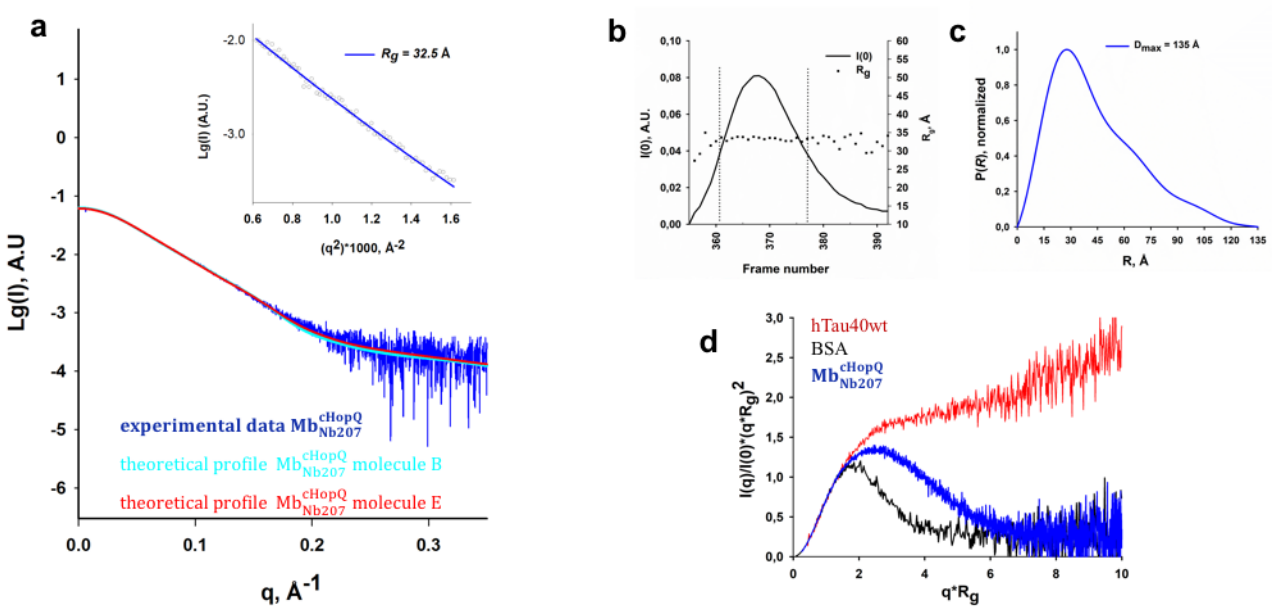
Supplementary Figure 2. Cloning, expression and purification of megabodies Mb^{cHopQ}_{Nb207} and $Mb^{cYgjKE2}_{Nb207}$. a, Gene fragments encoding β-strands B to G of a nanobody are amplified by PCR using TU89 and EP230 primers and cloned into pMESD2 (**b**) to turn a nanobody into the cHopQ-megabody format or cloned in pMESP3E2 (**c**) for the cYgjK format. The residues of nanobody, HopQ and YgjK are numbered according to IMGT, UniProtKB B5Z8H1 and UniProtKB P42592. d-e, Amino acid sequences of Mb^{cHopQ}_{Nb207} (**d**) and $Mb^{cYgjKE2}_{Nb207}$ (**e**). **f-g,** Size exclusion profiles (Superdex 200 PG 16/90) of Mb^{cHopQ}_{Nb207} (**f**) and $Mb^{cYgjKE2}_{Nb207}$ (**g**), purified from the periplasm of *E. coli* by Ni-NTA affinity chromatography. **h,** Representative melting curves of Nb207, Mb^{cHopQ}_{Nb207} and Mb^{cYgjK}_{Nb207} measured by thermal shift assays using the partition hydrophobic-binding dye SYPRO® Orange. Experiments were performed in triplicates and the raw data were fitted to the Boltzmann's equation using Prism 7 software (GraphPad) to calculate melting temperatures (T_m).



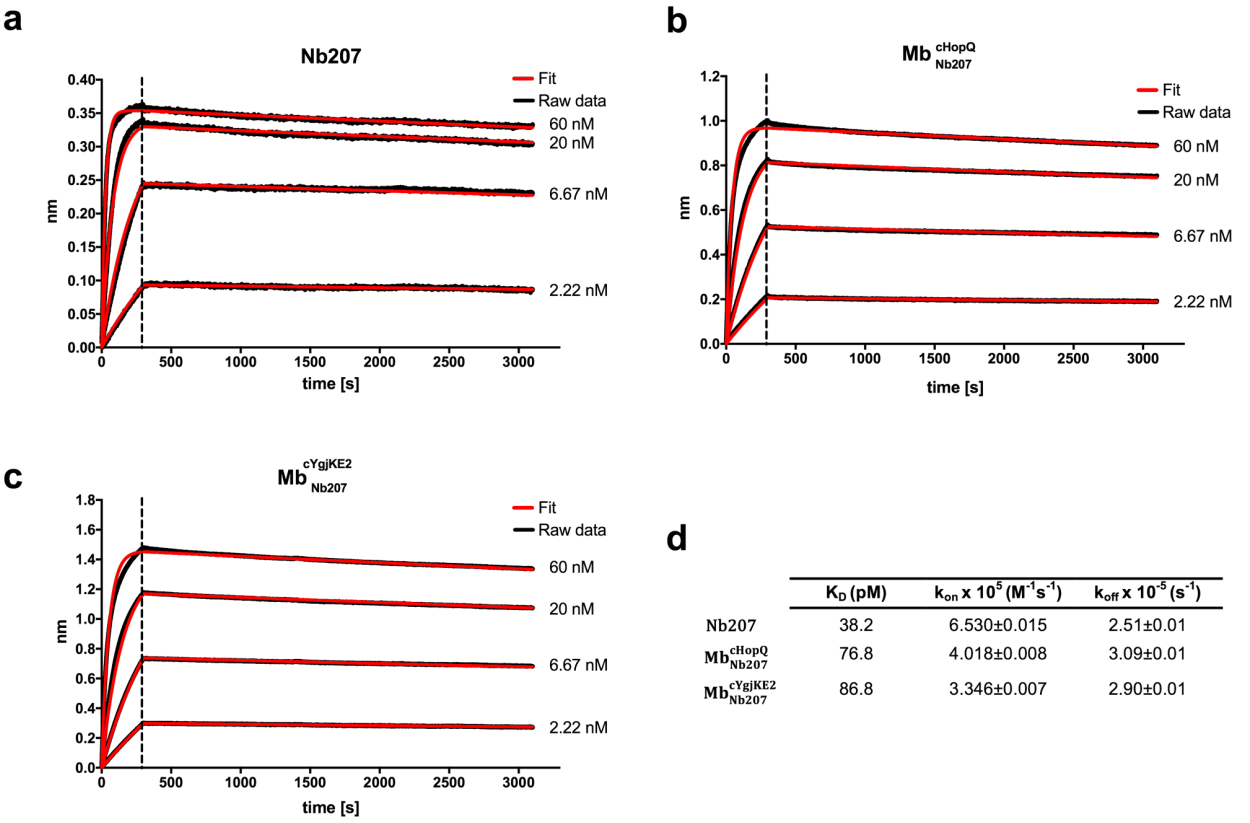
Supplementary Figure 3. Crystal structure of megabody Mb^{cHopQ}_{Nb207}. a, Comparison of the ten Mb^{cHopQ}_{Nb207} molecules present in the asymmetric unit (PDB ID: 6QD6). Molecules were aligned using the Cα atoms of the scaffold protein (cHopQ, grey) manifesting minor bending of the Nb207 part (green). **b,** Schematic representation of the β-sheet topology within the region connecting Nb207 to the scaffold. Hydrogen bonds between backbone atoms are indicated by dotted lines. **c,** 2Fo–Fc electron density map (contoured at 1.0 σ) containing the peptides connecting Nb207 (green) to cHopQ (grey) in molecule F. **d**, Structural comparison of molecules B and E that possess the most distinct bending of the nanobody part (residues 1-13 and 422-532 of Mb^{cHopQ}_{Nb207}). The rotation axis (gold stick) and angle are calculated for the nanobody part.



Supplementary Figure 4. Structural comparison of the parental *H. pylori* adhesion domain to the circularly permutated scaffold in Mb^{cHopQ}_{Nb207}. **a**, Alignment of each molecule of Mb^{cHopQ}_{Nb207} in the asymmetric unit (coloured in grey-green, PDB ID: 6QD6) onto the *H. pylori* adhesin domain (coloured in magenta, PDB code:5LP2). **b,** The RMSD values between the *H. pylori* adhesin domain (magenta) and the different Mb^{cHopQ}_{Nb207} molecules in the asymmetric unit (grey) were calculated from all corresponding C_α atoms that are refined in the respective electron density maps. The block diagrams describe the segments that are visible/invisible in the adhesin crystal structure and ten megabody molecules in the asymmetric unit, taking into account the circularly permutated arrangement of the scaffold protein.

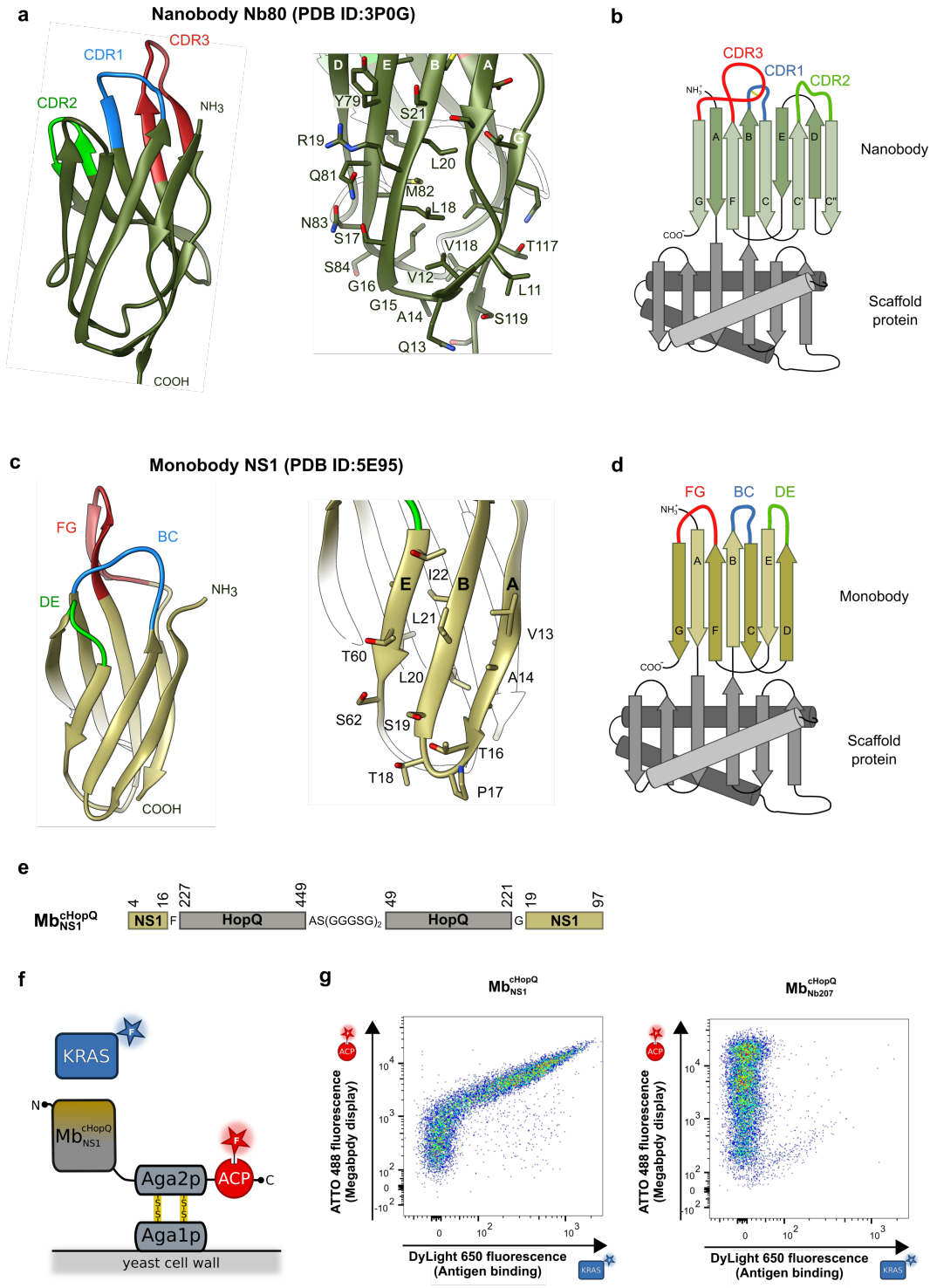


Supplementary Figure 5. SEC-SAXS analysis of megabody Mb^{cHopQ}_{Nb207}. a, Superposition of the experimental scattering profile of Mb^{cHopQ}_{Nb207} (blue) on the theoretical profiles of molecule B (cyan) and molecule E (red) calculated from the X-ray structure (PDB ID: 6QD6) using CRYSOL (χ^2 = 1.699 for molecule B and χ^2 = 2.033 for molecule E). The inset figure shows the linear Guinier region from the experimental scattering curve and is indicative of a non-aggregated protein sample. The respective R_g value is given. **b,** Elution profile of a SEC-SAXS experiment (black line, frame range of the peak 361-377). A stable R_g is observed over the entire elution profile (black squares). **c,** Normalised P(r) profile with derived D_{max} value. **d,** Dimensionless Kratky plot for Mb^{cHopQ}_{Nb207} (blue) in comparison with two reference proteins: the highly flexible hTau40wt (red) and the globular BSA (black).

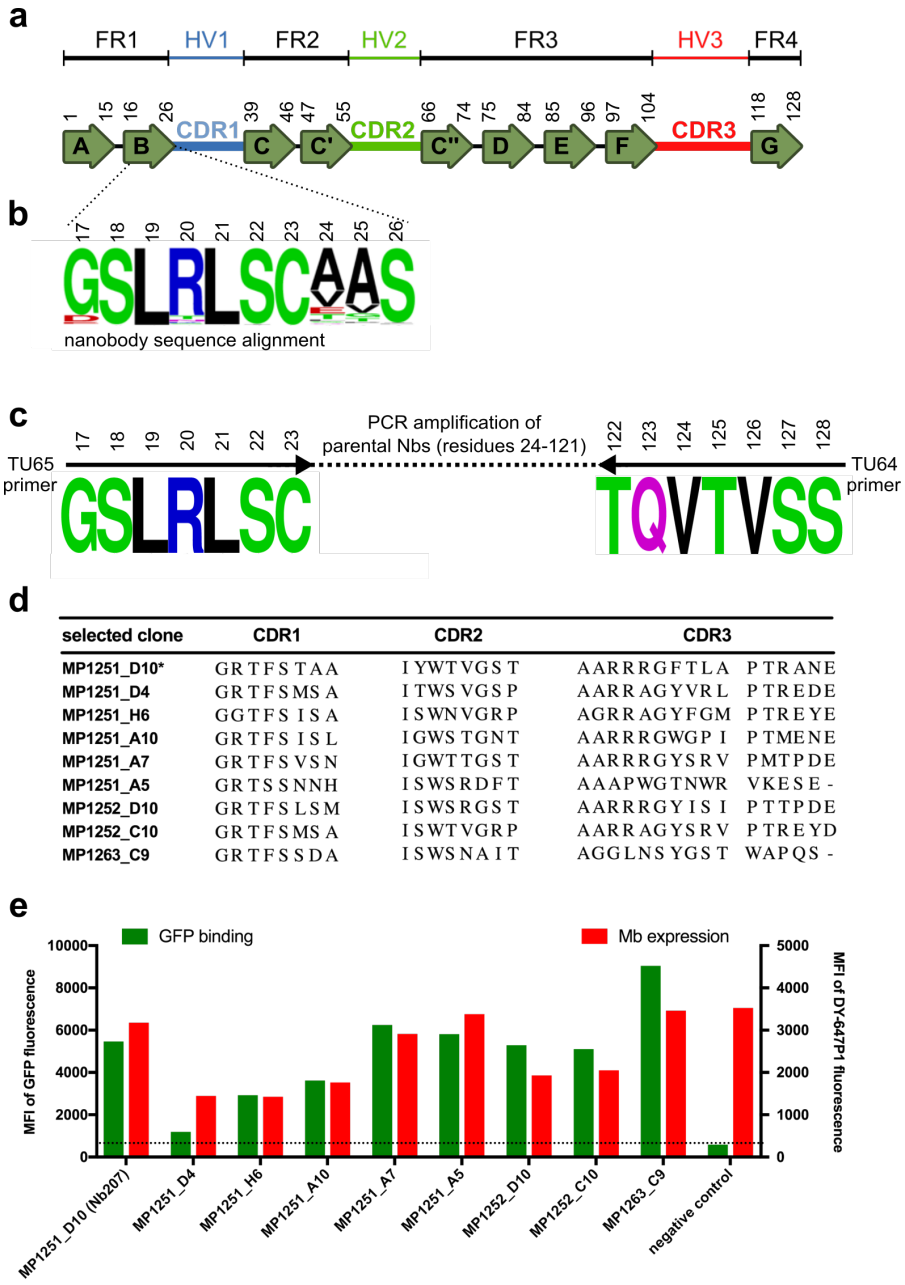


	K_D (pM)	k_{on} x 10^{-5} ($M^{-1}s^{-1}$)	k_{off} x 10^{-5} (s^{-1})
Nb207	38.2	6.530±0.015	2.51±0.01
Mb^{cHopQ}_{Nb207}	76.8	4.018±0.008	3.09±0.01
$Mb^{cYgjKE2}_{Nb207}$	86.8	3.346±0.007	2.90±0.01

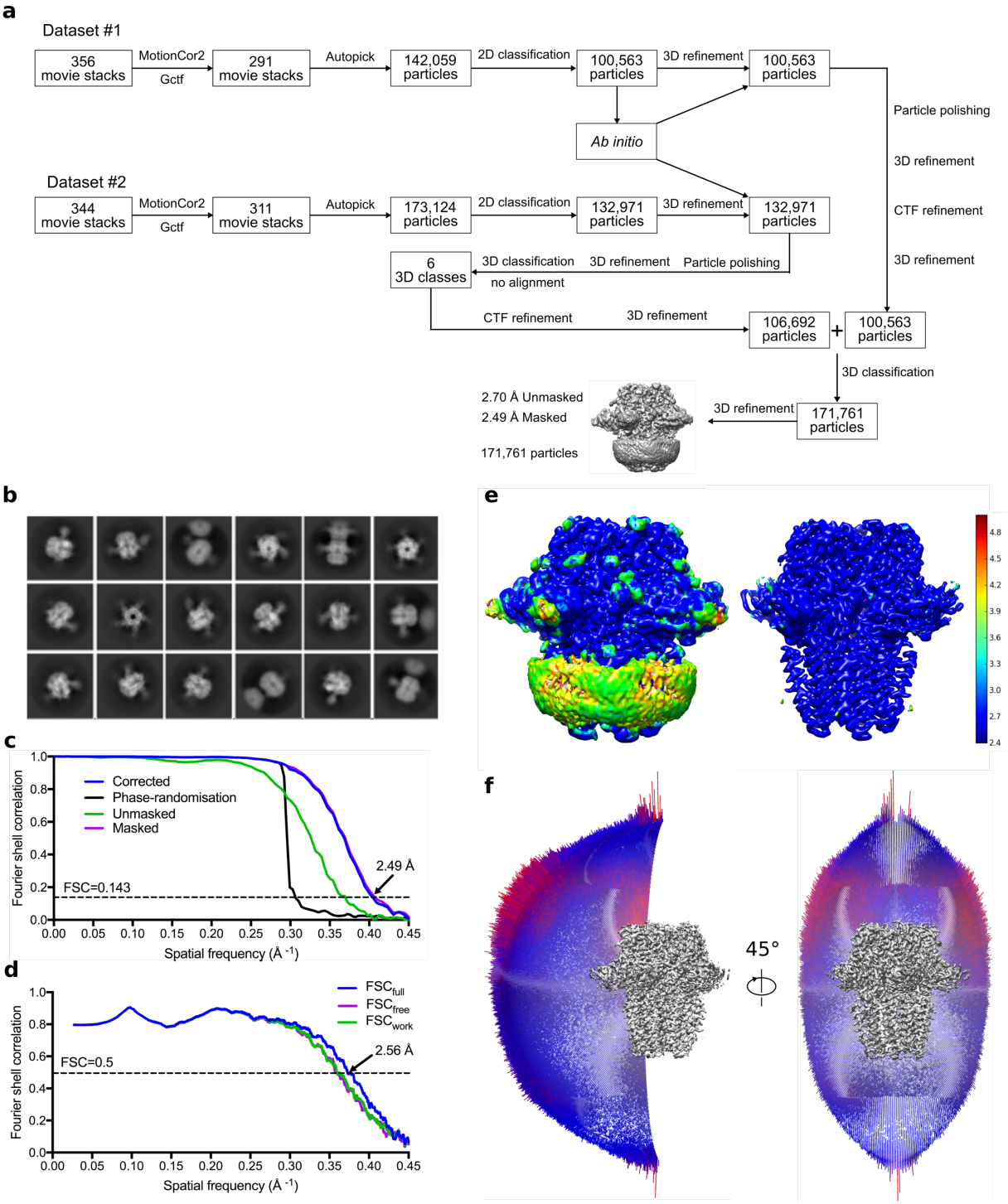
Supplementary Figure 6. Nb207, Mb^{cHopQ}_{Nb207} and $Mb^{cYgjKE2}_{Nb207}$ bind to the cognate antigen with similar affinities. Sensograms of the association and dissociation of Nb207 (**a**), Mb^{cHopQ}_{Nb207} (**b**) and $Mb^{cYgjKE2}_{Nb207}$ (**c**) onto immobilized GFP. Biotinylated GFP was immobilized on a Streptavidin (SA) bio-sensor and the binding kinetics were monitored by bio-layer interferometry (BLI) on OctetRED96 (ForteBio). The measured responses (black lines) were fitted to a monophasic 1:1 binding model (red lines). **d,** Calculated kinetic parameters are shown as mean standard error of the mean (s.e.m.) from n = 3 independent experiments.



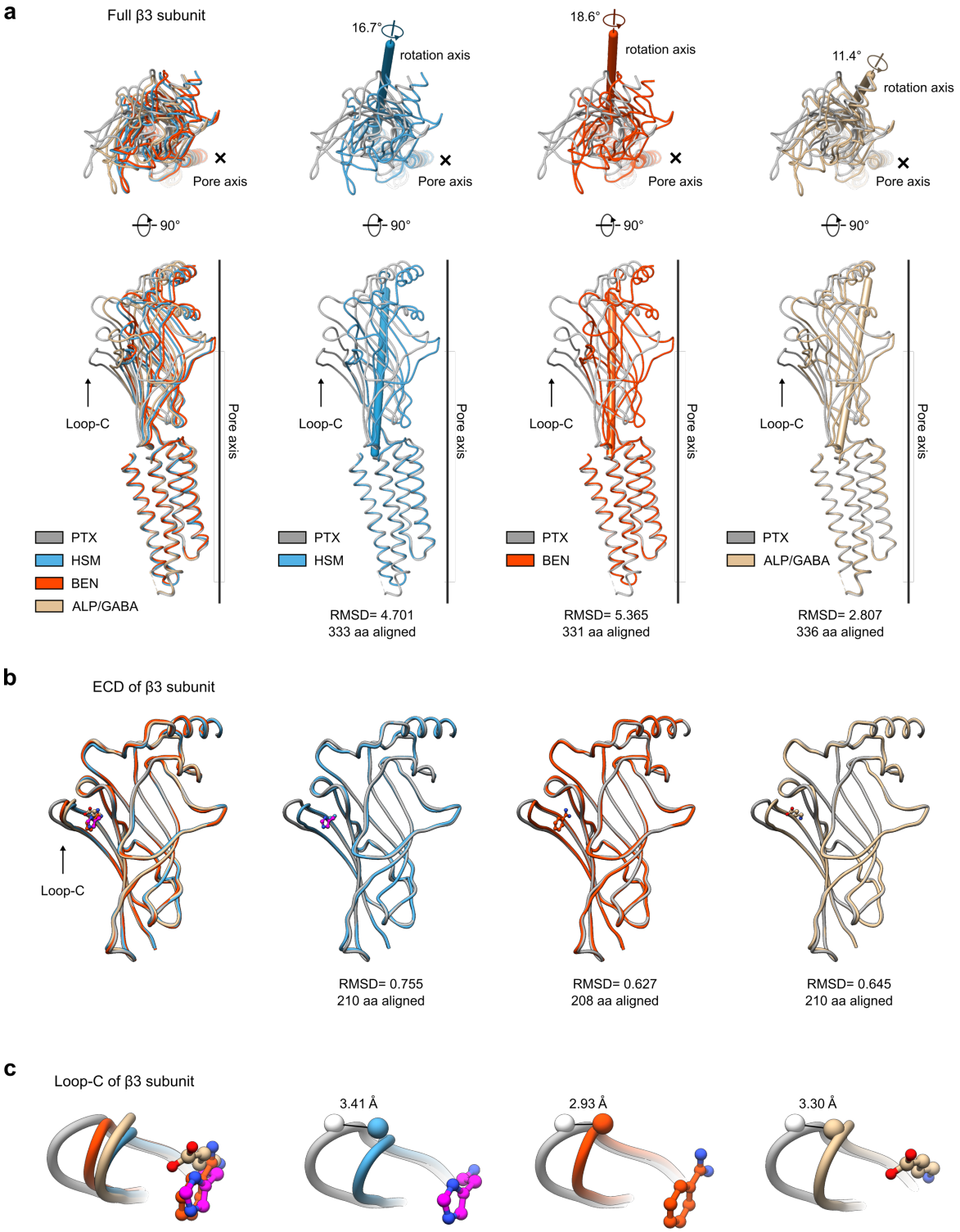
Supplementary Figure 7. Yeast surface display of megabody Mb^{cHopQ}_{NS1} built from the NS1 monobody that was grafted onto cHopQ. a, Tertiary structure of a nanobody, based on crystal structure of Nb80 nanobody (PDB ID: 3P0G). The side chain conformations of β-strand A and β-strand B are indicated. **b**, Molecular design of a megabody that is assembled from a nanobody and a scaffold protein. **c**, Crystal structure of monobody NS1 (PDB ID: 5E95). The side chain conformations of β-strand A and β-strand B are shown. **d**, Molecular design of a megabody that is assembled from a monobody and a scaffold protein. **e**, Schematic representation of the primary structure of Mb^{cHopQ}_{NS1}. **f**, Mb^{cHopQ}_{NS1} was displayed on the surface of yeast as a Mb^{cHopQ}_{NS1}-Aga2p-ACP fusion, and orthogonally stained with CoA-488 (red star) to monitor the display level. Binding of the antigen was monitored by incubating the yeast cells with 100 µM KRAS-DyLight 650 (blue star). **g**. Comparison of flow cytometric dotplots representing yeast cells displaying Mb^{cHopQ}_{NS1} (left panel) and cells displaying Mb^{cHopQ}_{Nb207} (right panel).



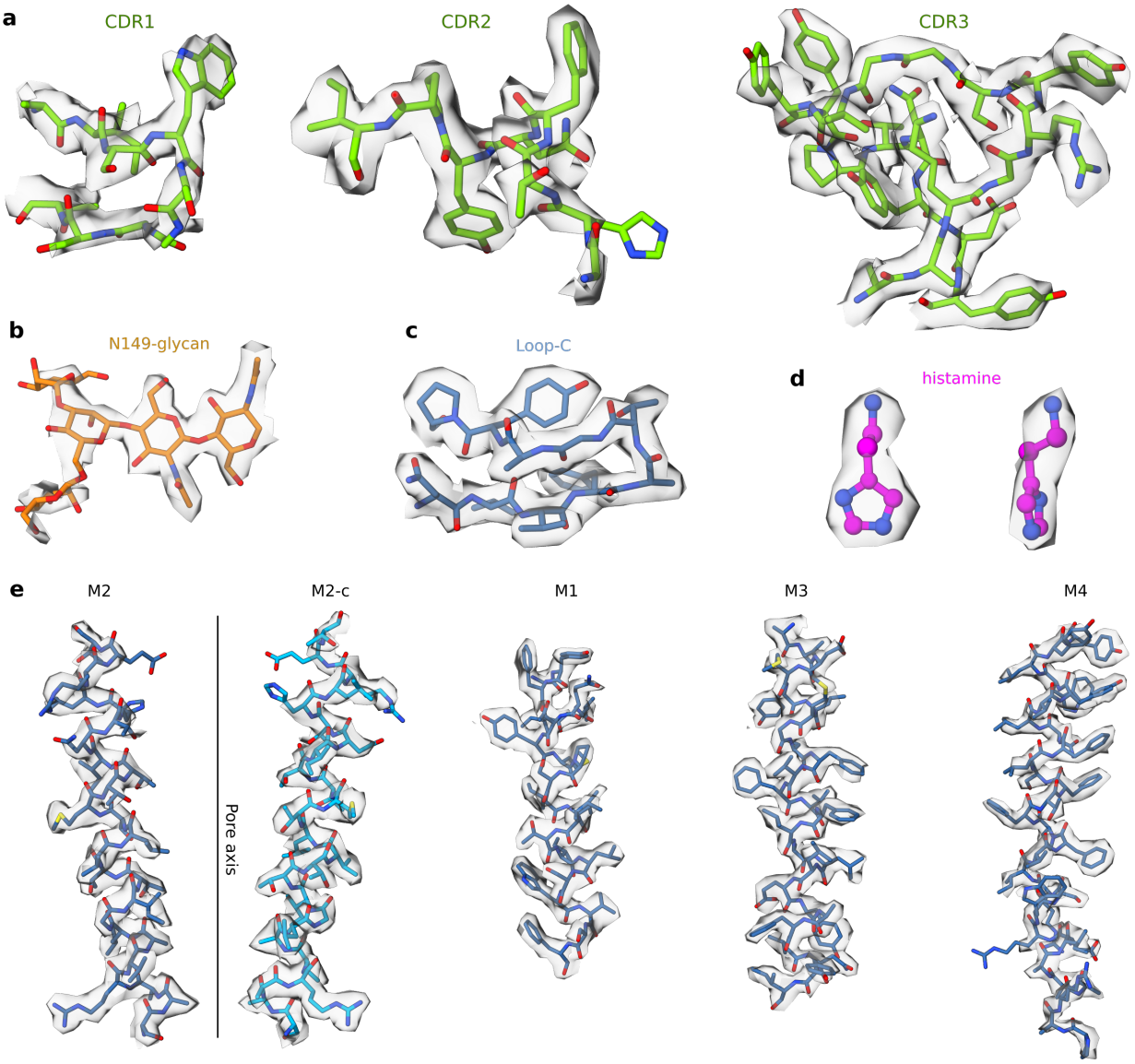
Supplementary Figure 8. Sequences and binding properties of a representative set of GFP-specific megabodies selected by yeast-display a, Schematic representation of a rearranged gene encoding a VHH domain (nanobody) in camelids. Conserved framework (FR, black) and hypervariable (HR, blue, green and red) regions are indicated and encode nine β-strand and three CDR regions, respectively. CDRs and β-strands of nanobodies are defined according to IMGT numbering. **b,** Alignment of β-strand B sequence, originated from 600 nanobody sequences available in-house (three different animals). **c**, PCR product of the *in vivo* maturated nanobody immune libraries amplified using TU65 and TU64 primers (**Supplementary Table 3**). **b,** CDRs composition of the nine megabodies selected by yeast display. CDRs are defined according to IMGT. Selected megabody clone MP12551_D10 contains the same CDRs composition as the nanobody Nb207, which was discovered by phage display (data not shown). **c,** Flow cytometric analysis of GFP binding for i yeast clones displaying nine selected megabodies. Individual yeast clones were orthogonally staining with Co-647 and incubated with 100 nM GFP. For each clone, the mean fluorescent intensities (MFI) of the DY-647P1 fluorescence (display level, red bars) and the GFP fluorescence (antigen binding, green bars) were calculated using the FlowJo software and compared to a cell displaying $Mb^{cHopQ}_{MP1031\_F2}$(the nanobody MP1031_F2 binds human coagulation Factor IX[8], negative control). The MFI of GFP fluorescence of a negative control is indicated as a dotted line.



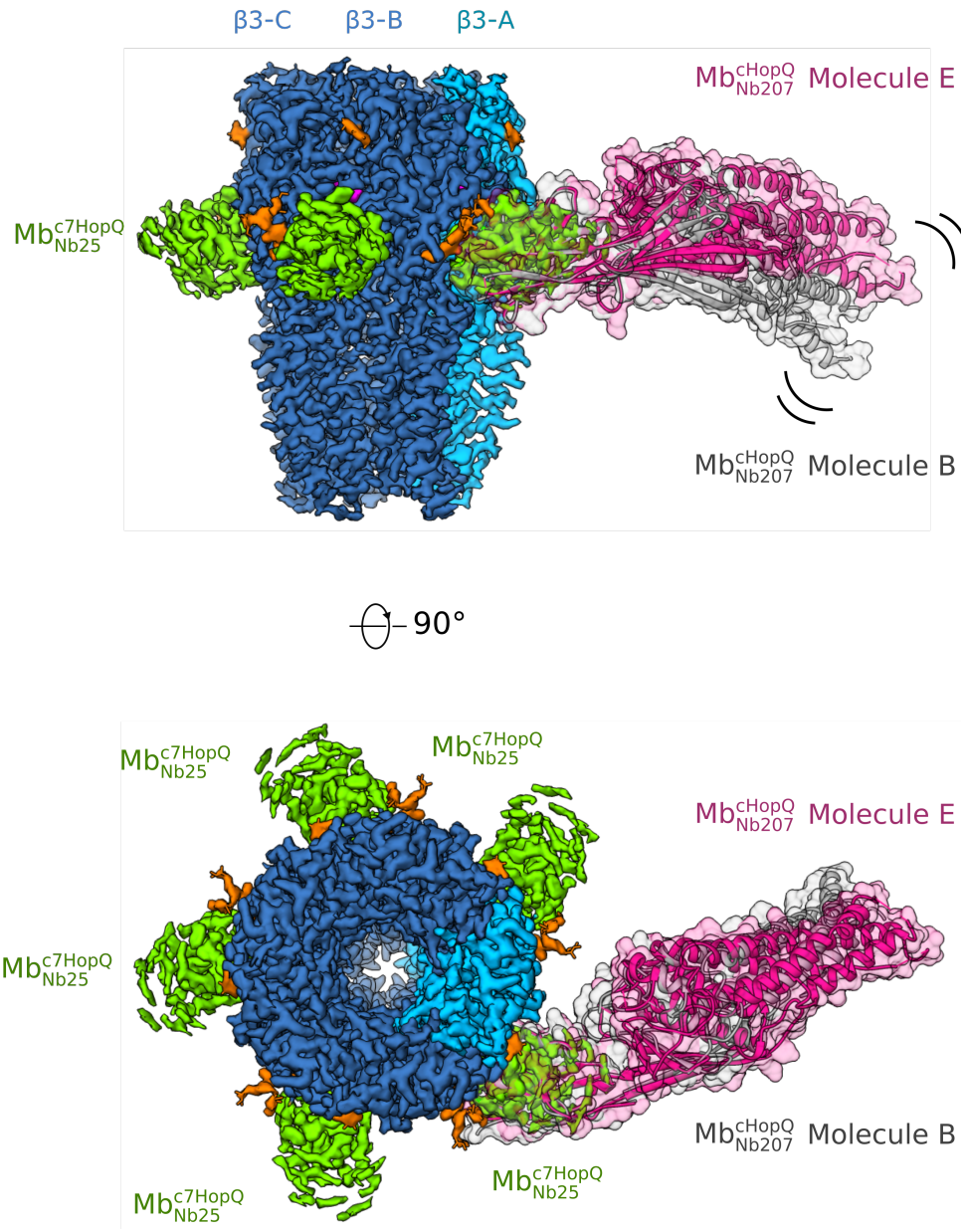
Supplementary Figure 9. Cryo-EM image processing procedure for high resolution reconstruction of β3 GABA$_A$R in complex with to Mb$^{c7HopQ}_{Nb25}$. a. Graphical overview of cryo-EM data collection and image processing (see Methods). **b,** 2D class averages used for cryo-EM map reconstructions. Aligned micrographs were obtained using FEI Titan Krios, Falcon3 detector and VPP (box size of 256 Å). **c,** FSC curves for the 3D reconstruction using gold-standard refinement in RELION. Data is shown for the phase randomisation, unmasked, masked and phase-randomisation-corrected masked maps. **d,** FSC curves for the atomic model refinement. Data is shown for model versus summed map (FSC$_{full}$), model refined in half-map 1 versus half-map 1 (FSC$_{work}$), and model refined in half-map 1 versus half-map 2 (FSC$_{free}$). **e**, Unsharpened cryo-EM map colored by local resolution (estimated using ResMap) shown at a lower contour level (left) and at a higher level (right). **f**, Angular-distribution histogram of particles used in calculating the final 3D reconstruction for the of histamine bound β3 GABA$_A$ receptor in a complex with Mb$^{c7HopQ}_{Nb25}$.



Supplementary Figure 10. Structural analysis of β3 subunits of PTX-bound, ALP/GABA-bound α1β3γ2L $GABA_A$ receptor and BEN-bound, HSM-bound β3 $GABA_A$ receptor structures. a-c Superposition of full β3 subunits (**a**), ECD (**b**) and Loop-C (**c**) of PTX-bound α1β3γ2L $GABA_A$ (grey, PDB ID: 6HUG), ALP/GABA-bound α1β3γ2L $GABA_A$ (khaki, PDB ID: 6HUO), BEN-bound β3 $GABA_A$ (orange, PDB ID: 4COF) and HSM-bound β3 $GABA_A$ (blue, PDB ID: 6QFA) receptors. **a** Superposition of full subunits on the basis of the global TMD alignment reveals the relative β3 ECD motions upon binding to PTX, ALP/GABA, BEN and HSM where rotation axis (sticks) and angles are indicated. The RMSD values are shown for full β3 subunits. **b** Superposition of ECDs (residues 1-217), where RMSD values are shown for ECDs. **c** Superposition of Loop-C, where differences in distances (Å) between the selected Thr202 C_α atoms (shown as spheres) are indicated with lines. The bound histamine (HSM), benzamidine (BEN) and GABA are indicated in magenta, orange and khaki, respectively.



Supplementary Figure 11. Histamine-bound β3 $GABA_A$ receptor model-map validation and electron microscopy density. a-e, Electron microscopy density segments of Mb^{c7HopQ}_{Nb25} CDRs (**a**) and β3 $GABA_A$ receptor N149-glycan (**b**), Loop-C (**c**), histamine (**d**), α-helices of TMD regions (**e**) (EMDB ID: 4542, PDB ID: 6QFA). Sharpened density maps are contoured at 0.08.



Supplementary Figure 12. Molecular docking of the Mb^{cHopQ}_{Nb207} crystal structure onto the cryoEM map of the β3 $GABA_A$ receptor in a complex with Mb^{c7HopQ}_{Nb25}. The two most distinct Mb^{cHopQ}_{Nb207} molecules from the asymmetric unit of the crystal structure (molecules B and E, PDB ID: 6QD6) are coloured in grey and magenta, respectively. They were aligned to the part of Mb^{c7HopQ}_{Nb25} that was refined in the cryo-EM structure of the β3 $GABA_AR$ in a complex with Mb^{c7HopQ}_{Nb25} (EMDB ID: 4542, PDB ID: 6QFA).

Supplementary Table 1. Amino acid sequence of Nb207.

Nb207
QVQLQESGGGLVQAGGSLRLSCAASGRTFSTAAMGWFRQAPGKERDFVAGIYWTVGSTY YADSAKGRFTISRDNAKNTVYLQMDSLKPEDTAVYYCAARRRGFTLAPTRANEYDYWG QGTQVTVSS

Supplementary Table 2. Data collection and refinement statistics.

	Mb^{cHopQ}_{Nb207}
Data collection	
Space group	P1
Cell dimensions	
a, b, c (Å)	71.17, 92.92, 244.22
α , β , γ (°)	92.05, 96.93, 112.15
Resolution (Å)	41.45-2.84 (2.90-2.84) *
R_{meas}	0.05 (0.66)
$I / \sigma I$	11.65 (1.45)
Completeness (%)	95.6 (94.5)
Redundancy	1.78 (1.77)
Refinement	
Resolution (Å)	38.85-2.84 (2.90-2.84)
No. reflections	230255 (13649)
R_{work} / R_{free}	0.225/0.251
No. Atoms	
Protein	33077
Ion Cl^-	1
Water	114
B factor	
Protein	99.9
Ion Cl^-	91.9
Water	91.7
R.m.s. deviations	
Bond lenghts (Å)	0.02
Bond angles (°)	1.87

*Values in parentheses are for highest resolution shell.

Supplementary Table 3. Cryo-EM data collection, refinement and validation statistics.

	β3 $GABA_AR$ - Mb_{Nb25}^{c7HopQ} complex EMDB: 4542 PDB: 6QFA
Data collection and processing	
Microscope, location	Krios-II, MRC-LMB
Magnification	75,000
Voltage (kV)	300
Detector	Falcon 3EC with VPP
Electron Dose ($e^-/Å^2$)	30
Exposure time (s)	60
Pixel Size (Å)	1.07
Dose rate (e^-/pixel/s)	0.4
Frame number	75
Defocus Range (μm)	-0.7 to -0.5
Micorgraphs collected (no.)	700
Micorgraphs selected (no.)	602
Initial particle images (no.)	315,183
Final particle images (no.)	171,761
Symmetry imposed	C5
Map resolution (Å)	2.49
FSC threshold	0.143
Map resolution range (Å)[a]	2.25-5.95
Refinement	
Initial model used (PDB code)	4COF, 5O8F
Model resolution (Å)[b]	2.56
FSC threshold	0.5
Model resolution range (Å)	2.56
Map sharpening *B* factor ($Å^2$)	-68
Model composition	
Protein residues	2,270
Non-hydrogen atoms	18,755
Protein atoms	18,240
N-linked glycan atoms	375
HSM atoms	40
R.m.s. deviations	
Bond lengths (Å)	0.005
Bond angles (°)	0.761
Validation	
MolProbity score	1.28
Clashscore	5.3
Poor rotamers (%)	0
Ramachandran plot	
Favoured (%)	98.17
Allowed (%)	1.83
Disallowed (%)	0

[a]Local resolution range. [b]Resolution at which FSC between map and model is 0.5.

Supplementary Table 4. Primer list.

	5' – 3' sequence
EP230	AGGACTGCTCTTCCACTGGAGACGGTGACCTGGGT
TU64	CCCTCCACCAGAGCCACCTCCCAAGCTTGAGACGGTGACCTGGG
TU65	GCATGTAACCACATCAAAGTATGGATCCCTGAGACTCTCCTG
TU89	CCTTGAGCTCTTCGTCCCTGAGACTCTCCTG