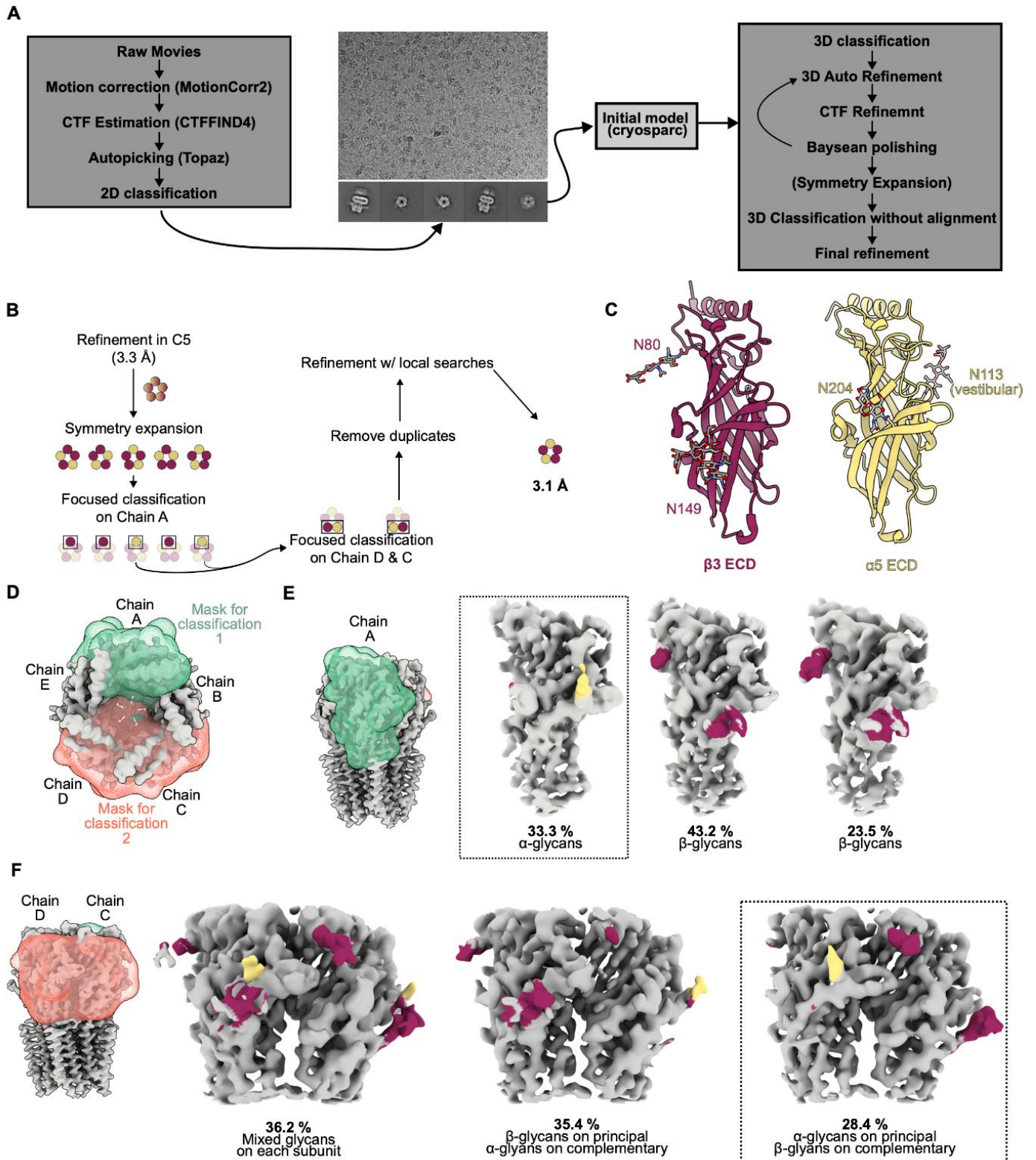


Supplementary



Supplementary Figure 1. Cryo-EM data-processing workflow for $\alpha 5\beta 3$ -EM structures.

(A) Generalized data-processing workflow for datasets in this study.

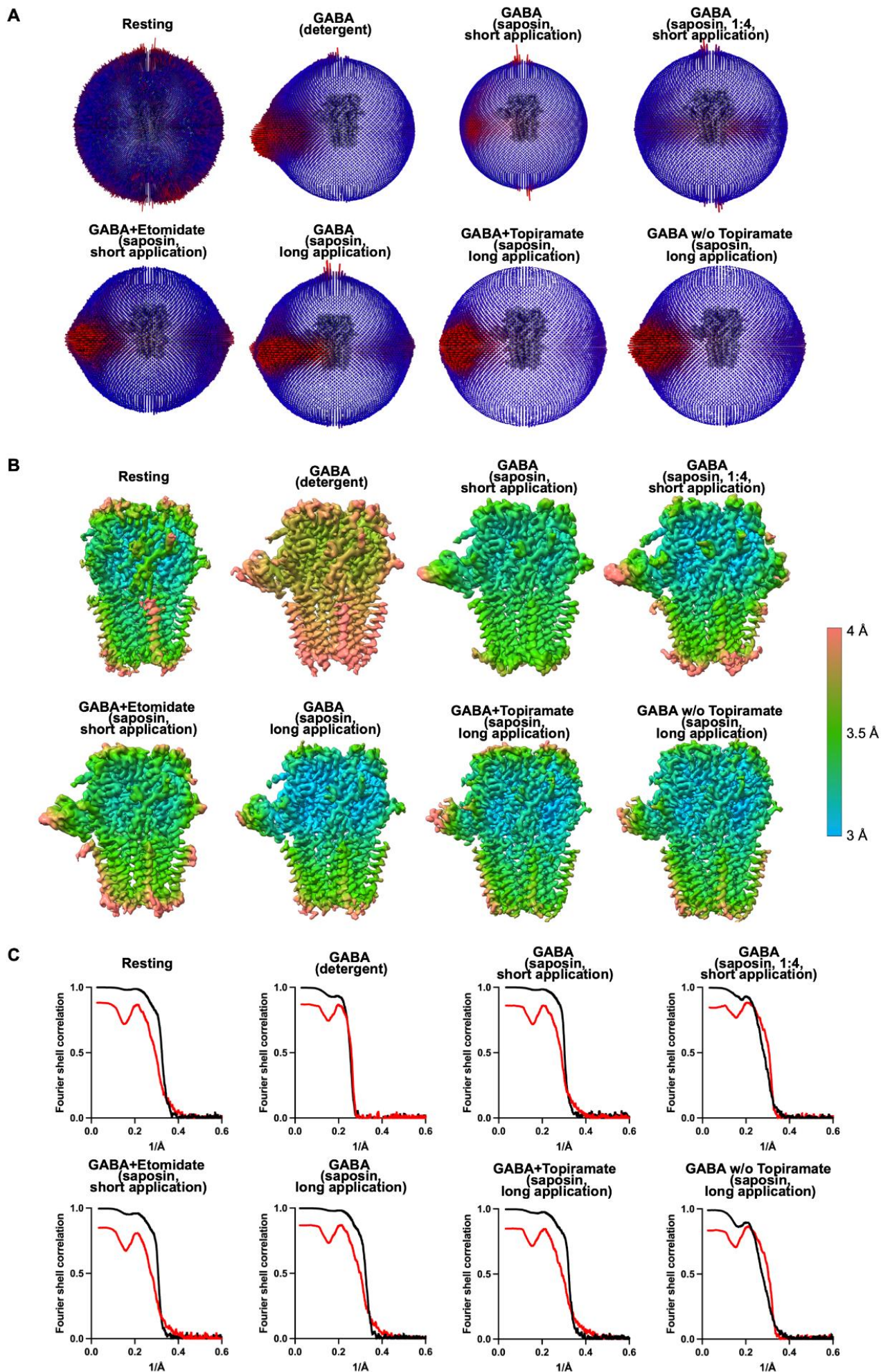
(B) Symmetry-expansion workflow for reconstruction of heteromeric receptors without fiducials, applied here to the structure in the resting-like state. The particle stack was first refined in C5 symmetry, then expanded 5-fold such that each particle was present in each of the 5 possible alignments, as shown by cartoons. Focused classification was then used to pull out particle replicates with $\alpha 5$ subunits the Chain A position, based on the differences in glycosylation relative to $\beta 3$ subunits. This subset of particle replicates was subjected to a second round of focused classification with a mask around the ECDs of Chains C and D. The class with a second $\alpha 5$ subunit as Chain D gave the cleanest separation of differentially glycosylated subunits, and was used for final refinement following removal of a small fraction of duplicated particles (<10% of total remaining).

(C) Individual $\beta 3$ (left, magenta) and $\alpha 5$ ECDs (right, yellow) showing the positions of glycosylation used to identify subunits during the focused-classification steps highlighted in panels d-f. Whereas most glycans faced the receptor surface, $\alpha 5$ -N113 occupied the extracellular vestibule of the channel.

(D) Masks used for first (green) and second (salmon) iterative classification steps to align symmetry-expanded particles, overlaid with the initial C5 refinement (gray).

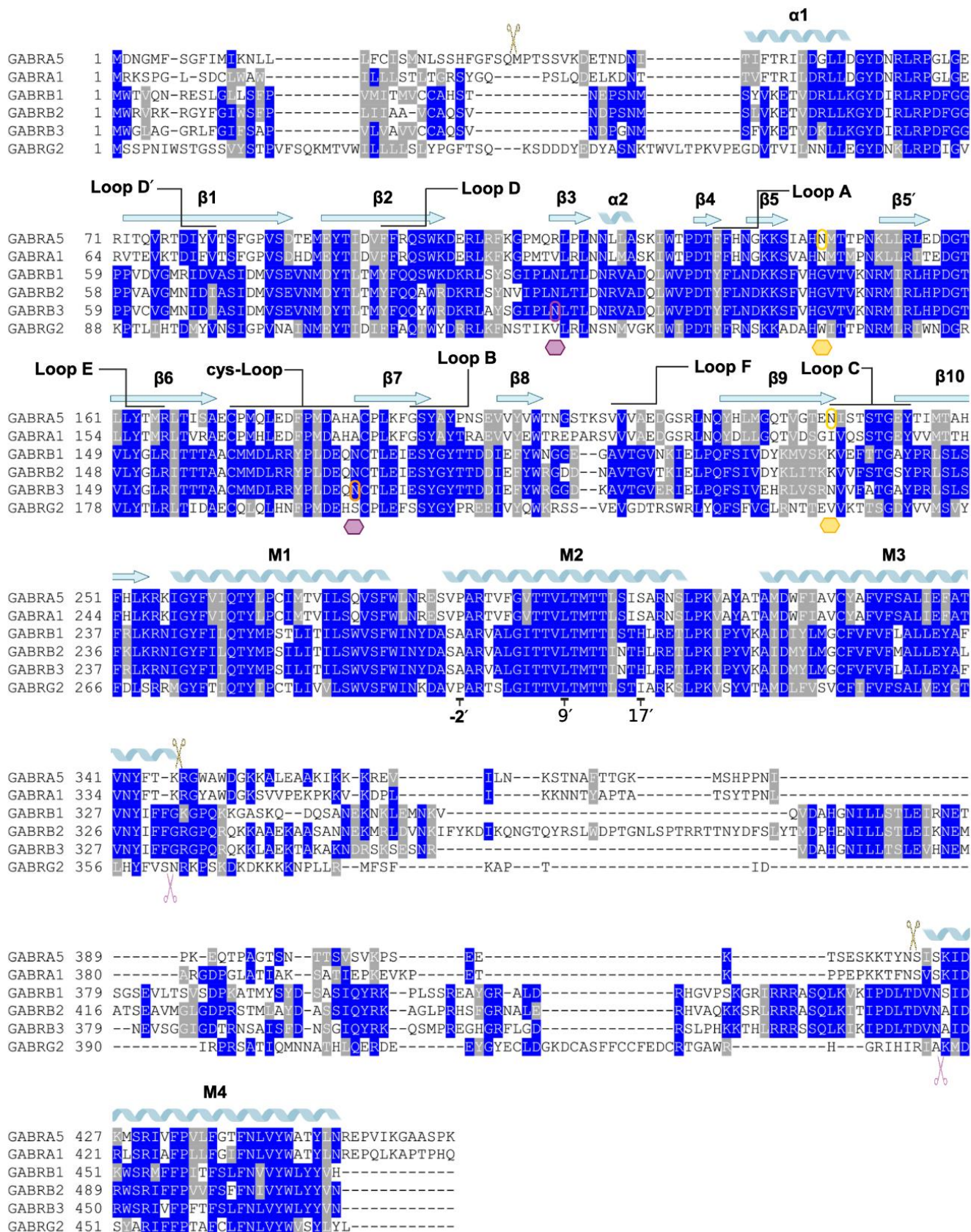
(E) First round of focused classification showing the mask used (left, green) and resulting classes (gray). Glycan densities are colored according to their linkage to either the $\alpha 5$ (yellow) or $\beta 3$ subunit (magenta). Models shown in panel c were merged and fitted into the density maps. The first class (33.3% of particles) was selected, as it showed clear glycan density at the N204 position expected for $\alpha 5$, with little density near positions expected for $\beta 3$.

(F) Second round of focused classification, with maps colored as in panel e. The third class, with $\alpha 5$ as Chain D and $\beta 3$ as Chain C, was used for the final refinement. 'Mixed glycans' indicates density at both $\alpha 5$ -specific (N204) and $\beta 3$ -specific (N80/N149) positions.



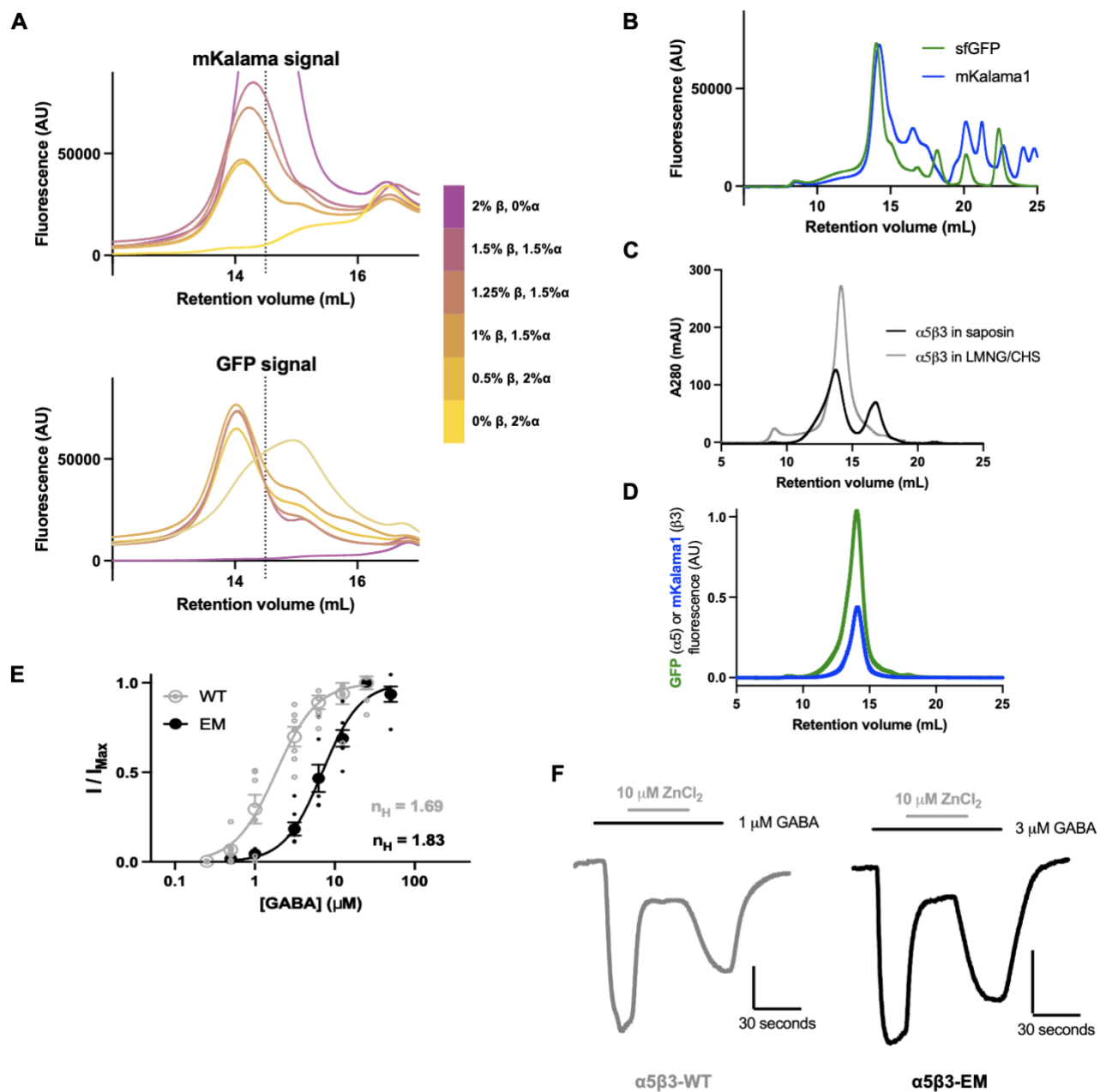
Supplementary Figure 2. Cryo-EM refinement parameters.

- (A) Angular distribution maps for the final reconstructions used in this study.
- (B) Local resolution of electron-density maps for each reconstruction.
- (C) Map-to-map (black) and model-to-map Fourier shell correlation curves (red) for each reconstruction.



Supplementary Figure 3. Sequence alignment of relevant GABA_AR subunits.

Sequence alignment between α5, β3 and common coassembling GABA_AR subunits. Glycans specific to α5 (yellow) and β3 (magenta) are marked with hexagons. Scissors above the alignment indicate modification to the α5 sequence, while scissors below the alignment indicate modification to β3.



Supplementary Figure 4. Biochemical and functional characterization of $\alpha 5\beta 3$ GABA_ARs.

(A) Titration of viruses used for transduction. Suspension cultures were transduced with six different ratios of $\alpha 5$ -EM and $\beta 3$ -EM viruses, colored according to legend at right. Expression and assembly was then assessed by FSEC using detectors for mKalamal-labeled $\beta 3$ -EM (top, 385 nm excitation, 456 nm emission) and sfGFP-labeled $\alpha 5$ -EM (bottom, 480 nm excitation, 510 nm emission) to determine an optimal ratio of viruses to use in large-scale cultures. Fluorescence intensity is shown in arbitrary units (AU).

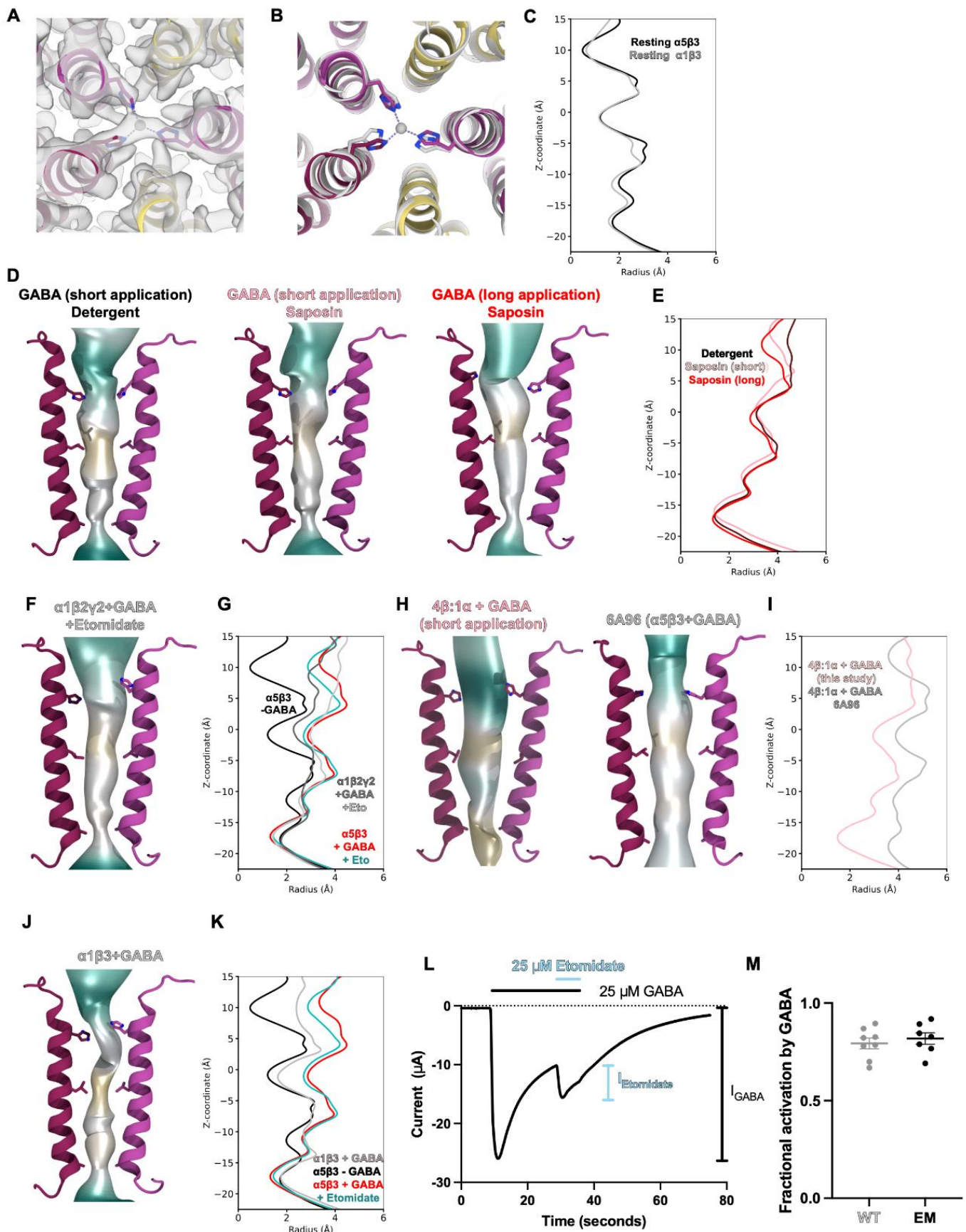
(B) FSEC profile of the condition used for large-scale expression of $\alpha 5\beta 3$ -EM, with the blue curve representing the fluorescence signal in the mKalamal detector, and green the sfGFP detector. Fluorescence intensity is shown in arbitrary units (AU).

(C) SEC profiles of $\alpha 5\beta 3$ -EM purified in detergent (gray) or nanodiscs (black), monitored by UV absorbance at 280 nm. Absorbance values are reported in milli-absorbance units (mAU).

(D) FSEC profile of $\alpha 5\beta 3$ -EM in nanodiscs showing co-elution of sfGFP (green) and mKalamal (blue), indicating a heteromeric assembly.

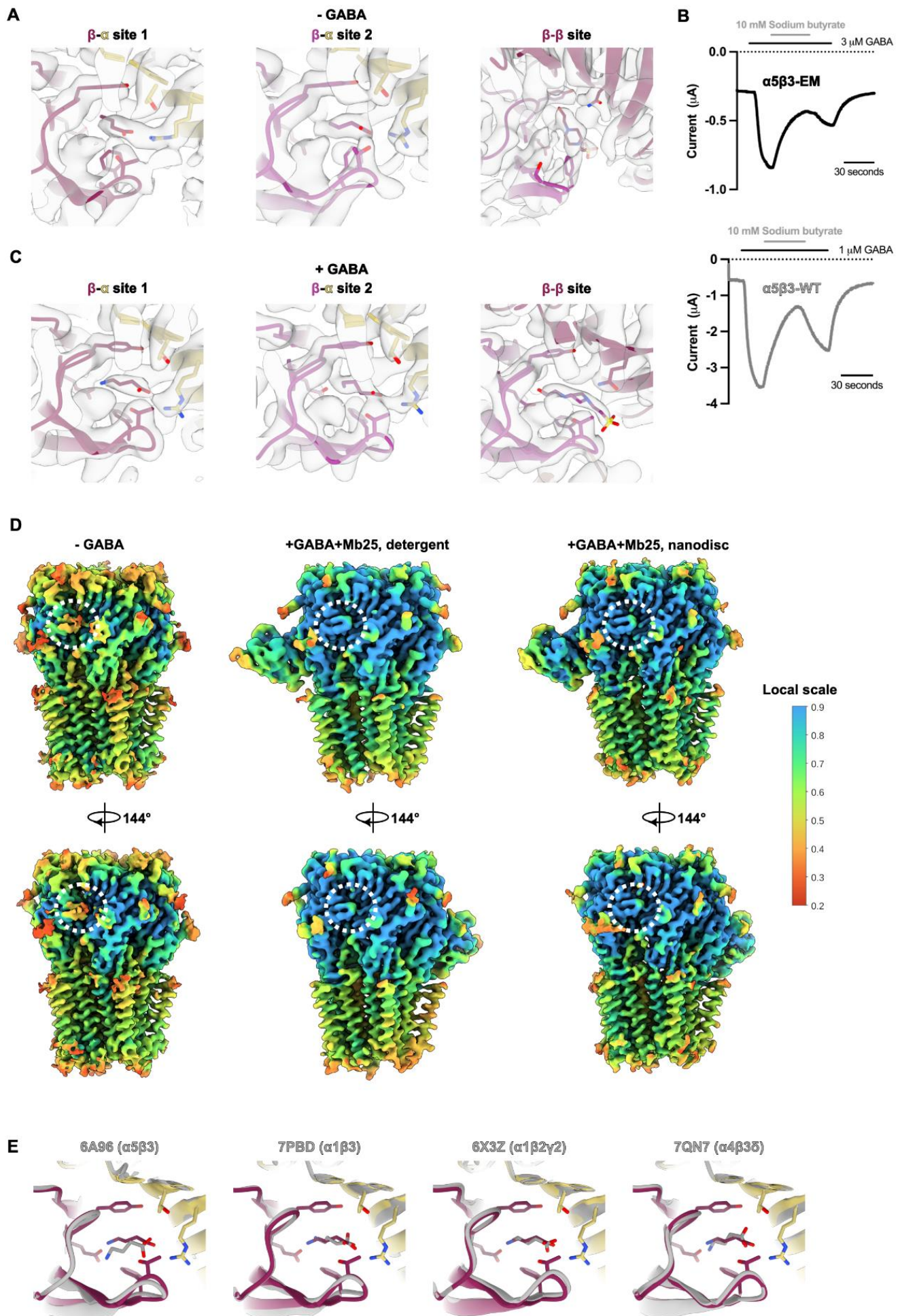
(E) GABA concentration-response curves for $\alpha 5\beta 3$ -EM (black) and $\alpha 5\beta 3$ -WT (gray) expressed in *Xenopus* oocytes. Larger circles indicate mean normalized current $\pm$ standard error of the mean for 7 independent oocytes. Smaller circles show individual values from each oocyte. *Inset* indicates Hill coefficients (n_H) for the Boltzmann equation fitted for each sample.

(F) Example traces for $\alpha 5\beta 3$ -WT (left, gray) and $\alpha 5\beta 3$ -EM (right, black) in response to $\sim EC_{20}$ GABA, with a transient co-application of 10 μM ZnCl₂. *Insets* indicate current versus time scales, with the vertical bars showing 50 nA of current.



Supplementary Figure 5. Comparison of pore properties in $\alpha 5\beta 3$ -EM versus other GABA_ARs.

- (A) Density (gray surface) overlaid with model for the pore of resting-like $\alpha 5\beta 3$ -EM (PDB-9HAA), viewed from the extracellular side with M2-helices in $\alpha 5$ (yellow) and $\beta 3$ subunits (magenta) as ribbons. Dashed lines indicate histidine coordination of a non-protein density in the pore, consistent with Zn^{2+} .
- (B) Resting-like structure of $\alpha 5\beta 3$ -EM, viewed and colored as in A. Inhibitor-bound $\alpha 1\beta 3$ (gray, PDB-7PC0)²⁸ is superimposed based on full alignment.
- (C) Pore radius as a function of z-coordinate relative to L9' for resting-like $\alpha 5\beta 3$ -EM (black) and $\alpha 1\beta 3$ ²⁸ (gray) structures.
- (D) Permeation pathways, depicted as in C, in GABA-bound $\alpha 5\beta 3$ -EM, in detergent (left, PDB-9HNQ) or nanodiscs with short (center, PDB-9HUM) or long (right, PDB-9HNS) applications of GABA.
- (E) Pore radius relative to L9' for GABA-bound $\alpha 5\beta 3$ -EM in detergent (black) or nanodiscs with short (pink) or long (red) GABA applications.
- (F) Permeation pathways, depicted as in C, in GABA-bound structures with 1:4 α : β stoichiometry reported here (left, PDB-9HNR) or previously (right, PDB-6A96)²⁵.
- (G) Pore radius relative to L9' for GABA-bound structures with 1:4 α : β stoichiometry reported here (pink) or previously (gray)²⁵.
- (H) Permeation pathway, depicted as in C, in $\alpha 1\beta 2\gamma 2$ with GABA and etomidate bound (PDB-6X3V)³³.
- (I) Pore radius relative to L9' for GABA-bound $\alpha 5\beta 3$ -EM in the presence (cyan, PDB-9HNT) or absence (red, PDB-9HUM) of etomidate, or $\alpha 1\beta 2\gamma 2$ ³³ in the presence (gray, PDB-6X3V) or absence (black, PDB-6X3Z) of etomidate.
- (J) Permeation pathway, depicted as in C, in a GABA-bound $\alpha 1\beta 3$ subtype (PDB-7PBD)²⁸.
- (K) Pore radius relative to L9' for GABA-bound structures of $\alpha 5\beta 3$ -EM in the presence (cyan, PDB-9HNT) or absence (red, PDB-9HUM) of etomidate, or of $\alpha 1\beta 3$ in the presence of GABA (gray, PDB-7PBD)²⁸.
- (L) Example trace for $\alpha 5\beta 3$ -EM showing estimation of the fractional activation by GABA, defined as the ratio of I_{GABA} (black bar) to the sum of I_{GABA} and $I_{Etomidate}$ (blue bar).
- (M) Fractional activation of $\alpha 5\beta 3$ -EM (black) and $\alpha 5\beta 3$ -WT (gray) by GABA. Bars indicate mean values $\pm$ standard error from 7 ($\alpha 5\beta 3$ -EM) or 8 ($\alpha 5\beta 3$ -WT) independent oocytes, also shown as points.



Supplementary Figure 6. Comparison of extracellular ligand-binding pockets across $\alpha 5\beta 3$ -EM and related structures.

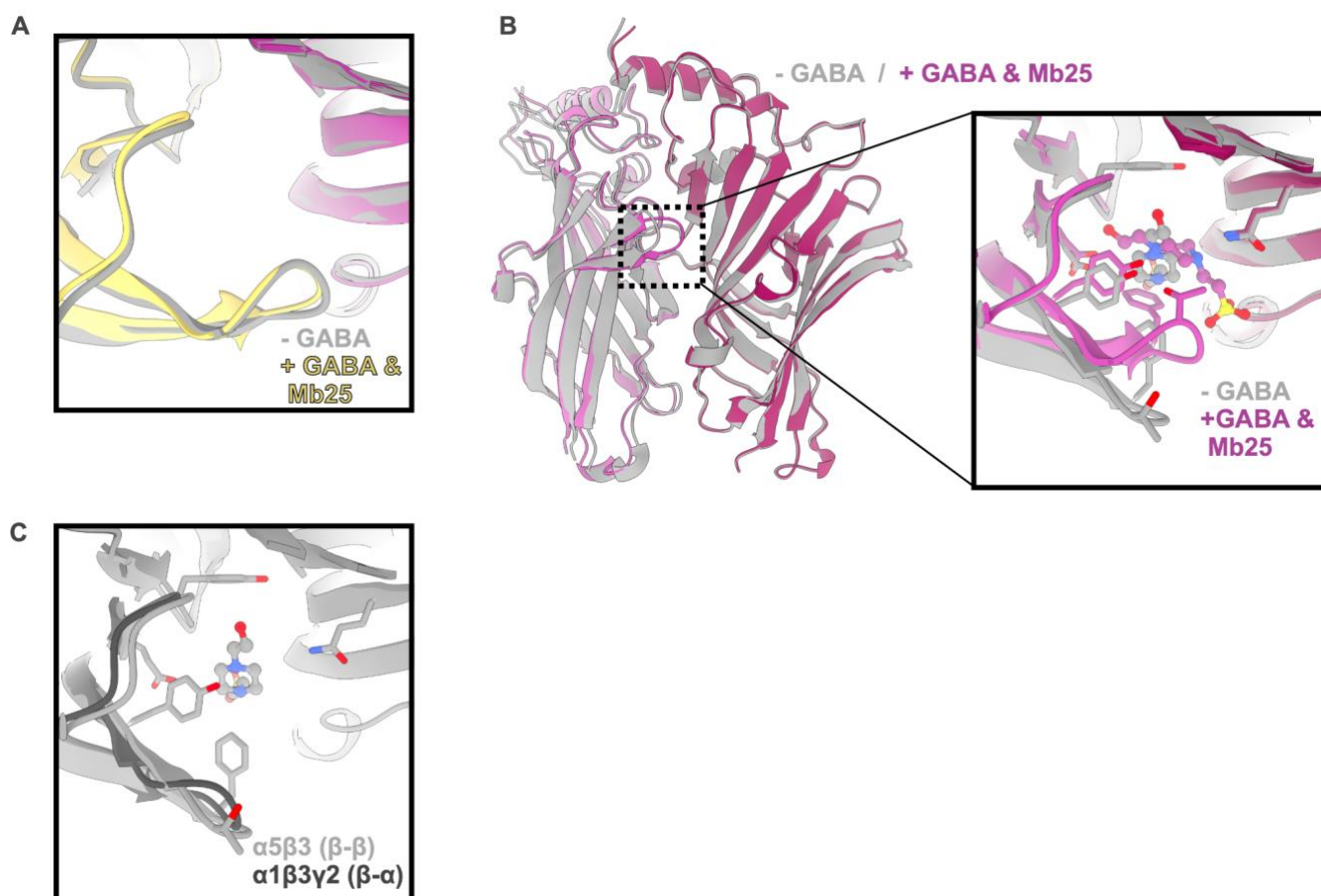
(A) Density map (gray surfaces) overlaid with atomic model at orthosteric site 1 (left), orthosteric site 2 (center) and an equivalent region of the β - β interface (right), all in resting-like $\alpha 5\beta 3$ -EM in micelles (PDB ID 9HAA). Each site is viewed from the extracellular side with $\alpha 5$ (yellow) and $\beta 3$ subunits (magenta) as ribbons. Butyrate and HEPES (magenta), modeled at β - α and β - β interfaces respectively, along with coordinating side chains, are shown as sticks and colored by heteroatom.

(B) Example traces for $\alpha 5\beta 3$ -EM (top, black) and $\alpha 5\beta 3$ -WT (bottom, gray) in response to $\sim EC_{50}$ GABA, with a transient co-application of 10 mM sodium butyrate.

(C) Density map (gray surfaces) overlaid with atomic model at sites depicted and colored as in panel a, but in GABA-bound $\alpha 5\beta 3$ -EM in micelles (PDB ID 9HNQ). Ligands at β - α and β - β interfaces are assigned to GABA and HEPES, respectively.

(D) OccyPy analysis of density maps for resting-like $\alpha 5\beta 3$ -EM (left, PDB ID 9HAA), GABA-bound $\alpha 5\beta 3$ -EM in micelles (center, PDB ID 9HNQ) and GABA-bound $\alpha 5\beta 3$ -EM in nanodiscs (right, PDB ID 9HUM). Maps are colored according to local scale according to the scale bar at left. White dashed circles indicate orthosteric sites 1 (top) and 2 (bottom).

(E) Orthosteric site 1 in previously reported GABA-bound structures of (left to right, gray) $\alpha 5\beta 3$ (PDB ID 6A96)²⁵, $\alpha 1\beta 3$ (PDB ID 7PBD)²⁸, $\alpha 1\beta 2\gamma 2$ (PDB ID 6X3Z)³³ and $\alpha 4\beta 3\delta$ GABA_ARs (PDB ID 7QN7)³⁵, viewed as in panel a, with GABA shown as sticks. For comparison, GABA-bound $\alpha 5\beta 3$ -EM is superimposed with each structure based on the complementary α -subunit, and colored as in panel c, with GABA and coordinating side chains shown as sticks.

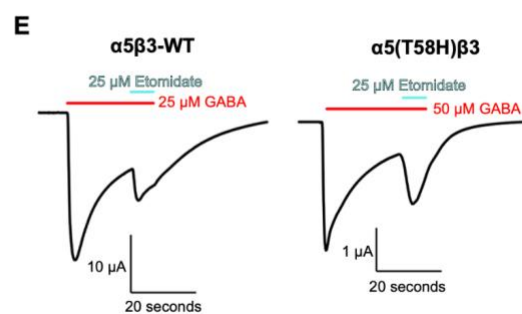
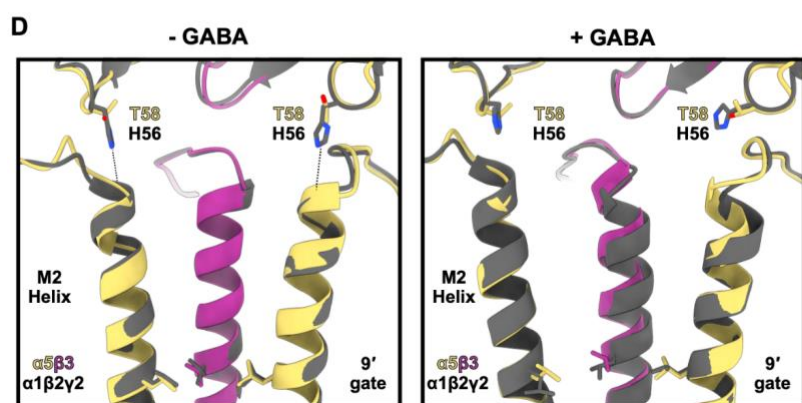
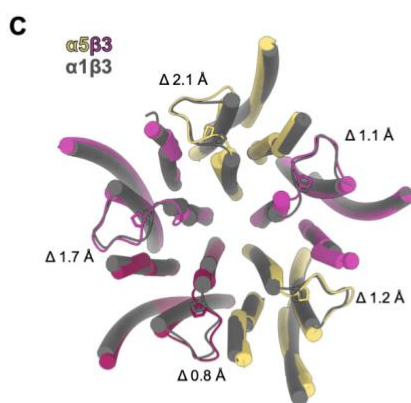
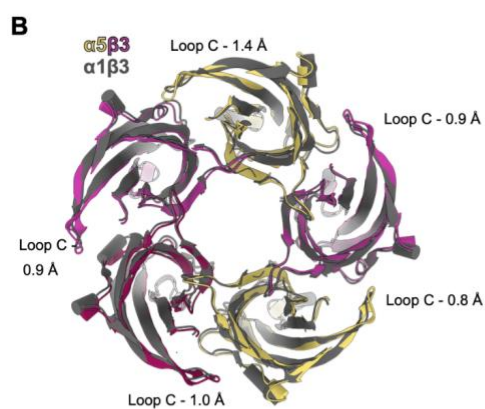
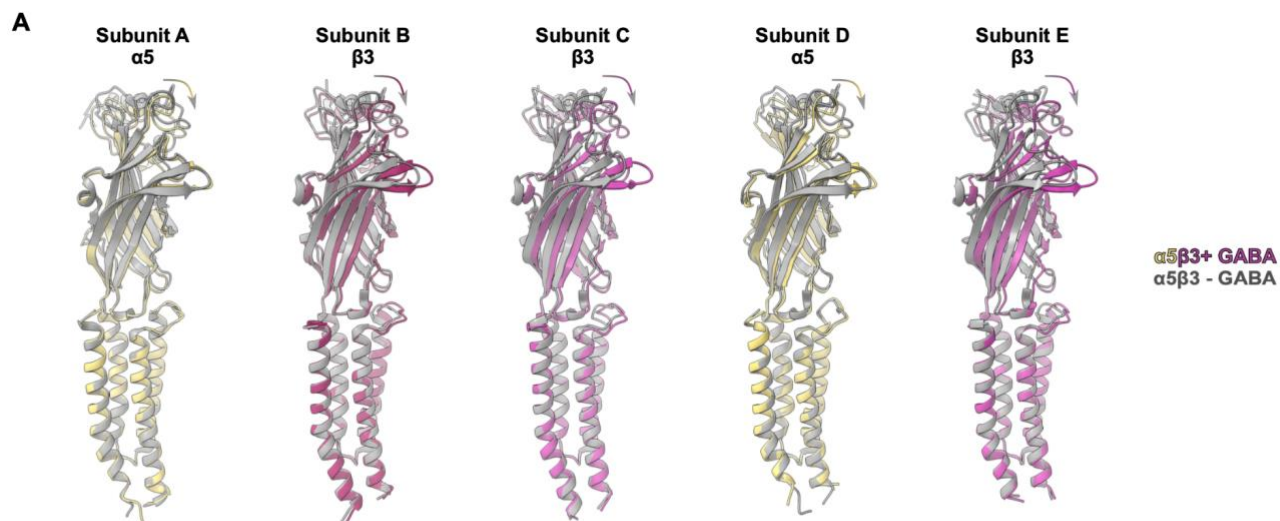


Supplementary Figure 7. Rearrangements at β - β and α - β ECD interfaces.

(A) An α - β ECD interface of GABA-bound $\alpha 5\beta 3$ -EM, viewed as in panel a-*inset*, showing $\alpha 5$ (yellow) and $\beta 3$ subunits (magenta) as ribbons. For comparison, the resting-like state (gray) is superimposed based on the complementary β -subunits.

(B) The β - β ECD interface of GABA-bound $\alpha 5\beta 3$ -EM (magenta, PDB ID 9HNQ) superimposed with the resting-like state (gray, PDB ID 9HAA) based on the complementary-subunit ECD, viewed from the membrane plane. *Inset* shows a zoom of the boxed region, viewed with 90° rotation from the extracellular side, with HEPES molecules shown as balls-and-sticks and neighboring side chains as sticks, colored by heteroatom.

(C) The β - β ECD interface of resting-like $\alpha 5\beta 3$ -EM (gray), depicted as in panel a-*inset*, superimposed with orthosteric site 1 at the β - α interface of a PTX-bound $\alpha 1\beta 3\gamma 2$ GABA_AR (black, PDB ID 6HUG)³⁶ based on the complementary subunits.



Supplementary Figure 8. Comparisons between $\alpha 5$ - and $\alpha 1$ -containing GABA_ARs.

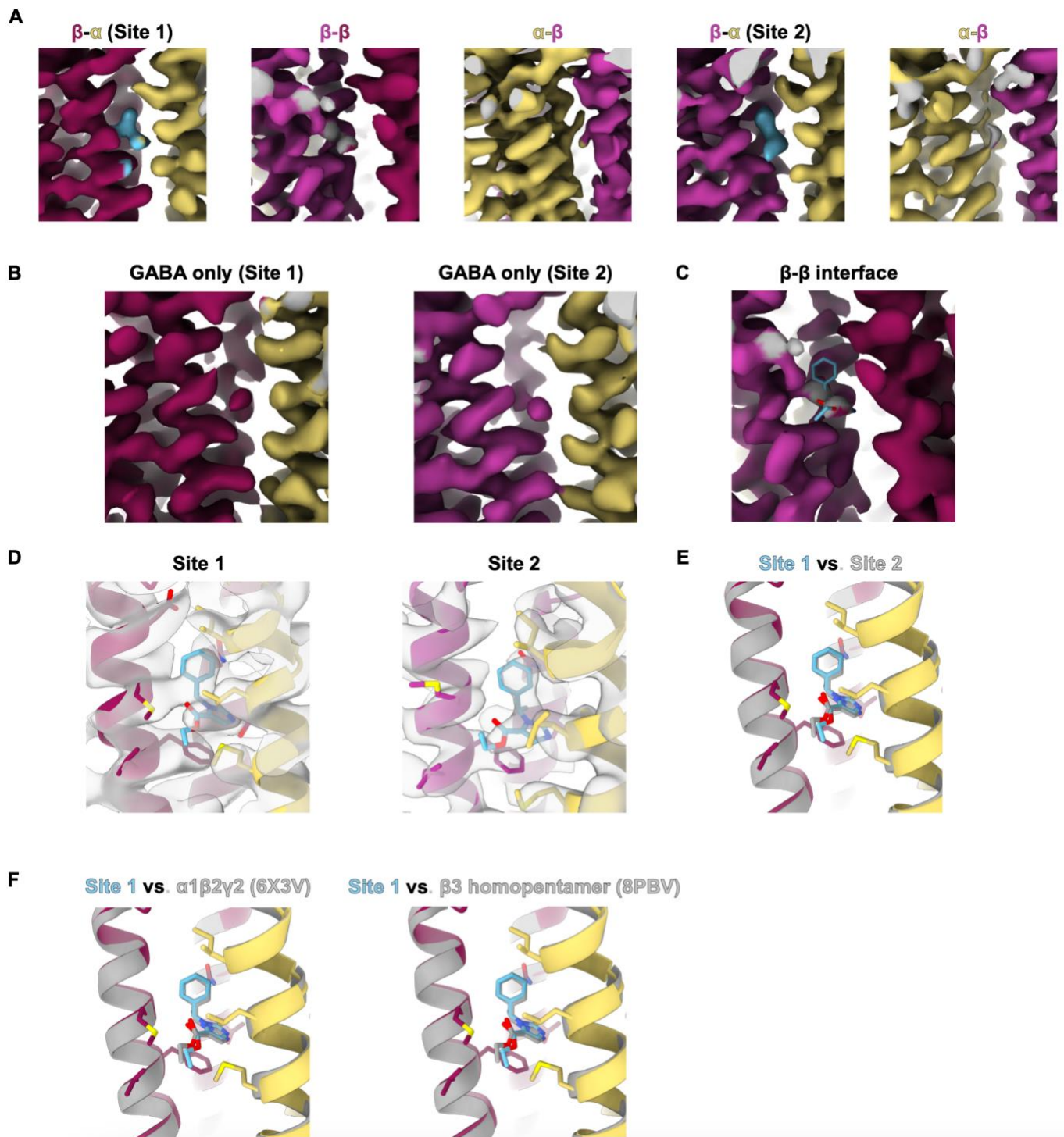
(A) Five individual subunits of GABA-bound $\alpha 5\beta 3$ -EM (PDB ID 9HNQ), with $\alpha 5$ (yellow) and $\beta 3$ (magenta). For comparison, the equivalent subunit in resting-like $\alpha 5\beta 3$ -EM (gray, PDB ID 9HAA) is superimposed based on the subunit TMD. Arrows indicate relative pivot of each subunit ECD relative to its TMD.

(B) Extracellular view of GABA-bound $\alpha 5\beta 3$ -EM (PDB ID 9HNQ), showing an ECD slice as in Fig. 4a, with $\alpha 5$ (yellow) and $\beta 3$ (magenta) subunits labeled according to chain ID. For comparison, a GABA-bound $\alpha 1\beta 3$ -GABA_AR structure (gray, PDB ID 7PBD)²⁸ is superimposed based on the five TMD subunits. Distances labeled below loop C in each subunit indicate displacement of C α atoms at the tip of each loop C ($\alpha 1/5$ -T209 or $\beta 3$ -T202) between $\alpha 5$ - and $\alpha 1$ -containing structures.

(C) TMD slice as in Fig. 4c of GABA-bound $\alpha 5\beta 3$ -EM and $\alpha 1\beta 3$ GABA_AR²⁸ structures, colored as in panel a. Distances labeled below the M2–M3 loop in each subunit indicate displacement of C α atoms at the midpoint of each M2–M3 loop ($\alpha 1/5$ -P281 or $\beta 3$ -P273) between $\alpha 5$ - and $\alpha 1$ -containing structures.

(D) Structures of $\alpha 5\beta 3$ -EM in the absence (left, PDB ID 9HAA) or presence (right, PDB ID 9HNQ) of GABA, depicted as in Fig. 4e. For comparison, structures of $\alpha 1\beta 2\gamma 2$ GABA_ARs (gray) with bicuculline (left, PDB ID 6X3S)³³ or GABA+etomidate (right, PDB ID 6X3V)³³ are superimposed based on the highlighted regions. Residues at the midpoint of the $\beta 1$ – $\beta 2$ loop and activation gate are shown as sticks and colored by heteroatom. Dashed lines indicate prospective hydrogen bonds between the side chain of $\alpha 1$ -H56 and backbone of the M2-helix in the bicuculline-inhibited state.

(E) Example electrophysiology traces for $\alpha 5\beta 3$ -WT (left) and $\alpha 5$ (T58H) $\beta 3$ (right) in response to a saturating pulse of GABA (25 μ M and 50 μ M respectively) followed by addition of 25 μ M etomidate and subsequent washout. *Insets* indicate current versus time scales.



Supplementary Figure 9. Etomidate binding to $\alpha 5\beta 3$ GABA_ARs.

(A) Density map at five subunit interfaces in $\alpha 5\beta 3$ -EM bound to etomidate (PDB ID 9HNT), colored as in Fig. 5b.

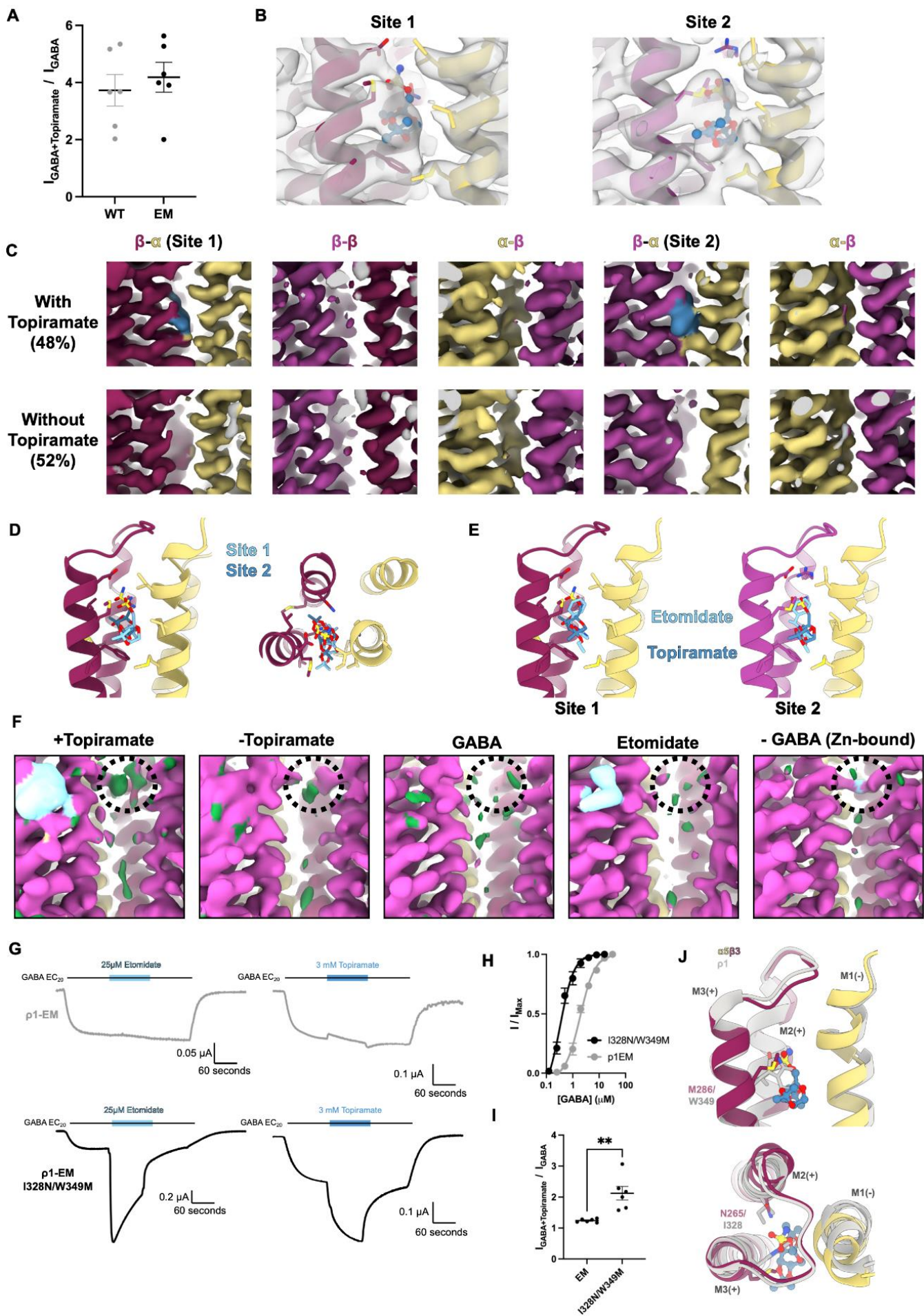
(B) Density map at β - α interfaces in GABA-bound $\alpha 5\beta 3$ -EM (PDB ID 9HUM), depicted as in panel a.

(C) Density maps at the β - β interface in $\alpha 5\beta 3$ -EM bound to etomidate, depicted as in panel a. An overlaid etomidate molecule (cyan sticks) fits poorly to the available density, and could not be reliably built.

(D) Density map (gray surface) overlaid with the atomic model at β - α interfaces in $\alpha 5\beta 3$ -EM bound to etomidate, depicted as in panel a.

(E) Overlay between TMD sites 1 (colored) and 2 (gray) in $\alpha 5\beta 3$ -EM bound to etomidate, overlaid on the full receptor and depicted as in panel d.

(F) TMD site 1 in $\alpha 5\beta 3$ -EM bound to etomidate, depicted as in panel e. For comparison, etomidate-bound structures (gray) of $\alpha 1\beta 2\gamma 2$ (left, PDB ID 6X3V)³³ or $\beta 3$ -homopentamer (right, PDB ID 8PVB)³⁹ GABA_ARs are superimposed based on the β - α interface, showing similar binding poses for etomidate.



Supplementary Figure 10. Topiramate binding to $\alpha 5\beta 3$ GABA_ARs pocket

- (A) Enhancement of GABA-evoked current by topiramate for $\alpha 5\beta 3$ -WT (gray) and $\alpha 5\beta 3$ -EM (black) using the protocol in Fig. 5a. Bars indicate mean values $\pm$ standard error from six oocytes, also shown as points.
- (B) Density (gray) overlaid with the atomic model at β - α interfaces in $\alpha 5\beta 3$ -EM bound to topiramate (PDB ID 9RL5), depicted as in Supplementary Fig. 9d.
- (C) Density map at five subunit interfaces in $\alpha 5\beta 3$ -EM structures with (top, PDB-9RL5) and without (bottom, PDB ID 9RPB) topiramate, resolved from the same dataset, depicted as in Supplementary Fig. 9a.
- (D) TMD-sites 1 (light blue) and 2 (dark blue) in $\alpha 5\beta 3$ -EM bound to topiramate, depicted as in Supplementary Fig. 9, viewed from the membrane plane (left) or from the extracellular side (right).
- (E) Overlay between etomidate (cyan) and topiramate (blue) binding poses in TMD-sites 1 (left) and 2 (right), shown with $\alpha 5\beta 3$ -EM bound to topiramate, depicted as in panel d.
- (F) Densities inside the pore of $\alpha 5\beta 3$ -EM structures determined (left to right) for Mb25+GABA+topiramate (PDB-9RL5); Mb25+GABA, topiramate not resolved (PDB-9RPB); Mb25+GABA (PDB-9HUM); Mb25+GABA+etomidate (bottom, PDB-9HNT); and in a resting-like state (PDB-9HAA). Non-protein density (green) is evident in the pore of structures determined with topiramate, in a similar position as Zn^{2+} (cyan) in the resting-like structure.
- (G) Example traces showing insensitivity of $\rho 1$ -EM GABA_ARs to 25 μ M etomidate or 3 mM topiramate (top) at GABA EC₂₀. The double I328N/W349M mutant is potentiated by both drugs (bottom).
- (H) GABA concentration-response curves for $\rho 1$ -EM (gray) and $\rho 1$ (I328N/W349M)-EM (black). Error bars indicate $\pm$ standard error from three independent oocytes.
- (I) Enhancement of GABA-elicited current by coapplication with topiramate for $\rho 1$ -EM and $\rho 1$ (I328N/W349M)-EM using the protocol in G. Solid bars indicate mean values $\pm$ standard error of the mean from six independent oocytes, also shown as individual points. Asterisks denote significance with $P=0.0025$ in two sided t-test.
- (J) TMD-site 1 in $\alpha 5\beta 3$ -EM bound to topiramate, depicted as in D and viewed from membrane plane (top) or extracellular side (bottom). An equivalent region of $\rho 1$ -EM bound to GABA (gray, PDB-8OP9)⁴³ is superimposed by the principal subunits.