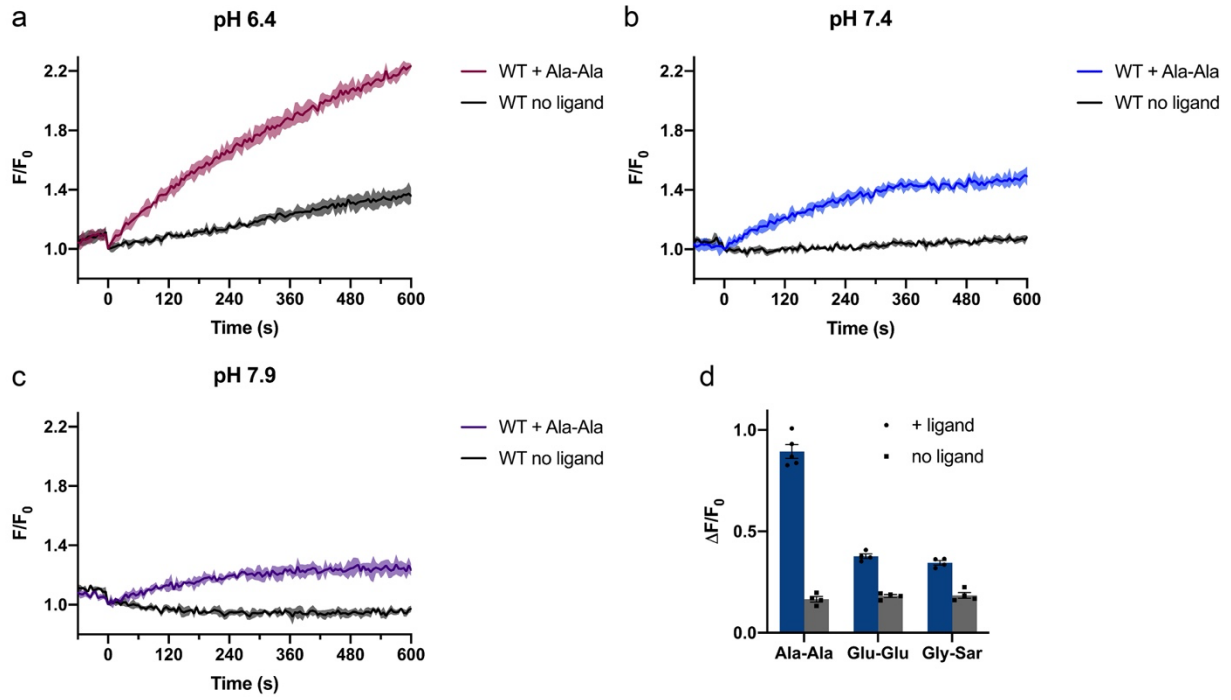

Figure S1. Substrate transport of PepT1 monitored by cytosolic pH, related to Figure 1. Time courses of pH changes in HEK293 cells induced by the peptide transport in the outside pH of 6.4 (**a**), 7.4 (**b**) and 7.9 (**c**). Solid lines represent the mean and shaded regions represent SD of four repeats. **d**, Cytosolic pH changes induced by different peptide ligands in the outside pH of 6.9.

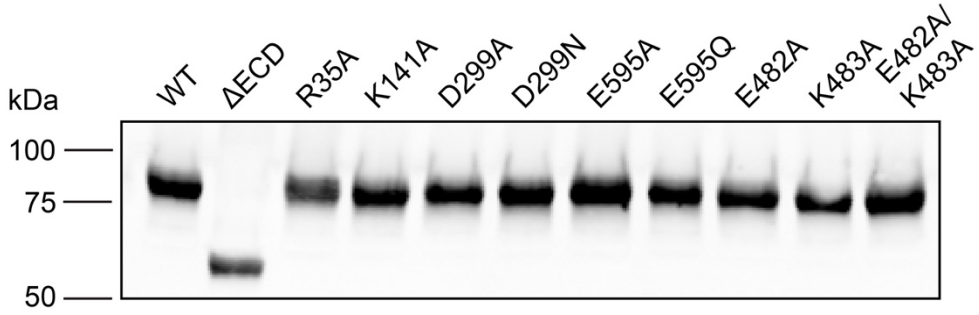

Figure S2. Assessment of expression level of GFP-tagged WT and mutant horse PepT1 in HEK293 cells by western blot, related to Figure 1.

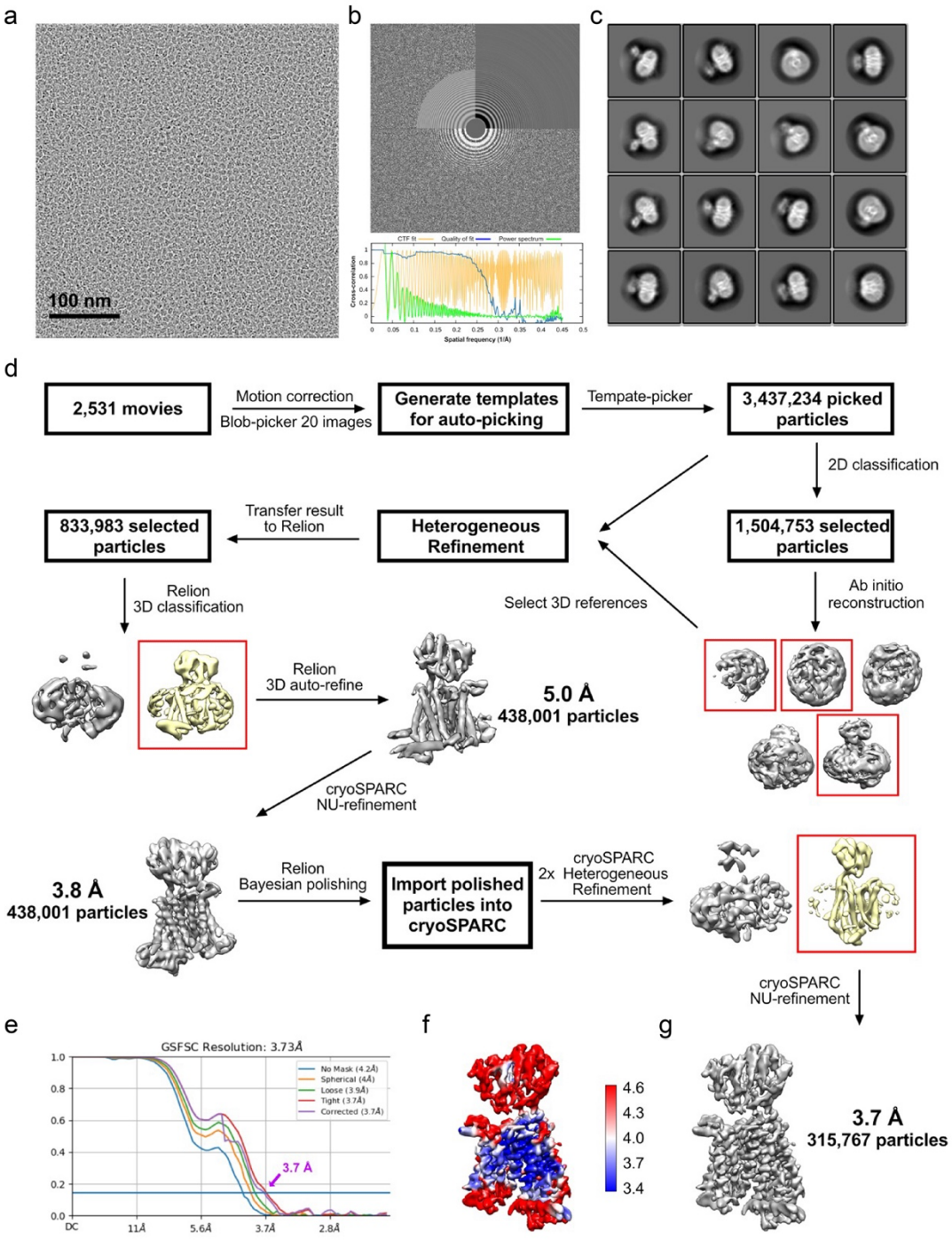

Figure S3. Cryo-EM data processing, related to Figure 2. a, A representative micrograph of horse PepT1, with **b**, its Fourier transform and contrast transfer function (CTF) fitting. **c**, Representative 2D class averages. **d**, The flow chart for data processing of PepT (methods). **e**, The

gold-standard Fourier shell correlation (FSC) curve for the final map shown in **f**, and the local resolution map of horse PepT1 shown in **g**.

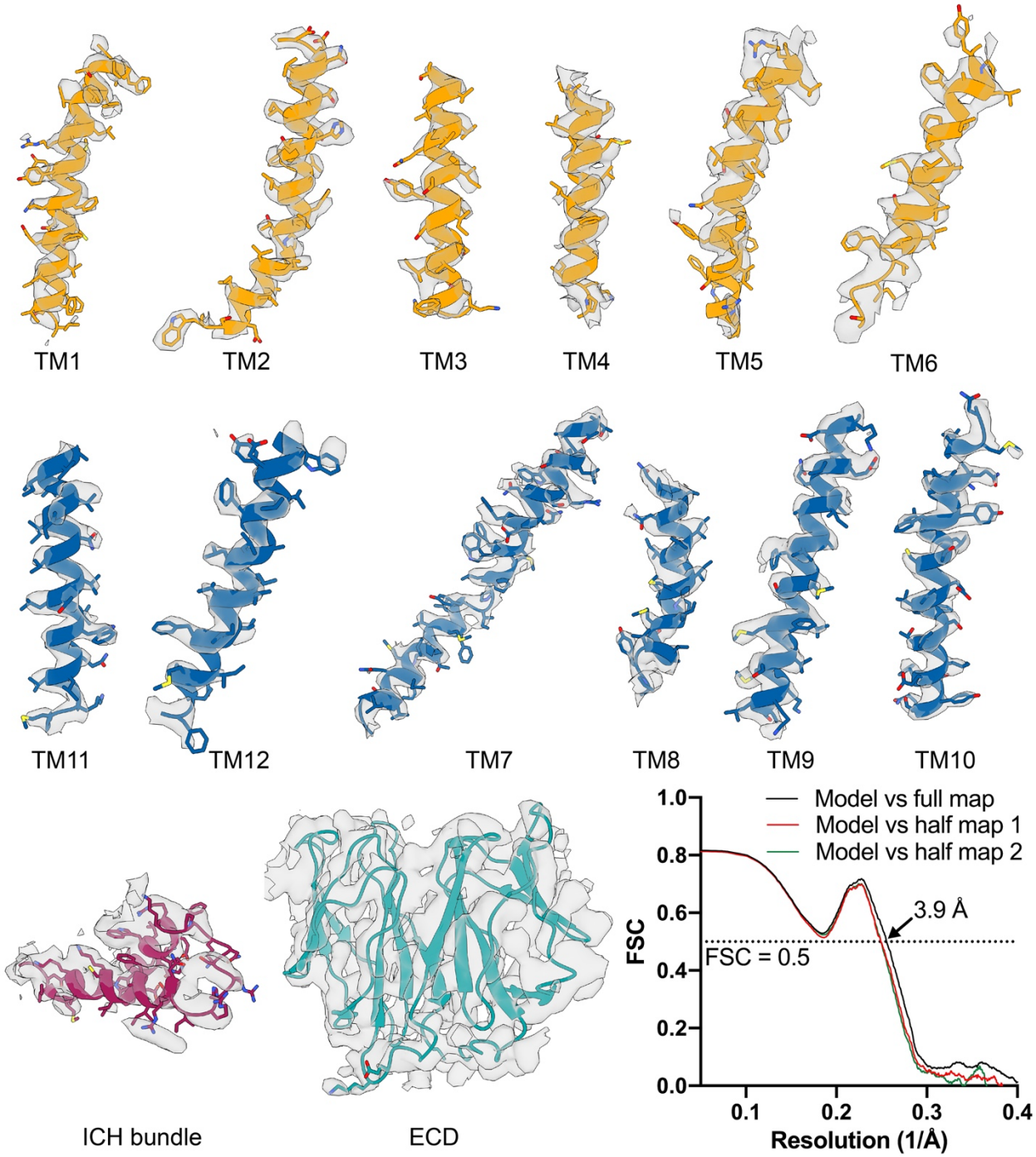

Figure S4. Cryo-EM map of TM helices, ICH bundle, and ECD of horse PepT1 in nanodisc, related to Figure 2. Lower right: FSC curves of the model versus the full map (black) and two half maps (red and green).

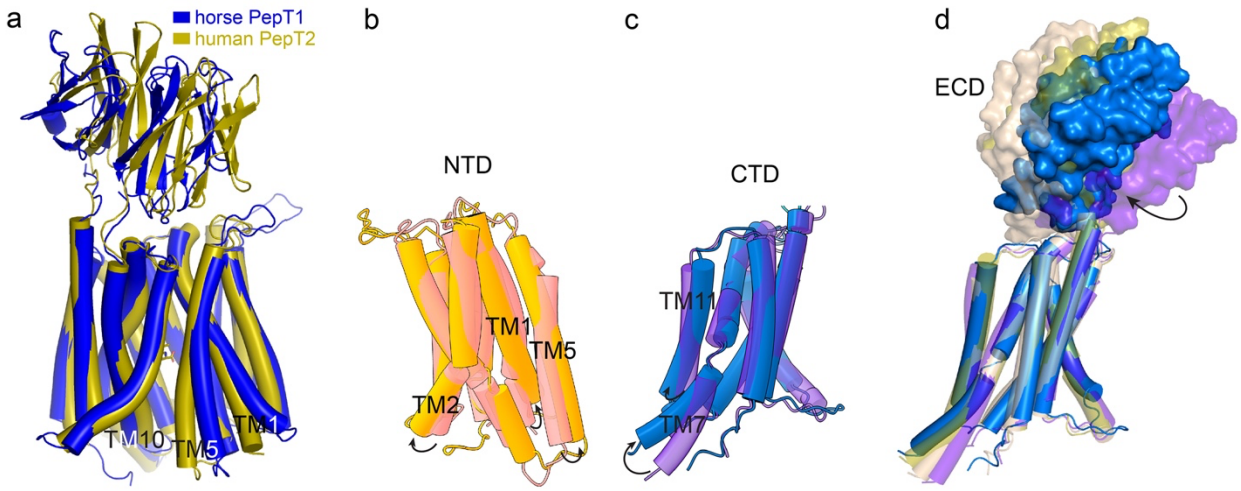

Figure S5. Structural comparison among mammalian PepTs, related to Figure 2. a, Overall structural comparison of horse PepT1 (blue) with the inward-occluded human PepT2 (olive, PDB ID: 7PMY). Structural alignments of **b**, NTDs and **c**, CTDs of horse PepT1 and the outward-open rat PepT2 (PDB ID: 7NQK) colored in translucent pink and purple for NTD and CTD, respectively. The horse PepT1 are colored as in **Figure 2c**. **d**, Variations in the relative position of ECD to CTD among mammalian PepT structures. Blue, inward-open horse PepT1 (this study); purple, outward-facing rat PepT2 (PDB ID: 7NQK); wheat, outward-facing human PepT1 (PDB ID: 7PMX); olive, inward-facing human PepT2 (PDB ID: 7PMY). Arrows indicate conformational differences of the aligned structures from horse PepT1.

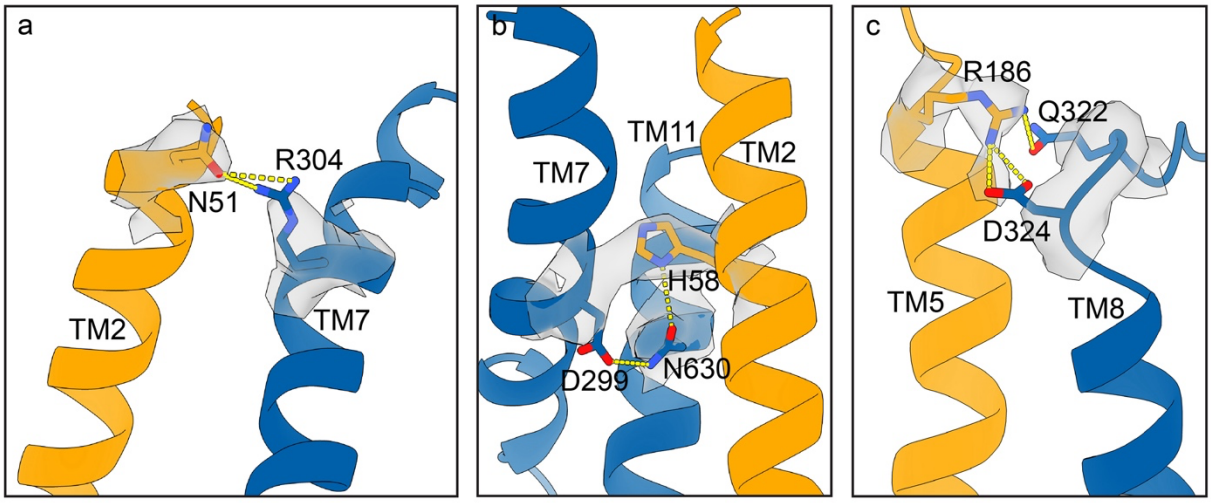

Figure S6. **Extracellular gates in TM domains of PepT1, related to Figure 3.** Hydrogen bonding and ionic interactions (yellow dash line) between NTD and CTD of horse PepT1. Electron density around the interacting residues is shown as translucent gray surface at 6σ.

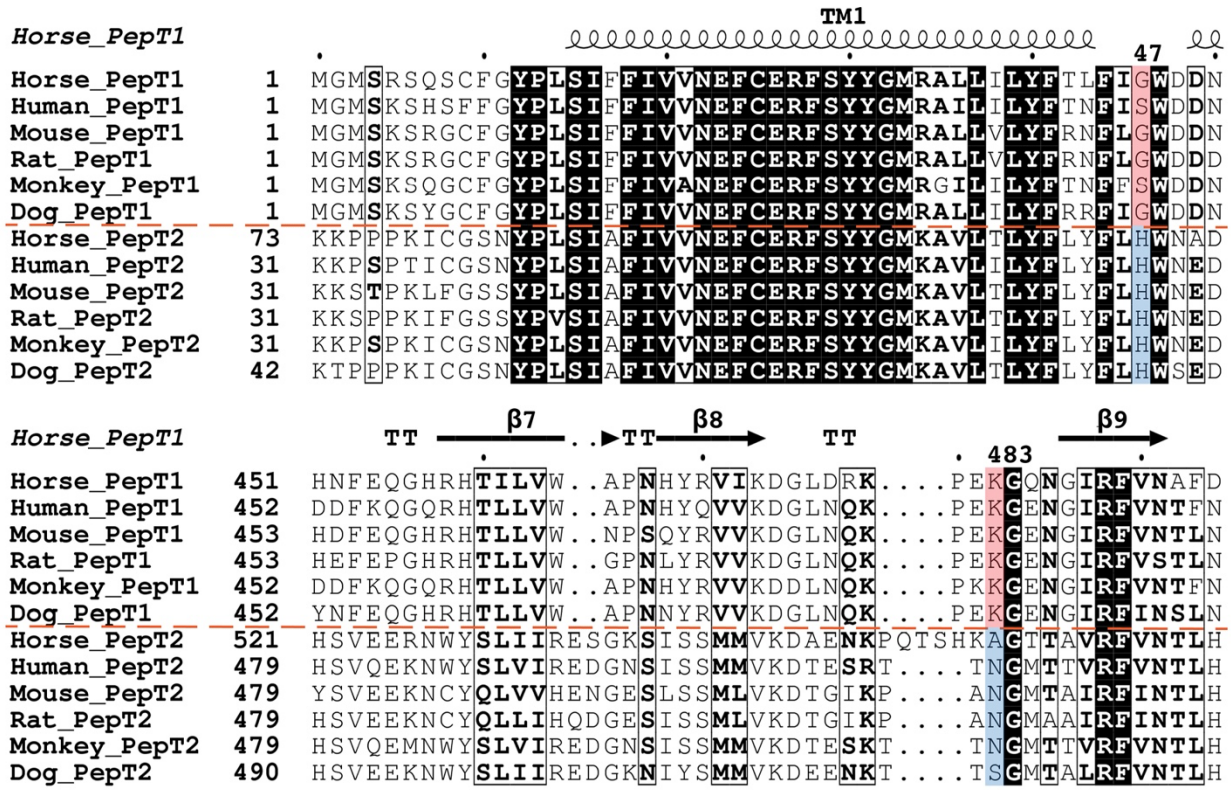

Figure S7. **Sequence alignment of mammalian PepT1 and PepT2, related to Figure 3.** PepT1 and PepT2 from horse (*Equus caballus*; UniProt ID: F6SG69 and F6R282), human (*Homo sapiens*; P46059 and Q16348), mouse (*Mus musculus*; Q9JIP7 and Q9ES07), rat (*Rattus norvegicus*; P51574 and Q63424), monkey (*Macaca mulatta*; F7H3Q3 and Q6WFZ7), and dog (*Canis familiaris*; F1PTV0 and E2QWX1) are aligned using the Clustal Omega server. Secondary structural elements of horse PepT1 are marked above the alignment, and an orange dash line separates the PepT1 and PepT2 group. Conserved residues in both PepT1 and PepT2 are indicated by black highlight and bold letter. Residue G47 and K483 of horse PepT1 and their equivalent residues are highlighted in red in the PepT1 group and in blue in the PepT2 group, respectively. The plot is prepared using the ESPript3 server.

Table S1 | Summary of cryo-EM data collection, processing, and refinement, related to STAR Methods.

Protein	Horse PepT1-MSP1D1 nanodisc
Cryo-EM Data Collection	
Voltage (kV)	300
Magnification (x)	105,000
Pixel Size (Å)	1.114
Electron exposure ($e^-/Å^2$/frame)	1.56
Defocus range (μm)	[-2.1, -1.9]
Number of image stacks	2,531
Number of frames per stack	32
Cryo-EM Data Processing	
Initial number of particles	3,437,234
Final number of particles	315,767
Symmetry imposed	C1
Map sharpening B factor ($Å^2$)	150
Map resolution (Å)	3.6
Map resolution range (Å)	3.4 – 4.6
FSC threshold	0.143
Model Refinement	
Number of amino acids	667
Total non-hydrogen atoms	5,210
Model-to-map resolution (Å)	3.9
Average B factor ($Å^2$)	97.45
Bond length RMSD (Å)	0.004
Bond angle RMSD (°)	0.840
Ramachandran Plot	
Favored (%)	91.28
Allowed (%)	8.57
Outliers (%)	0.15
Rotamer outliers (%)	0.35
EMRinger Score	2.21

Table S2 | Primers used in this study, related to STAR Methods.

Primer name	Sequence (5' → 3')
ΔECD_GGSA_For	CGTTCAAGTGGAGATCGACGGCGGCTCCGCCGTCAACATGGC CCTGCAG
ΔECD_GGSA_Rev	CTGCAGGGCCATGTTGACGGCGGAGCCGCCGTCGATCTCCAC TTGAACG
R35A_For	GATTCTCATACTACGGCATGGCCGCCCTGCTCATCTTGTAC
R35A_Rev	GTACAAGATGAGCAGGGCGGCCATGCCGTAGTATGAGAATC
K141A_For	CGGAACCGGTGGCATCGCCCCTTGCGTGTCGGCTTTC
K141A_Rev	GAAAGCCGACACGCAAGGGGCGATGCCACCGGTTCCG
D299A_For	GATGTTCTGGGCCCTCTTCGCCCAGCAAGGTTCTCGCTG
D299A_Rev	CAGCGAGAACCTTGCTGGGCGAAGAGGGCCCAGAACATC
D299N_For	GATGTTCTGGGCCCTCTTCAACCAGCAAGGTTCTCGCTG
D299N_Rev	CAGCGAGAACCTTGCTGGTTGAAGAGGGCCCAGAACATC
E595A_For	CTTCCTCTTGACTTGCGGAGCCGTGGTCTTCTCAGTGACAGG
E595A_Rev	CCTGTCACTGAGAAGACCACGGCTCCGCAAGTCAAGAGGAAG
E595Q_For	CTTCCTCTTGACTTGCGGACAGGTGGTCTTCTCAGTGACAGG
E595Q_Rev	CCTGTCACTGAGAAGACCACCTGTCCGCAAGTCAAGAGGAAG
E482A_For	CTTGGACAGGAAGCCGGCCAAGGGCCAGAACGGAATC
E482A_Rev	GATTCCGTTCTGGCCCTTGGCCGGCTTCCTGTCCAAG
K483A_For	GGACAGGAAGCCGGAAGCCGGCCAGAACGGAATCCG
K483A_Rev	CGGATTCCGTTCTGGCCGGCTTCCGGCTTCCTGTCC
E482AK483A_For	CTTGGACAGGAAGCCGGCCGCCGGCCAGAACGGAATCCG
E482AK483A_Rev	CGGATTCCGTTCTGGCCGGCGGCCGGCTTCCTGTCCAAG