Influence of Disease-Causing Mutations and Ivacaftor on the Dynamics of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Protein

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Abstract

Cystic fibrosis (CF), one of the most common life-shortening autosomal recessive diseases, arises from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene and leads to severe respiratory and digestive dysfunction. CFTR-targeting therapies such as ivacaftor have transformed CF care by directly modulating CFTR function. However, the precise mechanism by which ivacaftor alters CFTR's conformational dynamics remains incompletely understood. In this study, we refined and employed a CFTR model derived from the phosphorylated, ATP- and ivacaftor-bound cryo-EM structure (PDB ID 6O2P). Through 32.5 μs of molecular dynamics simulations, we investigated the structural and dynamic effects of the common F508del mutation and the gating mutations G551D, G1349D, and S549N. Our results show that mutations within the ATP-binding regions cause local rearrangements at the nucleotide-binding sites, affecting the interface between the nucleotide-binding domains (NBDs) relative to wild-type CFTR, rather than abolishing ATP binding entirely. We further indicate that, for the NBDs to approach one another productively, protonation of the aspartate side chains in G551D and G1349D CFTR may be necessary to mitigate charge repulsion. The F508del mutation induces increased rigidity within the protein core, impeding the rearrangement of transmembrane helices required for channel opening. Ivacaftor does not directly perturb the ATP-binding sites, consistent with emerging evidence that it exerts its effects allosterically on regions distal to the nucleotide-binding sites. Collectively, our findings enhance our understanding of CFTR dynamics and provide a versatile framework for exploring the molecular effects of disease-causing mutations and evaluating potential therapeutic agents.

Introduction

Cystic Fibrosis (CF) is a life-shortening autosomal recessive disorder that affects multiple organ systems, leading to significant morbidity and reduced life expectancy.[1] A hallmark of CF is the accumulation of thick, sticky mucus that obstructs epithelia-lined ducts and tubes, particularly in the lungs and pancreas, resulting in chronic infections, progressive lung damage, and severe digestive complications from pancreatic-duct obstruction.[2]

CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a chloride/bicarbonate channel in epithelial membranes that regulates fluid and electrolyte balance.[2] CFTR belongs to the ATP-binding cassette (ABC) protein family, but unlike typical ABC proteins, CFTR functions as an ATP-gated ion channel.[3] Its architecture comprises two membrane-spanning domains (MSD1 and MSD2), two nucleotide-binding domains (NBD1 and NBD2), and a distinct, intrinsically disordered regulatory (R) domain. In the dephosphorylated state, the R domain obstructs channel activation by preventing the close association or dimerisation of the NBDs, a process we refer to as dimerization for simplicity. Once phosphorylated by cAMP-dependent protein kinases, this inhibition is lifted, enabling ATP binding and subsequent NBD dimerisation that drives channel gating.[4]

To date, over 2,000 CFTR mutations have been identified,[5] each disrupting CFTR activity through one or more molecular mechanisms, including impaired protein trafficking, defective channel gating, reduced membrane stability, or aberrant protein synthesis.[6] The most common mutation, F508del (a deletion of phenylalanine at position 508), exemplifies this pleiotropy, causing protein misfolding, gating defects, and decreased cell-surface stability.[6]

The complexity and variability of CFTR mutations present challenges for effective CF treatment. Therapies must not only relieve symptoms but also correct the specific molecular defects associated with each mutation. Achieving this goal requires a deep understanding of CFTR's structure, dynamical behaviour, interactions with modulators, and the influence of its membrane environment. In recent years, CFTR modulators (small molecules that directly restore CFTR function) have transformed the CF therapeutic landscape.[7] These include potentiators (which enhance channel gating), correctors (which rescue protein folding/trafficking), stabilisers (which prolong

membrane retention), read-through agents (which bypass premature stop codons), and amplifiers (which increase overall CFTR protein production).[7] Combination regimens of one or two correctors with a potentiator have shown substantial clinical benefit for patients with responsive genotypes.[7]

High-resolution cryo-electron microscopy (cryo-EM) studies have provided unprecedented static snapshots of CFTR and its drug-bound conformations. The first high-resolution cryo-EM structure of zebrafish CFTR in its dephosphorylated, ATP-free state,[8] laid the foundation for subsequent human CFTR structures in various functional states, including ATP-bound and drug-bound forms.[9-12] Despite these advances, our understanding of CFTR's conformational dynamics remains limited. In particular, how modulators such as ivacaftor affect CFTR structure and function, especially in mutant backgrounds, and the role of the surrounding lipid membrane in stabilising CFTR and facilitating drug binding, are not yet fully resolved.

In this study, we use molecular dynamics simulations to investigate the behaviour of membrane-embedded CFTR in its phosphorylated, ATP-bound state. We constructed a detailed atomistic model of human CFTR based on the cryo-EM structure of the phosphorylated, ATP- and ivacaftor-bound state (PDB ID 6O2P)[9] and performed extensive simulations on wild-type (WT) CFTR, the common F508del mutant, and the gating mutations G551D, G1349D, and S549N. For G551D and G1349D, both protonated (G551DH, G1349DH) and unprotonated aspartate (G551D, G1349D) forms were simulated. Each system was simulated under both ivacaftor-free and ivacaftor-bound conditions to examine how these mutations and ivacaftor influence CFTR structure and dynamics.

Our simulations extend structural data to reveal mechanistic insights into CFTR's dynamic behaviour. We find that the glycine-to-aspartate mutations G551D and G1349D, which lie adjacent to the ATP-binding sites, likely require aspartate protonation to facilitate stable NBD dimerisation required for activity. Although these mutations, along with S549N, do not fully abolish ATP binding, they impair channel gating through allosteric disruption beyond the nucleotide-binding pockets. The F508del mutation increases core rigidity, hindering the conformational transitions needed for channel opening. Finally, our results support the growing view that ivacaftor enhances gating primarily via allosteric modulation of the membrane-spanning

domains rather than by directly affecting the ATP-binding sites. These findings contribute to a deeper, mutation-specific understanding of CFTR dynamics and may inform future therapeutic strategies.

Methods

System preparation

We constructed a human CFTR model (Figure 1), based on the cryo-EM structure of the phosphorylated, ATP- and ivacaftor-bound state (PDB ID 6O2P).[9] Missing loops (residues 410-436, 890-899, 1174-1201) were grafted from the AlphaFold2 model AF-P13569-F1-v4 (Figure S1).[13, 14] The regulatory (R) domain (>200 residues) was omitted due to low prediction confidence. The E1371Q mutation present in the cryo-EM structure was reverted to the WT residue and the resulting structure was validated with PROCHECK.[15] The protein, solvent, and ions were described with the AMBER99SB-ILDN force field,[16] and the lipid bilayer with the Slipids force field.[17] GROMACS-compatible parameters for the ATP and ivacaftor molecules were generated in ACPYPE[18] with the GAFF[19] force field, assigning Gasteiger partial charges for ATP and AM1-BCC charges for ivacaftor.

We embedded the protein in a 5:5:1 POPC:POPE:cholesterol bilayer using CHARMM-GUI.[20] The system was then solvated with TIP3P[21] water in an 11.957 × 11.957 × 18.480 nm simulation box. Water molecules within the bilayer region (identified by *z*-coordinates relative to phosphate averages) were removed with a custom Fortran program (courtesy of Dr Richard Sessions). Following solvation and membrane drying, the system was neutralised and brought to 0.15 M NaCl. A 20 ps unrestrained NPT equilibration (1 fs timestep) at 200 K and 1 bar (Nosé-Hoover thermostat,[22] τ_t = 0.5 ps; Berendsen barostat,[23] τ_p= 5.0 ps, compressibility = 4.5×10^{-5} bar^{-1}) was performed to facilitate lateral compression of the bilayer, sealing the voids generated by water removal, yielding a working box of 11.38 × 11.38 × 20.78 nm.

The complete system was energy-minimized using the steepest-descent algorithm[24] for up to 10,000 steps (or until the maximum force <200 kJ mol^{-1} nm^{-1}). No thermostat or barostat was active during minimization, and no new velocities were generated. Non-bonded interactions used a 1.4 nm cutoff and PME[25] electrostatics (0.16 nm grid spacing, fourth-order interpolation), and a Verlet[26] neighbour-list scheme.

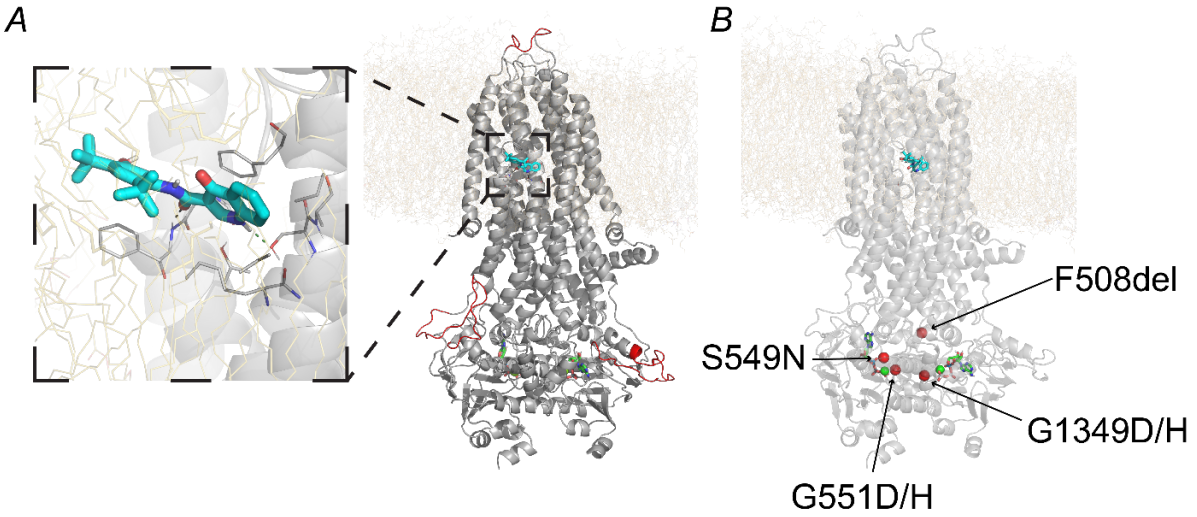

Figure 1. Structural views of the CFTR binding site and locations of studied mutations.

A, the insert depicts a side view of the ivacaftor binding site at the protein-lipid interface. Ivacaftor is shown as sticks with cyan carbon atoms, surrounding protein residues are shown as sticks with grey carbon atoms. Hydrogen bonds between the heavy atoms of S308 O-γ and ivacaftor N16, as well as between F931 backbone N atom and ivacaftor O13, are shown as green and black dashed lines, respectively. CFTR is displayed as a grey cartoon and is embedded within a mixed lipid bilayer (POPC, POPE and cholesterol) represented by yellow sticks. ATP is shown as sticks with green carbon atoms, and Mg^{2+} ions as green spheres. Loops modelled from AlphaFold2 structure predictions (AF-P13569-F1-v4)[13, 14] are coloured red. *B*, locations of CFTR mutations studied here (G551D/H, G1349D/H, F508del and S549N) are indicated as red spheres and labelled on the WT structure.

Finally, a 1 ns position-restrained NPT equilibration at 310 K and 1 bar (velocity-rescaling thermostat,[27] τ_t = 0.1 ps; Berendsen barostat,[23] τ_p = 2.0 ps, compressibility = 4.5×10^{-5} bar^{-1}) was performed with a 2 fs timestep (leap-frog integrator).[28] Bonds to hydrogen atoms were constrained with LINCS[29] (order 4, one iteration), and non-bonded interactions used a 1.4 nm cutoff for both van der Waals and Coulomb forces. Coulomb forces and velocities were regenerated at the target temperature and pressure.

To preserve the cryo-EM-identified binding position of ivacaftor while the lipid environment (which contributes ~60% of the binding site)[9] equilibrated, we imposed harmonic distance restraints on two key hydrogen bonds between the protein and ivacaftor (heavy atoms N of F931 with O13 of ivacaftor and O-γ of S308 with N1 of

ivacaftor) using the umbrella potential implementation (k = 1000 kJ mol^{-1} nm^{-2}, r_0 = 0.28 nm) in GROMACS.[30] These restraints were maintained throughout a 20 ns "pre-production" stage under the NPT ensemble at 310 K and 1 bar (Parrinello-Rahman barostat;[31] velocity-rescale thermostat[27]). After lipid equilibration, the restraints were released and 500 ns of unrestrained NPT production simulations (310 K, 1 bar) were carried out, during which ivacaftor remained stably bound in the cryo-EM-defined site.

Molecular dynamics production runs

Production MD simulations (500 ns each) were carried out in the isothermal-isobaric NPT ensemble using GROMACS (v 2022.6)[32] with periodic boundary conditions. We applied the AMBER99SB-ILDN[16] force field for the protein, solvent and ions; Slipids for the lipids;[17] and GAFF[18] for the ligands. A 2 fs integration timestep (leap-frog integrator)[28] was used throughout. Long-range electrostatics were treated with the particle-mesh Ewald (PME) method[33] with a 0.16 nm grid spacing and fourth-order interpolation, and a 1.2 nm cutoff was imposed for both direct-space Coulomb and van der Waals interactions (with long-range dispersion corrections). All bonds to hydrogen atoms were constrained with LINCS[33] (order 4, one iteration). Temperature was maintained at 310 K via the velocity-rescaling thermostat[27] (τ_t = 0.5 ps), and pressure was held at 1 bar using semi-isotropic coupling with the Parrinello-Rahman barostat[33] (τ_p = 2.0 ps; compressibility = 4.5×10^{-5} bar^{-1}). Trajectories were saved every 20 ps, with velocities written every 5 ns.

We simulated WT CFTR both with and without ivacaftor, as well as four mutants (Figure 1, B): the gating mutations G551D, G1349D, and S549N, and the processing mutant F508del. All point mutations were introduced in PyMOL 2.5.7[34] on the final frame of the WT 20 ns pre-production run (with ivacaftor restraints). For the Gly-to-Asp mutations at positions 551 and 1349, both protonated and unprotonated forms were simulated (designated 'DH' for the protonated state). The F508del construct was generated by grafting the loop from the phosphorylated, ATP- and Trikafta-bound F508del cryo-EM structure (PDB ID 8EIQ)[10] onto the WT model. MD simulations without ivacaftor bound were prepared by deleting ivacaftor from the corresponding final frames, followed by 1 ns NPT equilibration to allow the lipid bilayer to refill the binding site.

Simulation analysis

Trajectories were visualised in VMD 1.9.3[35] and PyMOL 2.5.7[34] and quantitative analyses (RMSD, RMSF, distances, etc.) were performed using GROMACS 2022.6 tools[36] and CPPTRAJ (part of AmberTools22).[37] Binding affinities of the ATP-Mg^{2+} complex to CFTR protein were estimated using molecular mechanics/generalized born surface area (MM/GBSA) calculations using the gmx_MMPBSA tool.[38]

Results

Exploring the structural stability of mutants in ivacaftor-free states

We calculated the root-mean-square deviation (RMSD) of C-α atoms for WT and mutant CFTR systems to assess structural stability and variations. All systems remained reasonably stable throughout the 500 ns simulations (Figure S2). To exclude artefacts from the inherently flexible intracellular and extracellular loops, we also computed RMSD values after aligning the structures to the transmembrane helices (M) of the membrane-spanning domains (MSDs). These M-aligned RMSDs remained below 2 Å across all trajectories, indicating that no large-scale conformational rearrangements occurred within the transmembrane regions over this timescale.

Close association of nucleotide-binding domains likely requires G551D and G1349D to be protonated

The G551D mutation lies within ATP-Binding site 2, adjacent to the negatively charged phosphates of ATP. In its deprotonated form, D551 is expected to electrostatically repel the γ-phosphate, creating an unfavourable environment for nucleotide binding and catalysis. To explore this, we simulated both protonation states of the D551 residue.

In initial simulations with unprotonated (charged) form of D551, the nucleotide-binding domains (NBDs) became more separated (Figure S3), exposing D551 to the solvent. In this configuration, the sidechain pK_a, as calculated by PROPKA[39, 40] on MD snapshots, was approximately 4, suggesting that D551 would remain charged at physiological pH. In contrast, when D551 was protonated, the NBDs stayed closer

together, creating a hydrophobic environment that shielded D551 from solvent exposure. Under these conditions, the calculated pK_a increased to ~10, implying that D551 would be neutral under physiological pH. This difference is reflected in our simulations (initiated from a conformation with the NBDs already closer together) by a larger distance between the C-γ atom of D551 and the nearest ATP phosphate (γ-phosphate) when the residue is deprotonated (Figure 2).

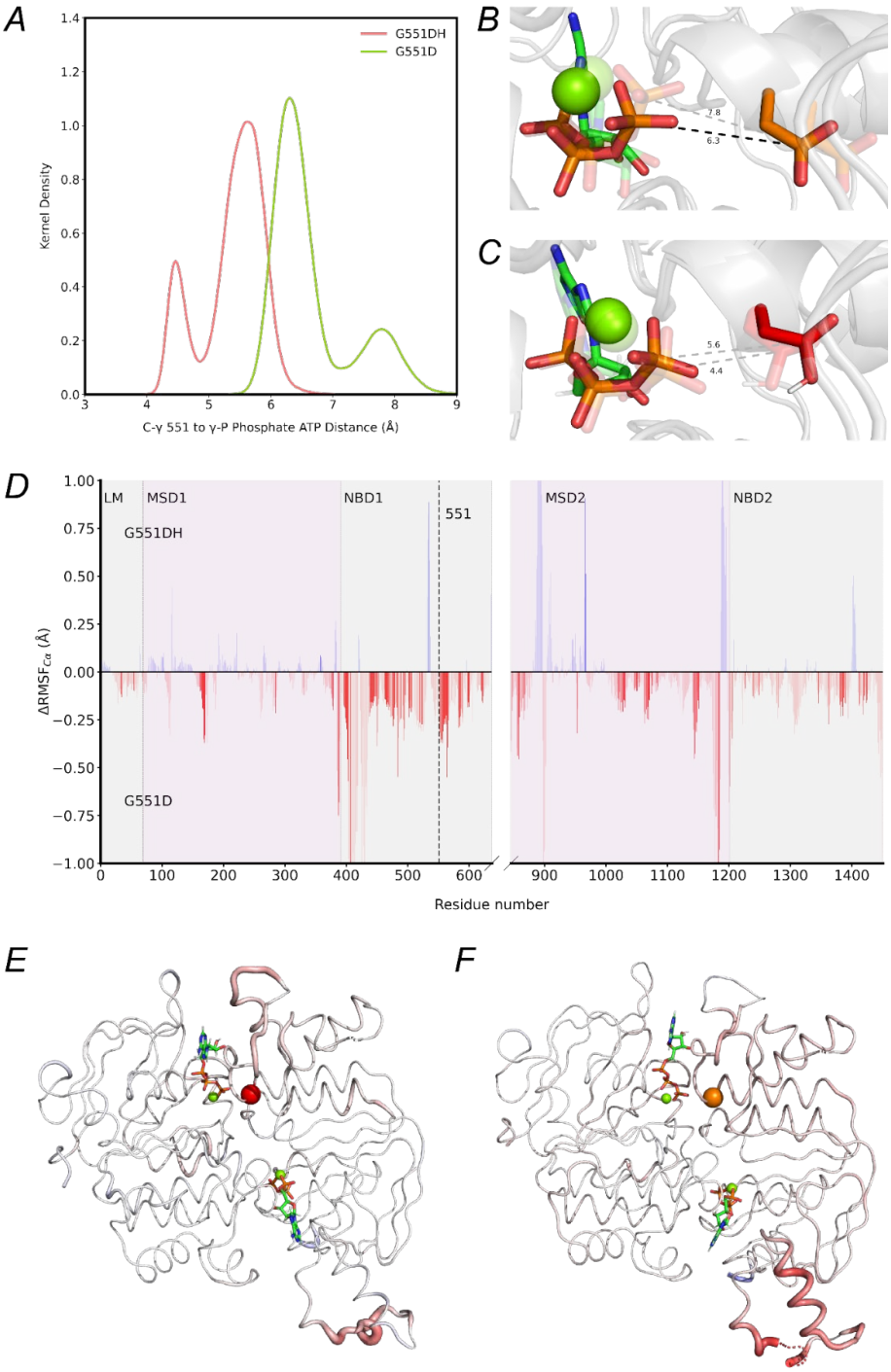

Figure 2. Simulation analysis of protonated (G551DH) versus deprotonated (G551D) CFTR mutant G551D.

A, kernel-density estimates (pooled from five independent 500 ns replicates per system) of the distance (Å) between the γ-carbon (C-γ) of residue 551 and the γ-phosphate of ATP in G551D (blue) and G551DH (pink) systems. *B, C,* representative structural conformations corresponding to the two major distance peaks identified in panel A. Structures from the major peak are depicted as opaque sticks, while structures from the minor peak are shown as transparent sticks. ATP is depicted as sticks with green carbons. G551D residues are shown as sticks with orange carbons (B), and G551DH residues

as sticks with red carbons (C). Dashed lines indicate measured distances, with numeric values annotated. *D*, differences in root-mean-square-fluctuation (ΔRMSF) for C-α atoms between G551DH and G551D. Blue bars indicate regions with greater flexibility in G551DH, while red bars indicate increased movement in G551D. Opacity of bars reflects statistical significance ($p < 0.005$). Residue 551 is marked by a vertical dashed line. Each CFTR domain is shaded in distinct colours and labelled above the plot. *E, F,* ΔRMSF (C-α) relative to WT mapped onto the NBDs of CFTR for G551DH (*E*) and G551D (*F*). RMSF values were mapped onto the cartoon representation as both colour and tube thickness. A blue-white-red colour scale was used in PyMOL,[34] with red and greater thickness indicating increased flexibility compared to WT. The position of residue 551 is shown as a red sphere (E) for G551DH and an orange sphere (F) for G551D. ATP is depicted as sticks with green carbons, and Mg^{2+} is shown as small green spheres. Abbreviations: LM, lasso motif; MSD, membrane-spanning domain; NBD, nucleotide-binding domain.

Comparison of root-mean-square fluctuations (ΔRMSF) for C-α atoms revealed greater local flexibility in the G551D mutant, particularly in NBD1, compared to its protonated counterpart G551DH (Figure 2D). This enhanced mobility indicates that the charged form of D551 destabilises the NBD positioning observed in WT CFTR, leading to increased local conformational fluctuations. Similarly, when compared to WT, the charged G551D exhibits significantly higher flexibility in NBD1 (Figure 2E; Figure S4). Together, these results suggest that G551DH best represents G551D-CFTR in conformations where the NBDs adopt a conformation close to that of catalytically active CFTR,[41, 42] with the NBDs in close proximity.

Simulations of the “mirror” mutation, G1349D, located at ATP-binding site 1 produced analogous results: the D1349 side chain remained closer to the ATP phosphates in its protonated state (< 7 Å) than in the deprotonated (charged) form (predominantly ~8 Å; Figure S5). Together, these data suggest that both D551 and D1349 likely need to be protonated to facilitate closer interactions with ATP and thus a more stable conformation of the NBDs. Therefore, from this point forward, we focus exclusively on the protonated forms of the mutants: G551DH and G1349DH.

<u>The effects of CFTR mutations on nucleotide binding domain interactions</u>

To assess differences in flexibility between the CFTR variants, we extended the ΔRMSF analysis ($\Delta RMSF = RMSF_{mutant} - RMSF_{WT}$) performed on protonated and

deprotonated G551D to the remaining mutants from this study: S549N, G1349DH, and F508del (Figure S6). S549N-CFTR exhibited increased flexibility compared with WT, particularly in the NBDs, with greater fluctuations than G551DH. Although the mutation lies in the opposing NBD, G1349DH showed similar increases in flexibility in NBD1 relative to WT, and, uniquely, in MSD1 at residues corresponding to transmembrane helices M5 and M6. These local increases in NBD mobility are related to global conformational shifts: the second principal component (PC2) from principal component analysis (PCA) of the combined trajectories discriminated WT from the S549N, G551DH, and G1349DH mutants (Figure 3). By contrast, F508del's PC2 distribution and NBD ΔRMSF resembled WT, apart from elevated flexibility at residues 505-507 and 509 adjacent to the deletion (Figure S6). Taken together, these data reveal that S549N and G1349DH perturb both local NBD dynamics and global inter-domain motion.

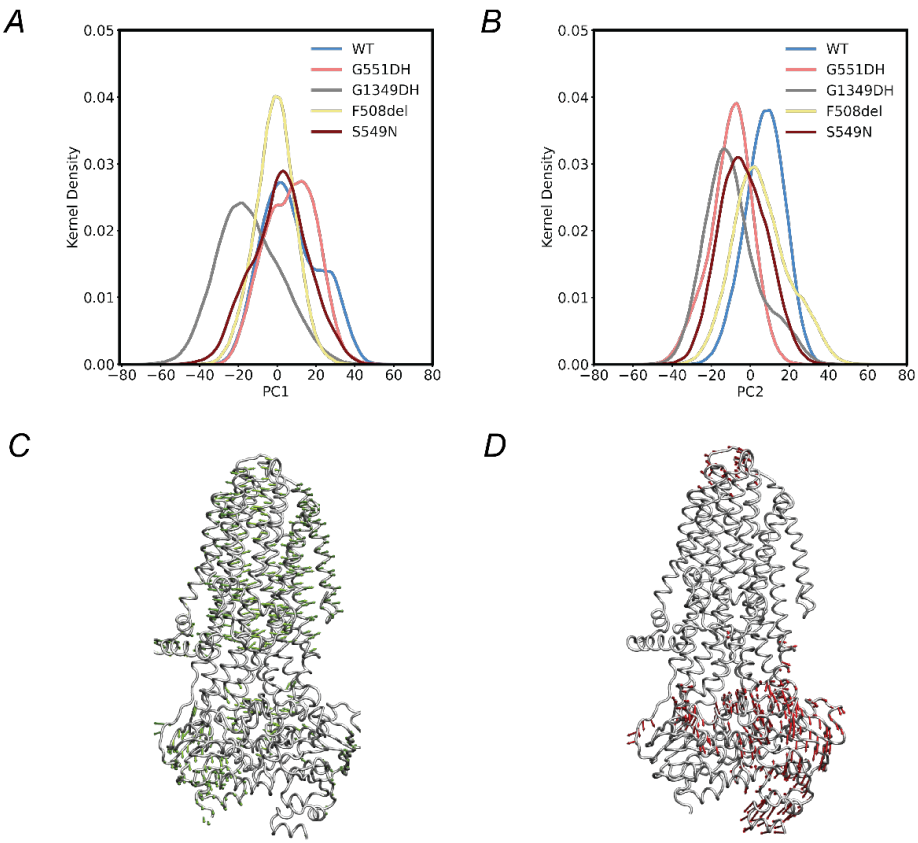

Figure 3 Principal Component Analysis (PCA) of WT and mutant CFTR systems.

A, kernel-density estimate (KDE) plots (pooled from five independent 500 ns replicates per system) of the first principal component (PC1) respectively, for WT and four mutants: G551DH, S549N,

G1349DH and F508del. *B*, KDE plot of the second principal component (PC2) for the same systems. *C*, *D*, conformational displacement along PC1 and PC2 mapped onto the CFTR structure. The CFTR protein is shown as a grey cartoon, and arrows indicate the principal directions of motion.

To quantify local and global differences in interactions between NBD1 and NBD2 across CFTR variants, we calculated three metrics for each system: (1) the distance between the C-α atom of residue 551 and the nearest ATP phosphate in binding site 2 (Figure 4A), (2) the distance between the C-α atom of residue 1349 and the nearest ATP phosphate in binding site 1 (Figure 4B), and (3) the centre-of-mass (COM) separation between NBD1 and NBD2, computed using only residues in α-helices and β-sheets to minimise bias from flexible loops (Figure 4C).

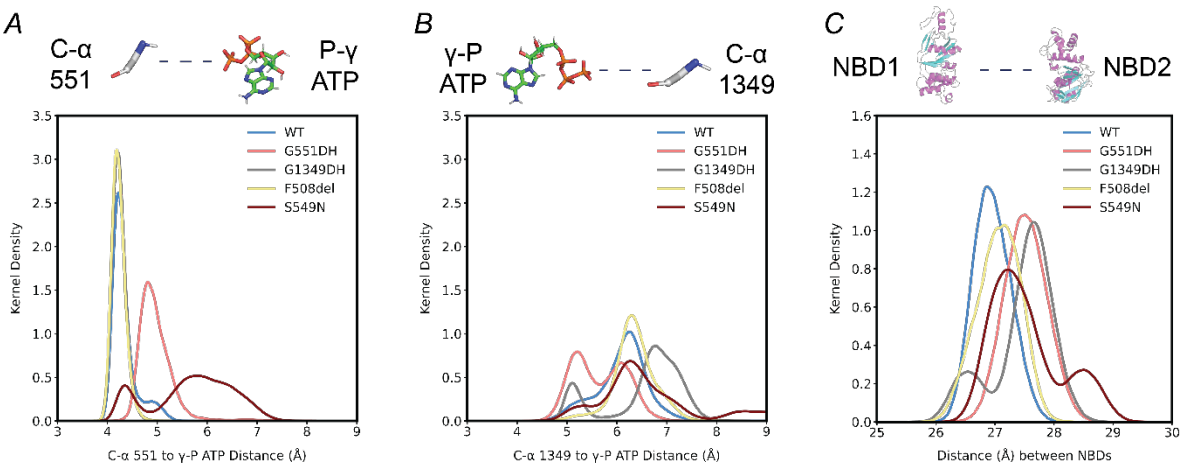

Figure 4. NBD inter-domain interactions in CFTR variants, quantified by distance distributions.

Kernel-density estimates (pooled from five independent 500 ns replicates per system) for*: A*, distance between the C-α atom of residue 551 and the γ-phosphate (γ-P) atom of ATP in the second ATP-binding site. *B*, distance between the C-α atom of residue 1349 and the γ-P atom of ATP in the first ATP-binding site. *C*, centre-of-mass separation between NBD1 and NBD2, calculated over α-helices and β-sheets only.

For the distance between residue 551 and ATP γ-phosphate in ATP-binding site 2, WT, G1349DH, and F508del display overlapping distributions centred at ~4 Å (Figure 4, A). In contrast, G551DH shows a moderate (but significant) increase in separation (~5 Å), and S549N displays the largest displacement and broadest range, indicative of a

destabilised interaction. In ATP-binding site 1, WT and F508del again coincide, whereas G1349DH, as expected, shows the greatest separation (Figure 4, B). Interestingly, a subpopulation of G551DH samples shorter distances than the main peak in WT, suggesting effects on ATP interactions in each site for this mutant. The COM separation between NBD1 and NBD2 is smallest for WT and only marginally higher for F508del, consistent with preserved inter-domain contacts (Figure 4C). All other mutants exhibit modestly increased separations, most pronounced in G551DH and G1349D, reflecting weakened NBD association (S549N has a minor sub-population with the largest NBD separation).

The increase in distance between the C-α atom of residue 551 and the γ-phosphate of ATP in the G551DH and S549N mutants suggests that ATP binding at site 2 may be compromised. To investigate if this can be observed in our simulations, we performed MM/GBSA calculations on the ATP+Mg^{2+} in this site. Compared to WT, G551DH simulations indicate an 18 kcal/mol reduction in estimated binding energy for G551DH compared to WT, a statistically significant difference (Table S1). This reduced ATP affinity in G551DH was analysed by decomposition per residue (Figure 5), showing that S549, G550, R555, and T1246 each contribute to reducing ATP affinity in G551DH, whereas interaction with D551 itself is favourable for affinity compared to G551, likely because the longer side chain at this position allows additional contacts without strong electrostatic repulsion. However, D551 may also compromise the interactions of the nearby residues (S549, R555, T1246) with ATP, leading to a less favourable ATP coordination overall. For example, the distance between the C-ζ atom of R555 and the γ-phosphate of ATP fluctuated significantly, compared to WT (Figure S7).

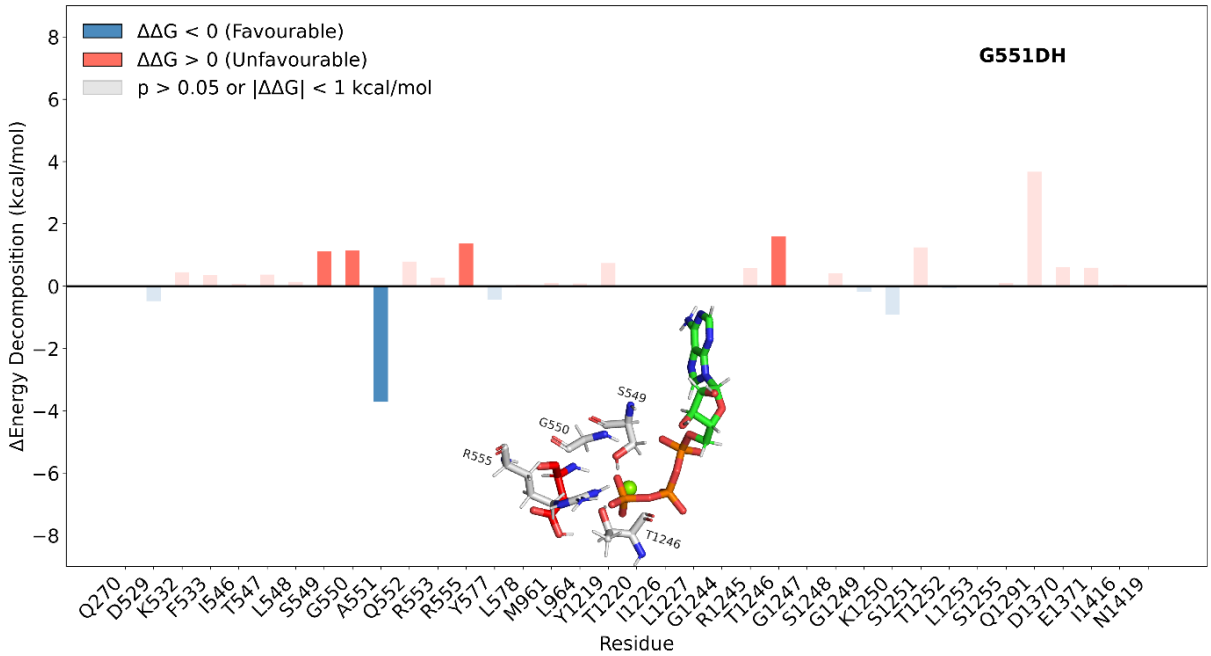

Figure 5. Per-residue energy decomposition of ATP+Mg^{2+} binding at site 2 in WT versus G551DH.

Bar plot of per-residue energy differences ($\Delta\Delta G = \Delta G_{G551DH} - \Delta G_{WT}$; kcal/mol), where positive values (light orange) indicate destabilising contributions in G551DH (less favourable binding), and negative values (blue) denote stabilising contributions (more favourable binding). Opacity reflects statistical significance across five 500 ns replicates (solid bars indicate $p \leq 0.05$, transparent bars $p > 0.05$). Bars for residues with $|\Delta\Delta G| < 1$ kcal/ mol are shown at reduced opacity to de-emphasise minimal effects. The inset shows the spatial organisation of residues with $|\Delta\Delta G| \geq 1$ kcal/mol, rendered as sticks with red carbons for residue 551, and grey carbons for other destabilising residues, green carbons for ATP, and Mg^{2+} as a green sphere.

For the other mutants investigated, differences in estimated ATP affinity to WT were not statistically significant (Table S1). Decomposition of residue contributions further indicated that there is essentially no effect on ATP interaction for F508del or G1349DH (Figure S8), likely because these mutations lie outside the second ATP-binding site. For the S549N mutant (which has a slightly higher estimated ATP affinity, but not significantly so; $p = 0.43$; Table S1), located within the same interaction sphere as G551DH, residues G551, Q552, K1250, and Q1291 contributed unfavourable effects on ATP binding ($\Delta\Delta G > 1$ kcal/mol), which are partially offset by contributions from S1251 and S1255 (Figure S8). These results suggest that S549N can similarly perturb local ATP contacts as G551DH, subtly disrupting ATP positioning.

Ivacaftor binding site is consistent across WT and mutant CFTR

We analysed the local lipid environment of the ivacaftor-binding site in WT and five mutant systems (indicated with the suffix "_IVA"). For each system, we identified the ten closest lipids of each type, namely 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), and cholesterol, and calculated the average percentage of each lipid type within 5 Å of ivacaftor (Figure 6). POPC was present most around ivacaftor, followed by POPE, then cholesterol. Visual inspection confirmed that ivacaftor contacts the lipid tails rather than the polar headgroups or ester linkages.

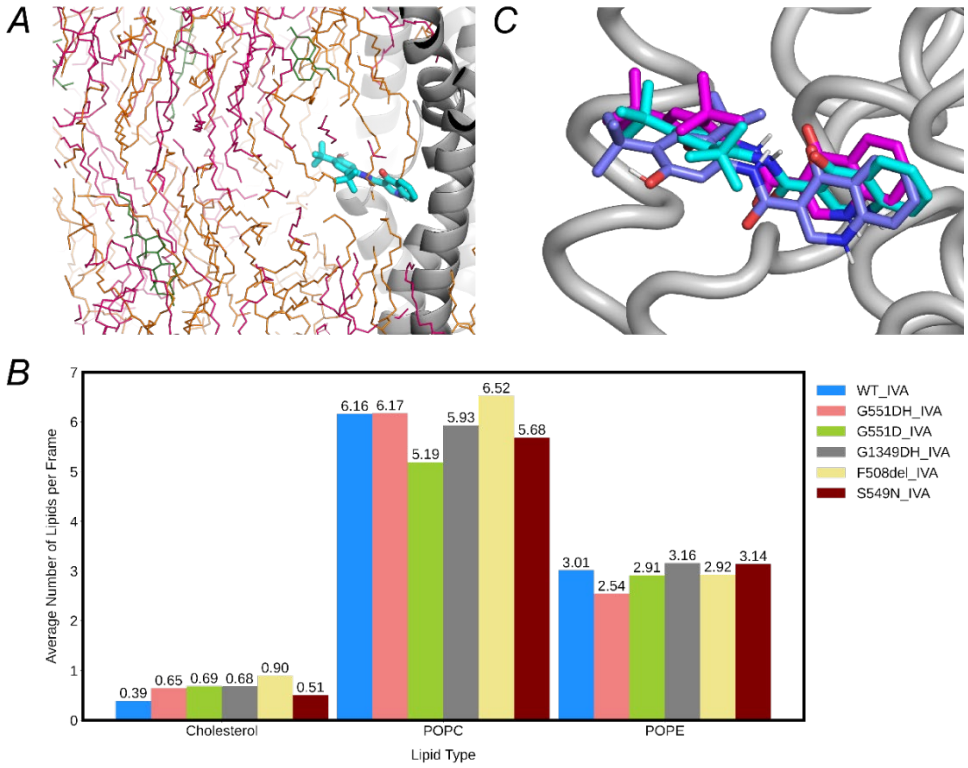

Figure 6 Lipid composition and binding-mode analysis of ivacaftor.

A, side view of ivacaftor at the protein-lipid interface (CFTR in grey cartoon; ivacaftor in cyan sticks; POPE in magenta, POPC in orange, and cholesterol in green sticks). *B*, bar plot of the average number per frame of each lipid type within 5 Å of ivacaftor, averaged across five 500-ns replicate simulations for each system. *C*, RMSD-based k-means clustering (k = 3) analysis of ivacaftor conformations, viewed from the bottom, with the mean structure across all systems overlaid as a ribbon. Trajectories were aligned based on the C-α atoms of the transmembrane segments (residues 81-110, 118-150, 177-218, 236-270, 292-329, 330-377, 860-880, 909-959, 966-1012, 1030-1063, 1081-1124, and 1125-1162), using the first frame as the reference. Cluster representatives are a

major state (cyan; 56.9% of frames), a similar secondary state (pink; 43.1%), and a minor outlier (purple; 0.003%).

All systems showed stable ivacaftor binding (Figure S9). Hydrogen-bond analysis (data not shown) revealed that two key interactions are conserved across all variants, namely between S308 O-γ to ivacaftor N16 (present in ~80% of frames) and F931 N to ivacaftor O13 (present in ~60% of frames). Ivacaftor also adopted comparable conformations in all simulations (Figure 6C). RMSD-based k-means clustering (k = 3), after alignment on the C-α atoms of transmembrane helices, revealed two slightly different conformational states, representing 56.9% and 43.1% of the sampled frames. Frame distributions among these clusters were virtually identical for WT and all mutants. Overall, therefore, ivacaftor binding in our simulations is essentially the same between WT and mutant CFTR systems.

Ivacaftor's influence on the NBDs and ATP binding is mutation dependent

To determine the impact of ivacaftor (IVA) on NBD inter-domain interactions in CFTR, we applied the same three metrics as described above (Figure 7). In WT, ivacaftor does not alter the 551-to-ATP distance, but it causes an increase in both the 1349-to-ATP distance and the NBD COM distance compared to the drug-free state. In G551DH, ivacaftor also does not substantially alter the 551-to-ATP distance (distribution similar in drug-free and drug-bound states). However, the COM distance shifts closer to that of drug-free WT, possibly indicating a partial rescue of NBD association. In G1349DH, ivacaftor appears to reduce the 1349-to-ATP distance towards the drug-free WT value, while the NBD COM distance remains similar to before. This suggests that ivacaftor primarily improves the local interaction at ATP-binding site 1 without significantly affecting overall NBD separation. In S549N, ivacaftor partially restores the 551-to-ATP distance towards the drug-free WT conformation (with limited change to the 1349-to-ATP distance). In contrast, the COM distance increases relative to both unbound S549N and unbound WT, indicating a more complex alteration in the NBD interface that may reflect incomplete or differential rescue of NBD association in this mutant. In F508del, the metrics are similar to those of drug-free F508del (and drug-free WT), indicating that ivacaftor does not significantly

affect NBD association or ATP-binding site geometry in this variant, which was already similar to WT.

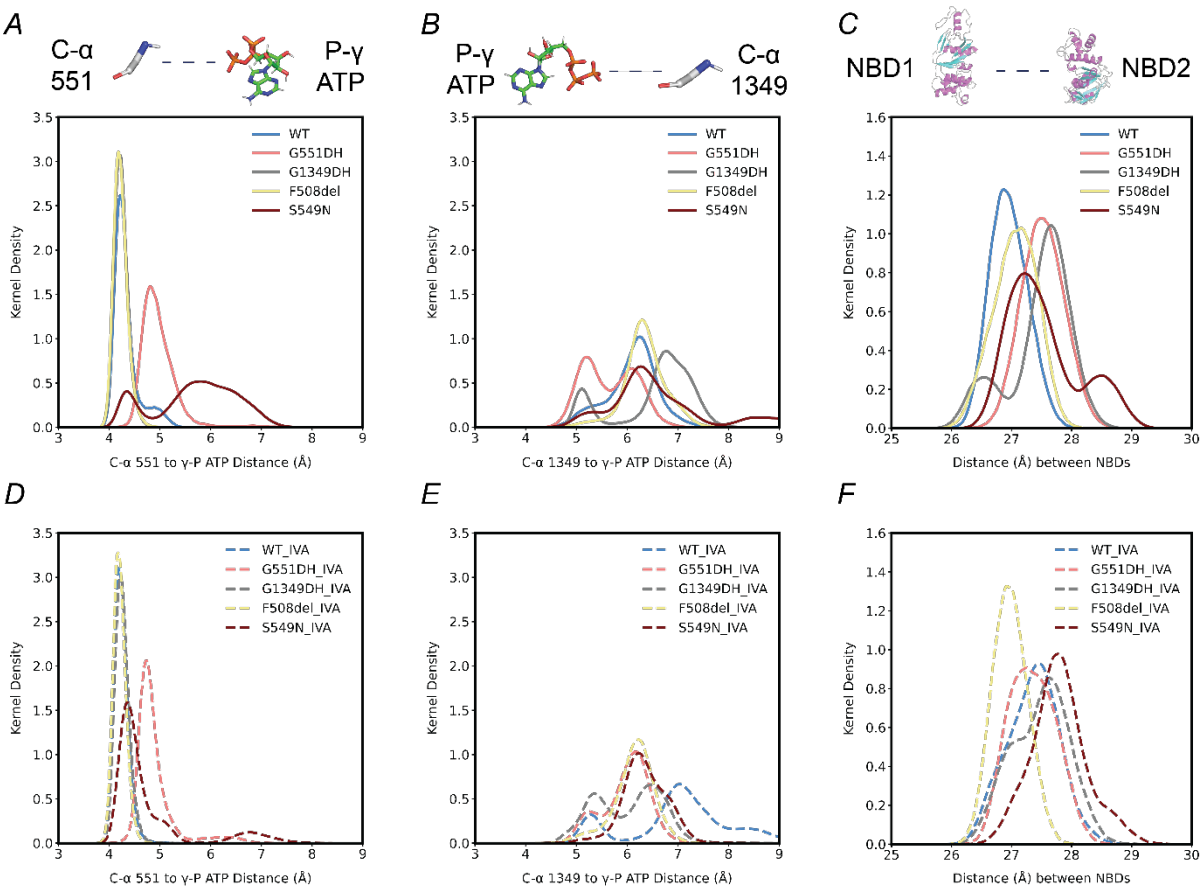

Figure 7. NBD inter-domain interactions in CFTR variants with and without ivacaftor, quantified by distance distributions.

Kernel-density estimates (pooled from five independent 500 ns replicates per system) for unbound systems (A-C) and ivacaftor-bound systems (D-F): *A*, *D*, distance between the C-α atom of residue 551 and the γ-phosphate (γ-P) atom of ATP in the second ATP-binding site. *B*, *E*, distance between the C-α atom of residue 1349 and the γ-P atom of ATP in the first ATP-binding site. *C*, *F*, centre-of-mass (COM) separation between NBD1 and NBD2 (calculated over α-helices and β-sheets only).

Notably, the predicted ATP binding affinity at the second ATP-binding site in G551DH with ivacaftor bound was not significantly different compared to WT-CFTR, whereas without ivacaftor, a significant reduction was observed (Table S1). Although this suggests partial rescue of ATP binding by ivacaftor, the difference in binding affinity between ivacaftor-bound and ivacaftor-free G551DH did not reach statistical

significance ($p > 0.1$). Thus, while ivacaftor may somewhat improve ATP binding compared to untreated G551DH, it does not fully restore it to WT. Residue-level energy decomposition (ΔΔG) further elucidates these findings. Comparing G551DH_IVA with drug-free WT revealed no significant change in R555's contribution to ATP binding (Figure S12), whereas drug-free G551DH did show a change. However, S549, F550 and T1246 remained destabilising, mirroring the pattern seen in drug-free G551DH. In a direct comparison of G551DH_IVA to G551DH (that is, ivacaftor-bound mutant versus ivacaftor-free mutant), only residue S549 exhibited a statistically significant difference. Taken together, these results indicate that ivacaftor may confer some improvement in ATP binding for G551DH, but underlying residue-level interactions, particularly around S549, F550, and T1246, still deviate from the WT, consistent with only partial restoration.

Interestingly, the S549N mutant with ivacaftor bound (S549N_IVA) exhibits a significantly reduced predicted ATP binding affinity compared to both the drug-free WT CFTR and S549N (Table S1). To better understand this effect, we compared the residue-level energy decomposition of S549N_IVA directly to S549N (Figure S13). Several residues significantly destabilise ATP binding in the ivacaftor-bound state (ΔΔG > 1 kcal/mol, $p < 0.05$), notably S1251 and S1255. It is unclear, however, if and how these changes are related to ivacaftor binding.

<u>The relationship between ivacaftor and two important pore salt bridges in CFTR</u>

Two pore-domain salt bridges, R347-D924 and R352-D993, have been identified as crucial for maintaining the open-pore architecture of CFTR,[43, 44] which are expected to be stable in WT CFTR (leading to long, stable channel openings[41]). In our simulations, we monitored these putative salt bridges through the distance between the functional-group carbon atoms (C-ζ to C-γ), using a threshold of 6 Å to define a "formed" salt bridge. Our results show that the R347-D924 salt bridge maintains a distance of 4-5 Å throughout the simulations (>95% of the frames, consistent with the cryo-EM structure used as starting point of the MD simulations) for both WT and mutant systems (Figure S14). In contrast, the R352-D993 interaction is dynamic in the simulations and only formed intermittently, present in about 30% of frames in WT and even less in some mutants (7% in F508del and G1349DH; Figure 8). Time-resolved analyses show

repeated formation and breakage throughout the trajectories (data not shown). Notably, ivacaftor appears to increase the proportion of R352-D993 salt bridge occupation in G551DH and F508del.

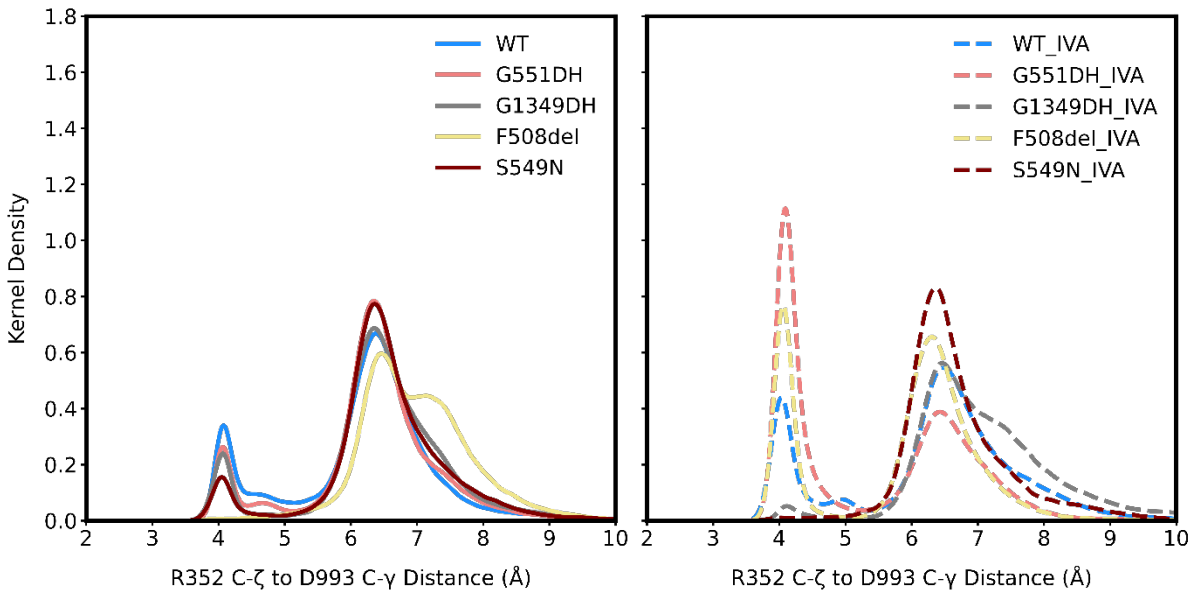

Figure 8 Pore-domain salt bridge R352–D993 formation in CFTR variants, quantified by distance distributions.

Kernel-density estimates (pooled from five independent 500 ns replicates per system) of the distance between the C-ζ atom of R352 and the C-γ atom of D993 for simulations without ivacaftor (left) and with ivacaftor (right).

Analysis of the pore across CFTR simulations reveals the passage of water molecules but not chloride ions.

The CFTR pore has an hourglass shape, with a shallow outer vestibule and a deep cytosolic vestibule separated by a narrow constriction, referred to as the selectivity filter.[9, 10, 45] Recent work from Levring and Chen[46] proposes that a hydrated chloride (Cl^-) ion enters via a lateral portal between transmembrane segments M4 and M6, where it is stabilised by a positive electrostatic surface. As the Cl^- ascends, it dehydrates to pass through the selectivity filter, where it is stabilised by interactions with G103, R334, F337, T338, and Y914. Upon exiting through a narrow lateral exit between M1 and M6, the Cl^- then rehydrates as it moves into the epithelial lumen.

In complementary microsecond-scale MD simulations conducted under a strong hyperpolarising electric field, spontaneous Cl^- permeation occurred in 2 of 10 runs, accompanied by subtle M1 and M6 rearrangements.[47] However, equivalent simulations without the electric field showed Cl^- binding in the channel pore, but no permeation.

We investigated chloride-CFTR interactions in our simulations of WT and mutant CFTR. The membrane region was defined by the phosphate-group *z*-coordinates and any ion entering this portion was followed. To focus on sustained, physiologically relevant events, only ions present in more than 10% of the frames were included in the analysis. In all our simulations, a single Cl^- occupies the pore for the majority of the time, although the specific ion frequently swaps (Figure 9, Figure S15; the latter shows chloride occupancy of the pore for each simulation replica). Consistent with previous work on WT CFTR[47], we did not observe a Cl^- crossing the upper membrane boundary in any of our WT or mutant CFTR simulations, indicating that the pore does not permit spontaneous ion movement into the extracellular space at this timescale (without applying an electric field). Levring and Chen suggest that positively charged residues attract Cl^- from the intracellular (bottom) side toward the extracellular (top) side of the pore,[46] a phenomenon that we also observed across these CFTR systems. In our simulations, Cl^- ions interact with R334, a residue located within the selectivity filter, indicating an upward attractive force (Figure 9).

Across all systems, we quantified interactions between Cl^- ions and pore residues by identifying any pore residue with at least one atom within 3.5 Å of Cl^- for more than 1% of the simulation frames. Notably, aside from the aforementioned R334, these interactions consistently involved residues Q353, W1145, Q1100, R352, R134, K95, N1148, R1097, Q1144, M348, W356, all located around the inner vestibule of the channel pore (Figure 9).

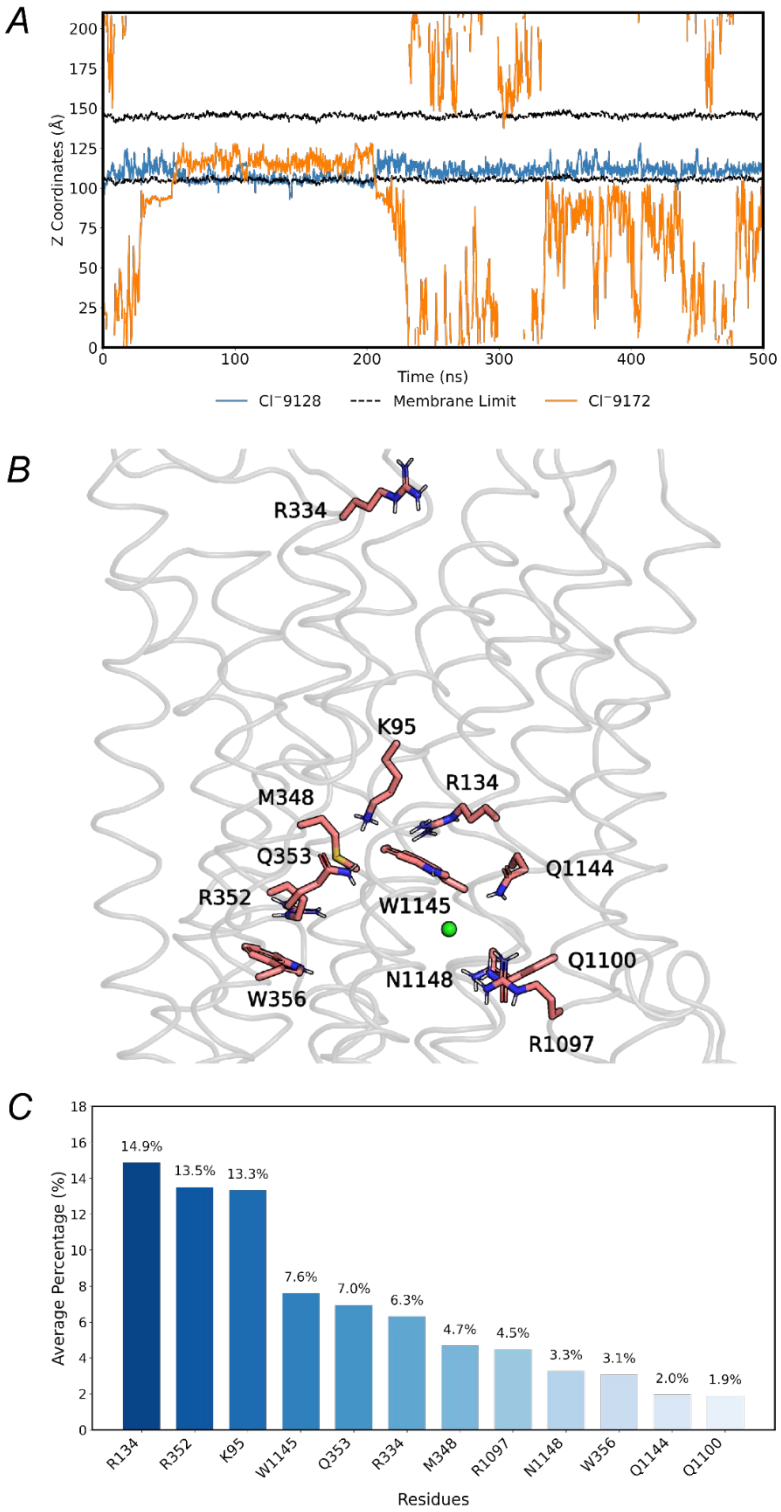

Figure 9 Chloride-ion occupancy and residue interactions in the CFTR pore.

A, time-resolved *z*-coordinate positions of Cl^- ions entering the membrane region (defined by the phosphate-group *z*-coordinates) during one 500-ns WT CFTR simulation (one of five independent replicates). Two individual ions are highlighted in blue and orange and the upper and lower membrane boundaries are shown as black dashed lines. *B*, CFTR pore residues that contact Cl^- in > 1% of frames are shown as pink sticks on the grey cartoon backbone; a representative pore Cl^- is shown as

a green sphere. Data are pooled across all systems, with and without ivacaftor, over five independent 500-ns replicates each. *C,* mean percentage of simulation frames in which each common residue interacts with Cl^- (any residue atom to Cl^- ion distance ≤ 3.5 Å), pooled across all systems, with and without ivacaftor, over five independent 500-ns replicates each.

We applied the same tracking approach for water molecules as used for Cl^-, observing frequent, bidirectional water movement through the CFTR pore in our simulations (Figure S16). The number of such passages differs per simulation, but reaches several hundred in each. There is no clear evidence of ivacaftor (or mutations) altering the rate or extent of water passage (Figure S17). Thus, the mutations and ivacaftor appear to have minimal impact on water permeability, or at least that any such effect is not readily discernible from our current dataset.

Discussion

In this work, we present extensive all-atom molecular dynamics (MD) simulations of the membrane-embedded cystic fibrosis transmembrane conductance regulator (CFTR) in both wild-type (WT) and disease-causing mutant forms, and either with ivacaftor bound or not. Using 32.5 μs of MD simulations (13 systems, each with five independent 500 ns repeats), we investigated how CF-associated mutations, including gating mutations G551D, G1349D and S549N, and the folding/gating F508del mutation, alter CFTR structure and dynamics. While individual 500 ns simulation runs may not capture large-scale conformational rearrangements away from the starting model, they are well suited to reveal local structural and flexibility changes relevant to CFTR function.

In WT CFTR, the binding of one ATP molecule (coordinated by a Mg^{2+} ion) at each of the two ATP-binding sites drives close association of the two nucleotide-binding domains (NBD), a process we refer to as NBD dimerisation. In the dimerised state, each ATP molecule simultaneously engages the Walker A (phosphate-binding) motif of one NBD and, from the opposing NBD, the ABC signature sequence (LSGGQ in NBD1 or its degenerate LSHGH in NBD2).[48] Gating mutations G551D and G1349D both substitute Gly with Asp within these signature motifs (resulting in LSGDQ in

NBD1, or LSHGH in NBD2, respectively). G551D is delivered to the cell surface at levels comparable to WT[49] but fails to activate the chloride channel.[50] In WT CFTR, the backbone amide nitrogen of G551 lies within hydrogen-bond distance of the ATP γ-phosphate in the dimerised state.[51] Replacing Gly with Asp could introduce a negative charge, that likely repels the γ-phosphate. Under saturating ATP (3 mM), single-molecule FRET between NBDs indicates an intermediate FRET efficiency for G551D (~0.37), distinct from both fully dimerised WT (~0.49) and the NBD-separated CFTR (~0.25), suggesting disrupted but not abolished NBD approach.[42] Our MD simulations start from the fully dimerised CFTR conformation. When Asp is treated as unprotonated (negatively charged), the distance between residue 551 and the nearby γ-phosphate is increased compared to WT. However, with Asp singly protonated (consistent with the expected pK_a at the dimerised conformation), the distance only increases by ~1 Å compared to WT CFTR. The potentiator ivacaftor appears to have minimal impact on this interaction, inducing at most a very slight reduction in the D551 to γ-phosphate distance. Flexibility analysis indicates that with Asp unprotonated, NBD dimerization is not stable and may indicate the start of NBD separation. Therefore, we suggest that two states of G551D CFTR exist in physiological conditions: one (partially) NBD-separated conformation with D551 unprotonated, and one NBD-dimerised state when it is protonated. Once the NBDs approach sufficiently (with ATP bound), the local microenvironment would shift the pK_a such that D551 becomes protonated, and full NBD dimerization can occur. Nevertheless, even when D551 is protonated, its interaction with ATP does not fully recapitulate the local NBD-dimerized conformation observed in WT CFTR, implying additional effects beyond simple charge repulsion. Indeed, binding affinity predictions (using MM/GBSA) confirm that ATP in NDB2 is bound with lower affinity with protonated D551 (G551DH) compared to WT. Recent cryo-EM data on G551D in complex with ivacaftor support the idea that ivacaftor enhances CFTR function by increasing the coupling efficiency between NBD dimerisation and pore opening, thereby prolonging the open state and enhancing chloride flux.[52] Although ivacaftor does not primarily promote NBD dimerisation, we propose that it may exert a secondary effect by helping to stabilize the NBD interface, therefore reducing the solvent exposure of D551, shifting its equilibrium towards the protonated state. Once protonated, productive interactions with ATP in binding site 2 can be maintained, as indicated by our simulations.

G1349D mirrors G551D in NBD2's (degenerate) signature motif.[50] Unlike G551D CFTR, which is largely ATP-insensitive, G1349D retains some ATP dependence but lowers the channel's open probability (P_o) approximately tenfold versus WT.[50] Although ivacaftor can potentiate G1349D CFTR (increase its P_o), genistein, another known CFTR potentiator that stimulates both WT and F508del-CFTR, does not do so.[53] Our simulations suggest that, as for G551D, protonation of D1349 is likely required to mitigate electrostatic repulsion with the ATP phosphate groups in the NBD-dimerised conformation. When protonated (G1349DH), the ATP binding to NBD2 (near G551) is essentially equal to WT CFTR, as is the flexibility of NBD2, where the mutation is located. However, increased flexibility in transmembrane segments M5 and M6 observed in our simulations supports the existence of an allosteric pathway between the NBDs and the pore that may underlie G1349DH's gating defect. Notably, ivacaftor reduces the distance between the protonated residue D1349 and ATP, restoring it towards WT values. This may indicate that interactions between ATP in NBD1 and the region with the mutation are strengthened, which in turn may contribute to the G1349D CFTR potentiation (and thus better CFTR function).

The F508del mutation in CFTR destabilises the protein folding, triggering endoplasmic reticulum quality-control recognition and leading to degradation before the channel can traffic to the cell surface.[54] Even when pharmacologically rescued to the plasma membrane,[55] F508del CFTR displays gating defects that require potentiator treatment. In this study, we observed that conformational dynamics and ATP binding of F508del CFTR were similar to WT, but noted a reduction in MSD1 flexibility that may hinder the structural transitions required for ion permeation. Ivacaftor binding did not appreciably modify NBD interactions, likely because our starting model represents a fully rescued CFTR (that is, properly folded, phosphorylated, and NBD-dimerised). However, ivacaftor did increase flexibility in both MSD1 and NBD1, which could underlie its potentiating effect by facilitating gating transitions, but can also explain why chronic treatment of F508del-carrying patients with ivacaftor can destabilize F508del CFTR.[56]

S549 lies within the conserved LSGGQ signature motif of NBD1, where its side chain hydroxyl normally hydrogen-bonds with the ATP γ-phosphate.[12, 57] The mutation disrupts this interaction, shifting the γ-phosphate further from position 549 and compromising the alignment needed for hydrolysis. Functionally, S549N is classified predominantly as a gating mutation,[57] with some mild processing defects.[58] ATP

binding affinity and functional efficacy (as measured by current passage) of S549N are diminished compared to WT.[58] When treated with ivacaftor and lumacaftor (a corrector that mitigates processing defects), S549N currents recover to near-WT levels. In our simulations, the γ-phosphate of ATP is indeed further from residue 551 in S549N compared to both WT and G551DH. Interestingly, with ivacaftor present, this distance is partially “rescued” to WT levels. Notably, despite these local improvements in ATP binding, ivacaftor increases the overall NBD COM distance somewhat, suggesting a nuanced allosteric effect. This supports the notion that ivacaftor does not directly promote increased gating in CFTR by causing closer NBD association, but through other dynamic effects (e.g., affecting the pore). Although the S549N mutation affects the ATP local environment, our binding affinity calculations indicate that it likely does not prevent ATP binding. However, the increased flexibility in NBD1 we observe may be an indication that conformations required for efficient ATP hydrolysis are not easily maintained.

Although our simulations showed that (at least) one chloride ion remains within the pore throughout, the ion did not pass through the selectivity filter to reach the extracellular side. Conversely, bidirectional water passage through the channel was observed in all systems. This observation suggests that additional conformational changes, likely linked to gating transitions, are required for chloride conduction, and these transitions are not captured within our simulation timescales. This aligns with previous studies suggesting that CFTR undergoes complex structural rearrangements to transition between permissive and non-permissive states for chloride conduction.[46] The presence of ivacaftor in our simulations affected neither chloride nor water passage. We identified several key residues that consistently interact with chloride ions, including several positively charged residues that were indicated to be important for anion conduction, such as R352,[59] R134,[60] K95.[59,60]

By conducting long-timescale MD simulations on our (Cryo-EM-based) CFTR model in the open-channel conformation, we gained new insights into how several CF-associated mutations can subtly disrupt channel dynamics and (thereby) function, and how the modulator ivacaftor influences (and partially rescues) these processes. Importantly, our extensively tested model provides a starting point for future investigations into CFTR dynamics, which may guide the development of next-generation therapeutic strategies. In this study, we observed that mutations in the ATP-

binding sites such as G551D, S549N and G1349D disrupt local ATP-binding geometries and NBD interactions, with protonation of aspartate in G551D and G1349D being a critical factor for maintaining a conformation conducive to proper NBD engagement. We also noted that ivacaftor's primary mechanism of action likely involves modulating the local environment of the transmembrane helices rather than directly tightening NBD association. Together, these insights provide a more nuanced understanding of CFTR dynamics and the molecular mechanisms underlying its mutation-induced functional alterations.

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