

Supplementary material for:

The polybasic puzzle: how N-terminal charges modulate Gα_{i1} membrane behavior and signaling output

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1. Additional experimental results

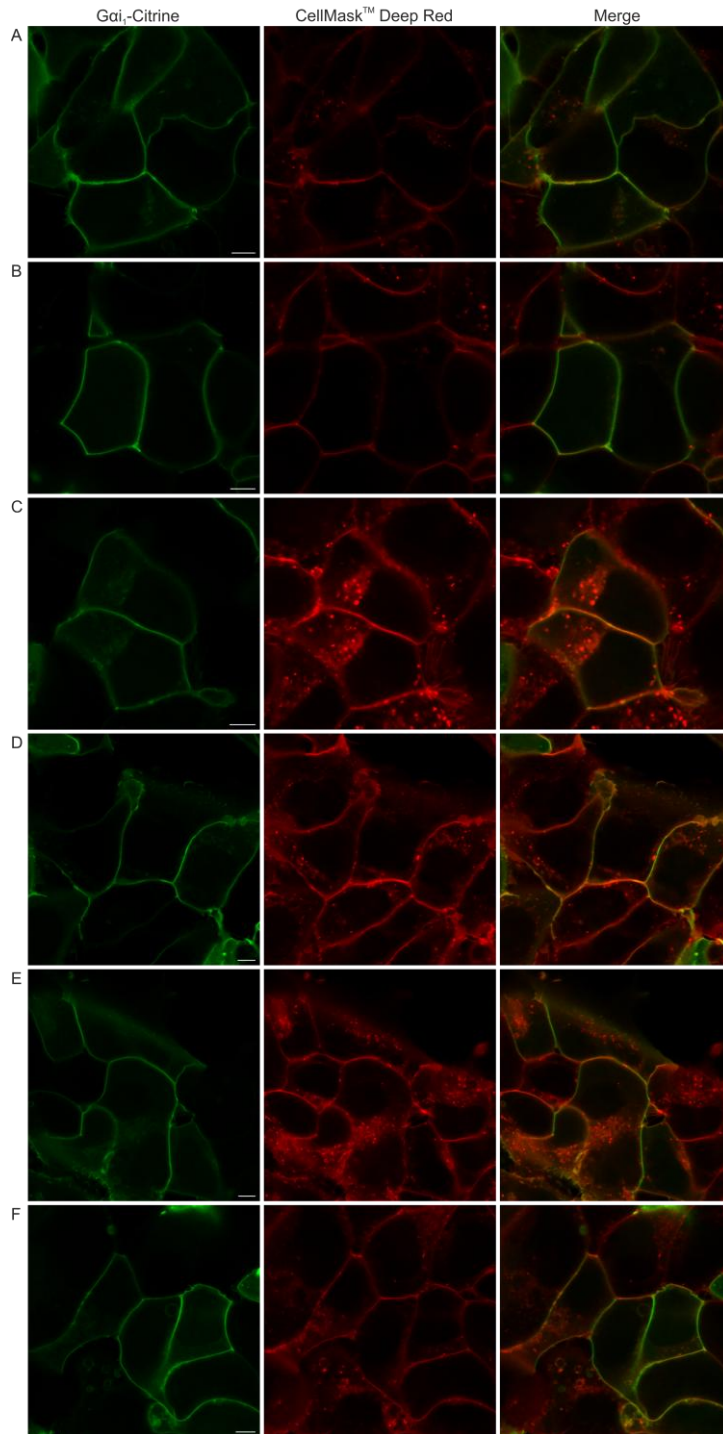


Figure S1. Cellular localization of all investigated *Gai*₁ subunit mutants with Deep Red plasma membrane marker. Representative confocal images show FP-tagged *Gai*-mCitrine subunits (green) and Deep Red plasma membrane marker (red) in transiently cotransfected HEK293 cells. Localization of the investigated proteins: (A) *Gai*₁, (B) *Gai*₁R21K, (C) *Gai*₁R32K, (D) *Gai*₁R21K/R32K, (E) *Gai*₁R21A, and (F) *Gai*₁R21A/R32A. Scale bar, 5 μ m.

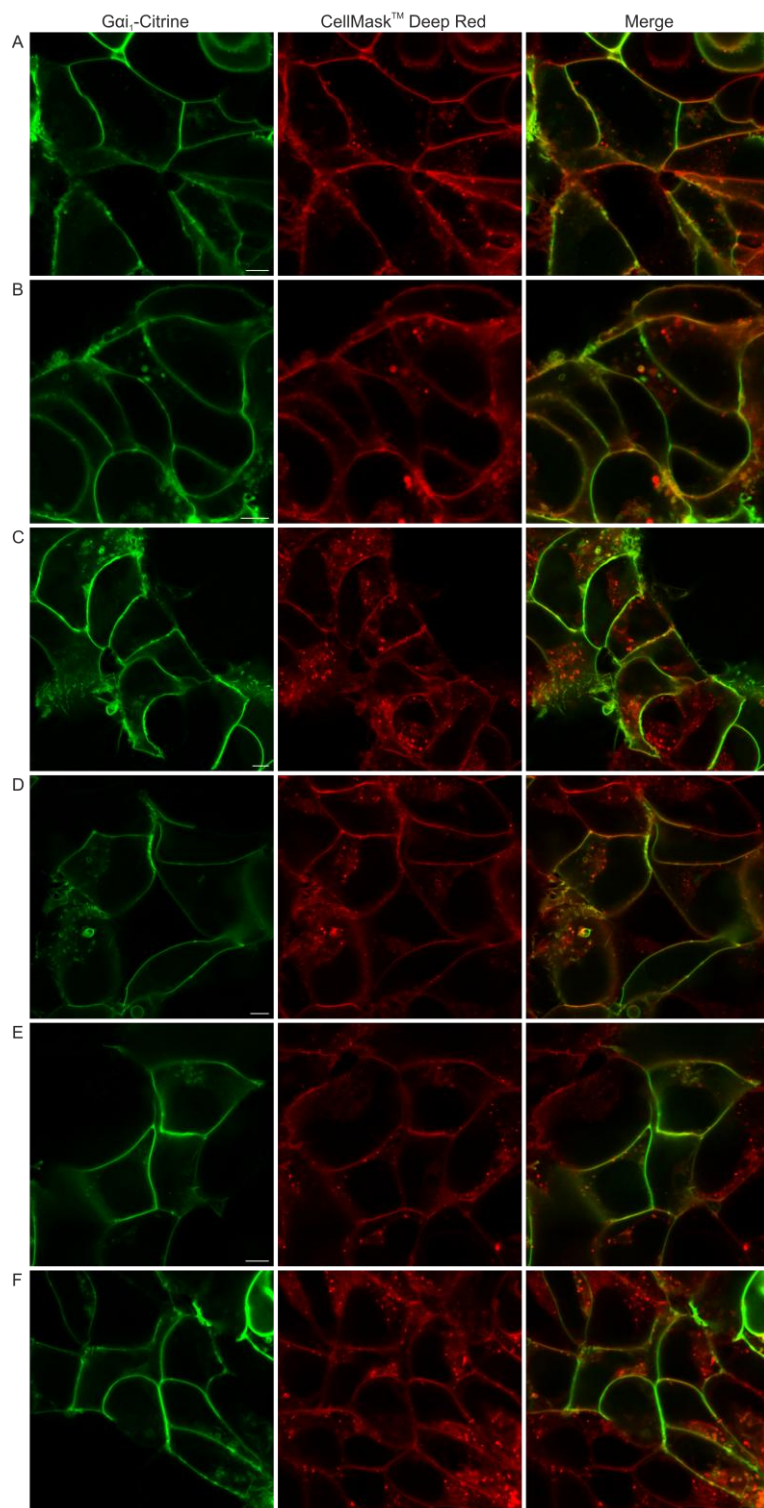


Figure S2. Cellular localization of all investigated Gai₁ subunit mutants with the Deep Red plasma membrane marker. Representative confocal images show FP-tagged Gai-mCitrine subunits (green) with Gβ₁γ₂ dimer and Deep Red plasma membrane marker (red) in transiently cotransfected HEK293 cells. Localization of the investigated proteins: (A) Gai₁ Gβ₁γ₂, (B) Gai₁R21K Gβ₁γ₂, (C) Gai₁R32K Gβ₁γ₂, (D) Gai₁R21K/R32K Gβ₁γ₂, (E) Gai₁R21A Gβ₁γ₂, and (F) Gai₁R21A/R32A Gβ₁γ₂. Scale bar, 5 μm.

2. Additional simulation results

A) Additional simulation details

To investigate the interactions between the Gai₁ subunit and lipid membranes, as well as the effects of specific mutations and lipid modifications, we performed two sets of atomistic molecular dynamics (MD) simulations using GROMACS 2022 (see Table S1). The first set included simulations of the wild-type (WT) Gai₁ and five of its mutants (R21K, R32K, R21K/R32K, R21A, and R21A/R32A) in the presence of a POPC lipid bilayer. The protein was initially placed 1–2 nm from the membrane surface, with its orientation relative to the bilayer chosen randomly. Each system was solvated with explicit water molecules and 0.15 M KCl, with additional K⁺ ions added to ensure charge neutrality. To ensure adequate sampling, five independent repeats were performed for each system.

To assess the impact of Gai₁ mutations on the stability of the heterotrimeric Gai₁β₁γ₂ complex, we conducted the second set of simulations for both WT and mutant complexes in the absence of a membrane. The systems were built from the crystal structure of the full heterotrimeric G protein (PDB ID: 6CRK), placed in a cubic box, solvated with explicit water, and neutralized with 0.15 M KCl. Each system was simulated in three independent repeats to ensure statistical robustness and reproducibility.

Table S1. Molecular compositions, simulation lengths, number of repeats, and initial box dimensions for the two sets of molecular dynamics simulations conducted in this study: **Set I** – wild-type Gai₁ and five of its mutants interacting with a lipid membrane; and **Set II** – wild-type Gai₁β₁γ₂ complex and five of its mutants simulated in aqueous solution without a membrane.

Set	Protein	POPC	Water	Ions (K ⁺ /Cl ⁻)	number of repeats	simulation time of each repeat	initial size of the simulation box
I	Gai ₁	1000	121310-131254	348-376/342-370	5	1 μs	XY: 16.6-7.3nm, Z: 16.2-18.2nm
	Gai ₁ R21A	1000	131253	377/370	5	1 μs	XY: 17.0-7.3nm, Z: 17.2-17.9nm
	Gai ₁ R21A/R32A	1000	131252	378/370	5	1 μs	XY: 17.0-7.2nm, Z: 17.4-17.9nm
	Gai ₁ R21K	1000	131254	376/370	5	1 μs	XY: 17.0-7.3nm, Z: 17.3-18.0nm
	Gai ₁ R32K	1000	131254	376/370	5	1 μs	XY: 17.2-7.3nm, Z: 17.4-17.6nm
	Gai ₁ R21K/R32K	1000	131254	376/370	5	1 μs	XY: 17.0-7.2nm, Z: 17.4-17.9nm
II	Gai ₁ Gβ ₁ γ ₂	-	99426	293/281	3	1 μs	XYZ: 14.5nm
	Gai ₁ R21A Gβ ₁ γ ₂	-	99425	294/281	3	1 μs	XYZ: 14.5nm
	Gai ₁ R21A/R32A Gβ ₁ γ ₂	-	99424	295/281	3	1 μs	XYZ: 14.5nm
	Gai ₁ R21K Gβ ₁ γ ₂	-	99426	293/281	3	1 μs	XYZ: 14.5nm
	Gai ₁ R32K Gβ ₁ γ ₂	-	99426	293/281	3	1 μs	XYZ: 14.5nm
	Gai ₁ R21K/R32K Gβ ₁ γ ₂	-	99426	293/281	3	1 μs	XYZ: 14.5nm

B) Additional simulation results using unanchored G α _{i1} (simulation sets I and II)

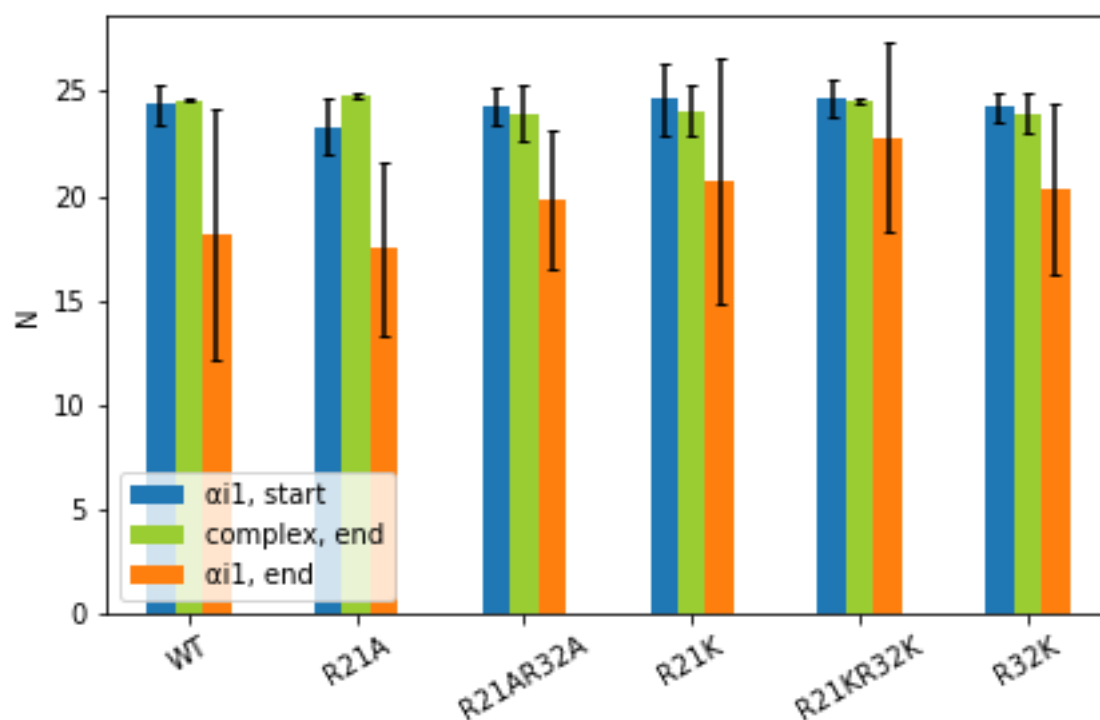


Figure S3. Secondary structure dynamics of the G α _{i1} N-terminus in mutant variants. The helicity of the N-terminal 35 amino acids of G α _{i1} (Set I) was analyzed via molecular dynamics simulations. Helicity was calculated during the initial 200 ns (blue) and the final 200 ns (orange) of the simulation trajectories. The helicity of the G α _{i1} β ₁ γ ₂ heterotrimers (Set II) is shown in green. N – number of residues.

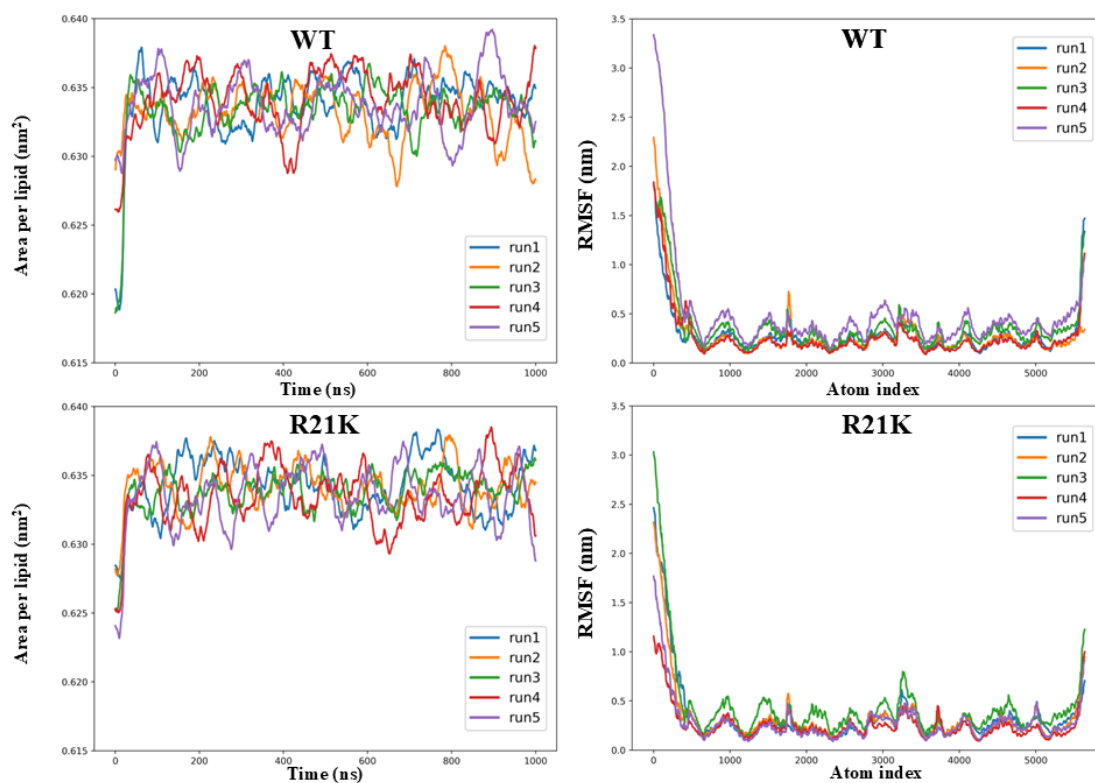


Figure S4. Convergence and reproducibility of the Gai₁-membrane simulations. (*left column*) Time evolution of the area per lipid (APL) for five independent simulation repeats of the wild-type (WT) and R21K mutant systems, showing fast equilibration and consistency across MD trajectories. (*right column*) Root mean square fluctuation (RMSF) profiles of protein backbone atoms for the same systems, illustrating the reproducibility of protein dynamics across independent runs.



Figure S5. Average frequencies of salt bridges formed between charged residues in the N-terminal region of the protein (Set I). A frequency value of 1.0 (black) indicates that a residue consistently formed a salt bridge with another charged residue throughout the simulation, while a value of 0.0 (white) indicates that no such interactions occurred.

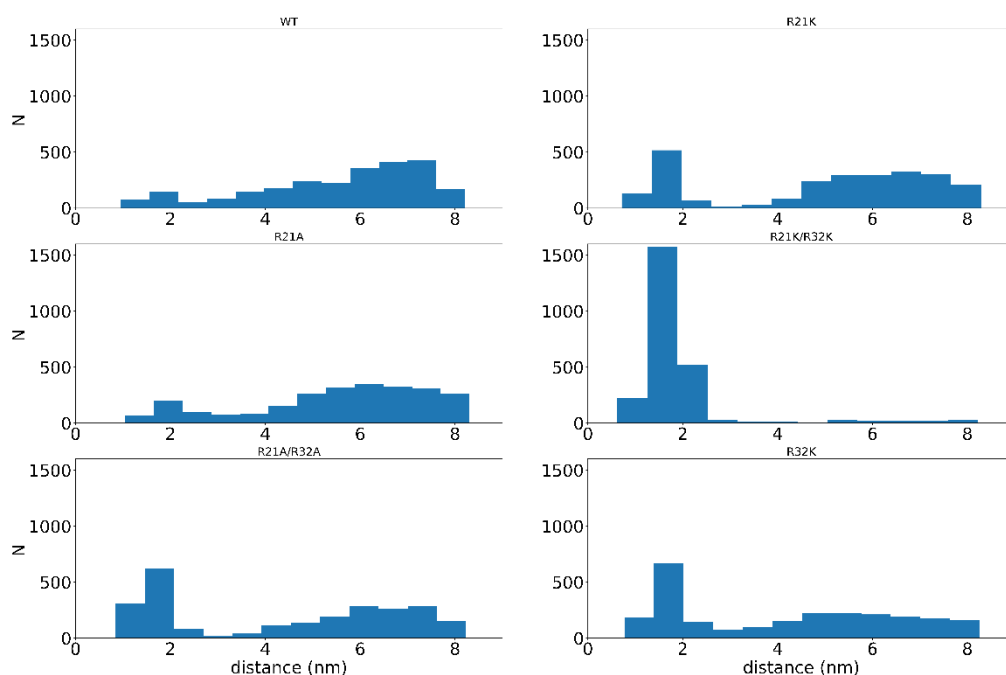


Figure S6. Distributions of N-terminal proximity to the bilayer midplane (Set I). Histograms show the absolute distance between the center-of-mass of the first two N-terminal residues of G α i1 and the bilayer midplane (0 nm), computed along the membrane normal (z). Panels depict WT and the five mutants as indicated, using identical binning and axis ranges to enable direct comparison. For each system, data are pooled across five independent repeats over the 500 ns analysis window.

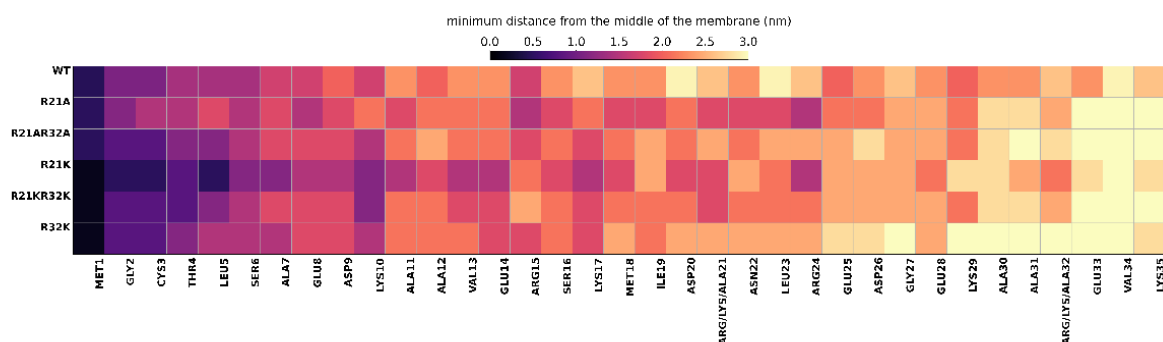


Figure S7. Deepest membrane insertion depth of each residue observed during the MD simulations (Set I). For each residue, the insertion depth was calculated as the minimum distance (along the z-axis) between the residue's center-of-mass and that of the lipid bilayer across all simulation repeats. The values represent the closest approach of each residue to the bilayer midplane, rather than averaged positions.

C) Additional simulation results using lipidated Gai₁ (simulation set III)

To evaluate how covalent lipid anchoring influences protein–membrane interactions, we conducted a third set of simulations using lipid-modified Gai₁ subunits (see Table S2). The myristoylation and palmitoylation modifications were introduced at the N-terminal glycine and cysteine residues, respectively, using the CHARMM36m force field parameters. These modified Gai₁ variants were randomly placed near the same POPC bilayer configuration, solvated with water and 0.15 M KCl, and simulated in five independent repeats for each variant.

Table S2. Molecular compositions, simulation lengths, number of repeats, and initial box dimensions for the three sets of molecular dynamics simulations conducted in this study: **Set III** – lipidated wild-type Gai₁ and five of its mutants interacting with a lipid membrane.

Set	Protein	POPC	Water	Ions (K ⁺ /Cl ⁻)	number of repeats	simulation time of each repeat	initial size of the simulation box
III	lipGai ₁	1000	131253	377/370	5	1 μs	XY: 17.2-17.3, Z: 17.3-17.6nm
	lipGai ₁ R21A	1000	131252	378/370	5	1 μs	XY: 17.2-7.3nm, Z: 17.3-17.5nm
	lipGai ₁ R21A/R32A	1000	131251	379/370	5	1 μs	XY: 17.3nm, Z: 17.3-17.4nm
	lipGai ₁ R21K	1000	131253	377/370	5	1 μs	XY: 17.2-7.3nm, Z: 17.3-17.5nm
	lipGai ₁ R32K	1000	131253	377/370	5	1 μs	XY: 17.2-7.3nm, Z: 17.3-17.6nm
	lipGai ₁ R21K/R32K	1000	131253	377/370	5	1 μs	XY: 17.2-7.3nm, Z: 17.3-17.5nm

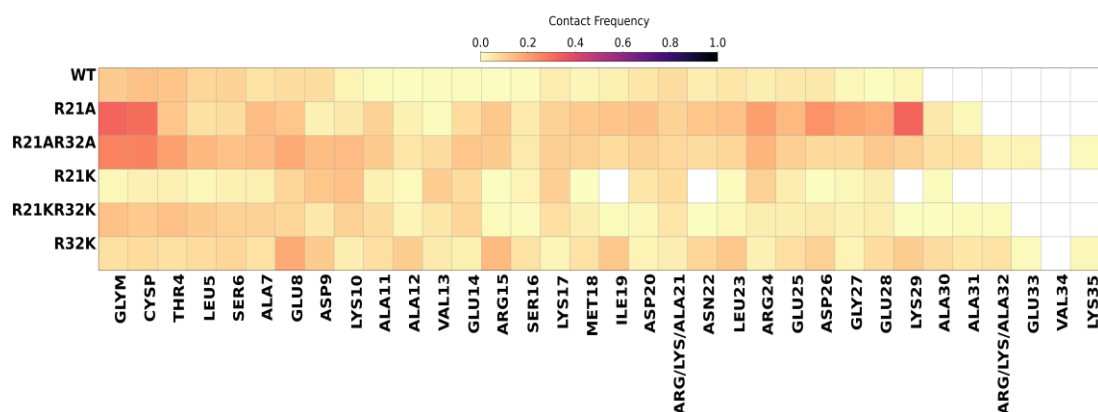


Figure S8. Average contact frequency formed between the lipidated protein's N-terminal and the POPC bilayer (Set III). A frequency of 1.0 (black) indicates that a given residue was always in contact with the membrane when the protein was bound, whereas a frequency of 0.0 (white) means the residue never made contact during binding.



Figure S9. Average frequencies of salt bridges formed between charged residues in the lipidated N-terminal region of the protein (Set III). A frequency value of 1.0 (black) indicates that a residue consistently formed a salt bridge with another charged residue throughout the simulation, while a value of 0.0 (white) indicates that no such interactions occurred.

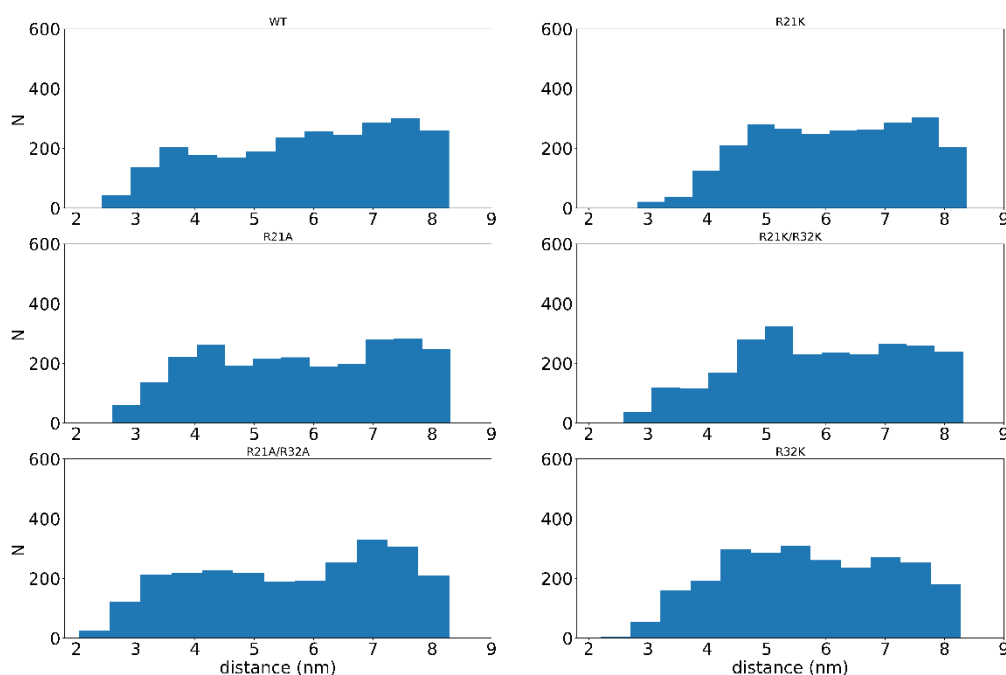


Figure S10. Distributions of N-terminal proximity to the bilayer midplane (Set III). Histograms show the absolute distance between the center-of-mass of the first two N-terminal residues of Gai₁ and the bilayer midplane (0 nm), computed along the membrane normal (z). Panels depict WT and the five mutants as indicated, using identical binning and axis ranges to enable direct comparison. For each system, data are pooled across five independent repeats over the 500 ns analysis window.

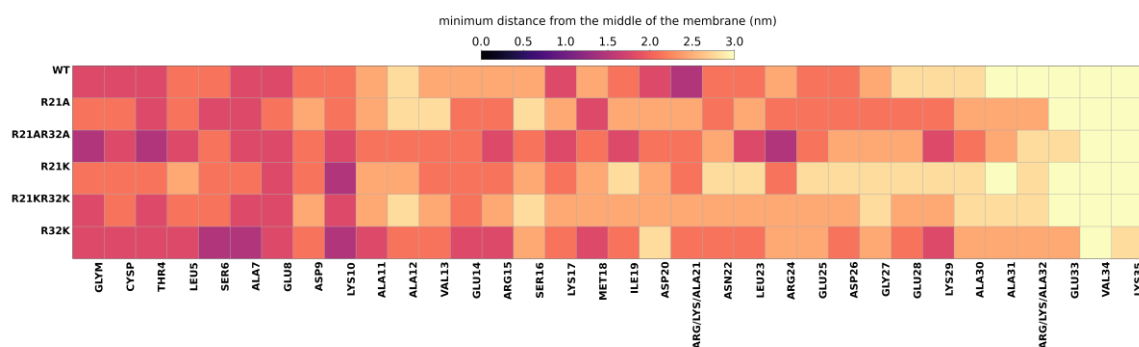


Figure S11. Deepest membrane insertion depth of each residue in the lipidated Gai₁ subunit observed during the MD simulations (Set III). For each residue, the insertion depth was calculated as the minimum distance (along the z-axis) between the residue's center-of-mass and that of the lipid bilayer across all simulation repeats. The values represent the closest approach of each residue to the bilayer midplane, rather than averaged positions.