

1 **Characterization of the dynamic resting state of a pentameric ligand-gated ion** 2 **channel by cryo-electron microscopy and simulations**

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13 **Abstract**

14 Ligand-gated ion channels are critical mediators of electrochemical signal
15 transduction across evolution. Biophysical and pharmacological development in this
16 family relies on high-quality structural data in multiple, subtly distinct functional
17 states. However, structural data remain limited, particularly for the unliganded or
18 resting state. Here we report cryo-electron microscopy structures of the *Gloeobacter*
19 *violaceus* ligand-gated ion channel (GLIC) under resting and activating conditions
20 (neutral and low pH). Parallel models were built either manually or using recently
21 developed density-guided molecular simulations. The moderate resolution of
22 resting-state reconstructions, particularly in the extracellular domain, was improved
23 under activating conditions, enabling the visualization of residues at key subunit
24 interfaces including loops B, C, F, and M2–M3. Combined with molecular dynamics
25 simulations, the cryo-electron microscopy structures at different pH describe a
26 heterogeneous population of closed channels, with activating conditions condensing
27 the closed-channel energy landscape on a pathway towards gating.

28

1 Introduction

2 Ligand stimulation of receptors enabling ion flow across cell membranes is a critical
3 mechanism of electrochemical signal transduction in all biological kingdoms.
4 Pentameric ligand-gated ion channels are major mediators of fast synaptic
5 transmission in the mammalian nervous system, but are also found throughout
6 evolution in a variety of biological roles [1]. Members of this protein family share a
7 five-subunit architecture assembled surrounding a central permeation pathway. The
8 extracellular domain (ECD) of each subunit consists of 10 β -strands, with loops A–F
9 enclosing a canonical ligand-binding site [2] at the intersubunit interface. The
10 transmembrane domain (TMD) contains four α -helices (M1–M4), with the M2 helices
11 lining the channel pore, and an intracellular domain of varying length (2–80 residues)
12 inserted between M3 and M4 [3].

13 Starting from resting (apo-closed) conditions, agonist binding is thought to favor
14 subtle structural transitions to a ‘flip’ or other intermediate state(s) [3], opening of the
15 transmembrane pore, and in most cases refractory desensitization [4]–[7].
16 Accordingly, a detailed understanding of pentameric channel biophysics and
17 pharmacology requires high-quality structural templates in multiple functional states
18 [8]. Over the last ~12 years several X-ray and cryo-electron microscopy (cryo-EM)
19 structures representing family members from prokaryotes to eukaryotes [3], [9]–[13]
20 have been established. However, few have been solved in the absence of
21 modulatory ligands, leaving substantial unanswered questions as to the structural
22 and dynamic properties of the resting state. The few resting-state structures reported
23 this far have been solved to lower resolution than their liganded counterparts [11],
24 [14], [15], suggesting inherent mobility of this state, which demands models taking the
25 dynamics into account in order to understand its properties and employ it e.g. for
26 drug design.

27 One of the best studied members of this protein family is the *Gloeobacter violaceus*
28 ligand-gated ion channel (GLIC) [16]–[21]. This proton-gated prokaryotic receptor
29 has proved amenable to crystallization under activating conditions (pH ~4) [22],

1 producing structures in apparent open states in the presence of various ligands,
2 modulators [23], and mutations [24]. Additional structures of GLIC have been
3 reported in lipid-modulated [25] and a so-called “locally closed” state [26], possibly
4 representing gating intermediates. However, only a single GLIC X-ray structure has
5 been reported under resting conditions (pH 7), and only to relatively low resolution
6 (4.4 Å) [27].

7 Here, we report single-particle cryo-electron microscopy structures of GLIC under
8 multiple pH conditions. To facilitate structure determination and comparison of the
9 same protein in different states, we also employed techniques for density-guided
10 molecular dynamics (MD) simulations [28], validated against manually built models.
11 Although most of the collected cryo-EM data displayed apparently nonconducting
12 pores, comparisons between the reconstructions revealed striking differences in
13 global and local resolution, including local differences in interfacial loops stabilized at
14 different pH. Atomistic simulations of current cryo-EM and previous X-ray structures
15 supported elevated flexibility under resting conditions, particularly in the extracellular
16 domain (ECD). We find increased stability, ECD contraction, and domain twist upon
17 protonation. These results support a broad distribution of conformations under
18 resting conditions, separated by low free energy barriers that produce a dynamic,
19 heterogeneous population of resting-state channels with a closed pore. The
20 distribution narrows upon ligand (e.g. proton) binding to enable transition of a subset
21 of molecules, potentially leading to pore opening.

22 **Results**

23 **Cryo-EM structures of closed GLIC by manual and automated modeling**

24 To characterize the resting state of the prokaryotic pentameric channel GLIC, we first
25 obtained single-particle cryo-EM data under resting conditions (~pH 7), resulting in a
26 4.1 Å resolution map (Fig. 1A–B - supplements 1-2, Tab. 1). The map resolution was
27 between 3.5 - 4 Å in the TMD, including complete backbone traces through the
28 C-terminal M4 helix. Side chains in the TMD core were also generally well resolved,
29 including a constriction at the so-called 9'-hydrophobic gate (I233, 2.9 Å Cβ-atom

radius) (Fig. 1 supplement 1C), consistent with a closed pore. However, side chains of polar residues E222, N224, and E243 in the pore-lining M2 helix were not as well resolved, and could not be confidently built in the final model. We similarly refrained from building some side chains on the TMD surface, including outward faces of M1 (I198), M3 (M261, E272), and M4 (8 residues), as well as five loop residues between transmembrane helices. Compared to the TMD, the ECD was less well resolved, including some backbone traces that could not be confidently built at the extracellular end (N-terminus and β 2– β 3, β 5– β 6, and β 7– β 8 loops), and incomplete side chains distributed throughout the β -strands.

GLIC has been thoroughly documented as a proton-gated ion channel, conducting currents in response to low extracellular pH with an EC50 around pH 5 [16]. Taking advantage of the capacity of cryo-EM to capture structures under different buffer conditions, we obtained additional reconstructions under partial and maximal (pH 5 and pH 3) activating conditions, producing relatively high-resolution (3.4 Å and 3.6 Å) maps (Fig. 1C-D - supplements 1-2, Tab. 1). To make a more quantitative comparison, a subset of particles was also selected randomly from each dataset collected at pH 5 and 3, containing the same number of particles as the autorefined set from pH 7. Refinements from these low-pH subsets again produced reconstructions to higher resolution than at neutral pH, particularly in the ECD (Fig. 1 - supplement 3), indicating that the varied resolution across pH could not be immediately attributed to particle numbers.

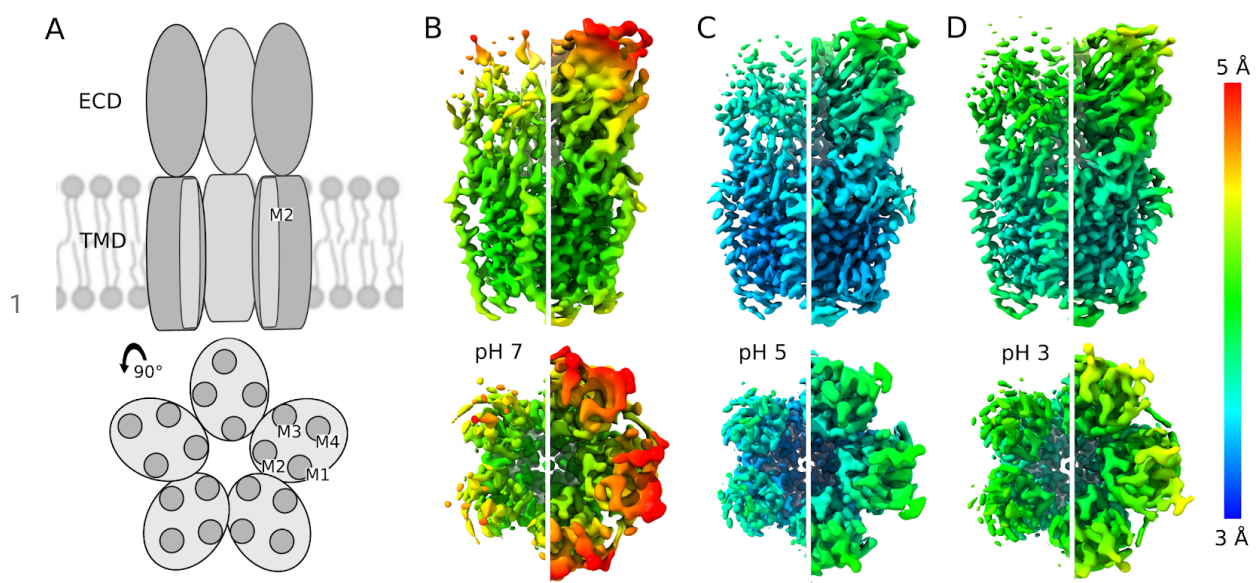


Figure 1: Improvement in local resolution of GLIC cryo-EM densities with decreasing pH. (A) Cartoon representations of GLIC. Upper image is viewed from the membrane plane (side view), with proximal two subunits removed for clarity, revealing the channel axis through the ECD (ovals) and M2 helices within the TMD (cylinders). Lower image shows a 90° rotation viewed from the extracellular side (top view), with transmembrane helices M1–M4 labeled in one subunit. Remaining panels show cryo-EM reconstructions from data collected at (A) pH 7 to 4.1 Å overall resolution, (B) pH 5 to 3.4 Å resolution, or (C) pH 3 to 3.6 Å resolution. Upper and lower images show side and top views respectively, as in panel A. Each density is colored by local resolution according to scale bar at far right, and contoured at both high (left) and low threshold (right) to reveal fine and coarse detail.

To standardize model building under parallel pH conditions, we also took advantage of new density-guided methods [28], [29] recently implemented in the GROMACS MD package [30], [31]. After equilibrating our pH 7 cryo-EM structure in a lipid bilayer with 150 mM NaCl, we fit this system to masked half-maps obtained at each pH using an automated adaptive-force scaling approach (Fig. 3A). Output models were evaluated relative to the independent half-map at 100 ps intervals, and for all three conditions they converged to stable ensembles within 20 ns (Fig. 3 supplement 1). Final models were obtained by simulated annealing of the best-fit configuration. Because symmetry was not applied during automated fitting, variations could be observed particularly in side chain orientations in different subunits. Given the relative novelty of this automated approach, we also manually built independent

1 models in each condition, which correspond closely (within 2.0 Å Cα
2 root-mean-square deviation; RMSD) to simulation-guided versions (Fig. 3D, Tab. 2).

3 Regardless of pH, the majority of cryo-EM data reconstructed a constricted
4 hydrophobic gate, likely reflecting predominantly closed-state particles. Still, the
5 relatively high-resolution maps at pH 5 and 3 revealed local rearrangements relative
6 to the maps at neutral pH, including densities that could be fully built for side chains
7 throughout helices M1, M2, M3, and the bulk of M4.

8 **Local rearrangements associated with protonation**

9 In the ECD, several residues missing backbone density became visible in cryo-EM
10 maps obtained at lower pH, especially in the β2–β3 and β5–β6 loops at the
11 extracellular surface. Furthermore, side chains with poor densities at pH 7 could be
12 confidently built in every β-strand and the β6–β7 (Pro) loop at both pH 5 and 3. At pH
13 3, additional residues or side chains could be built especially in the β4 strand, β7–β8
14 (loop B) and β9–β10 (loop C), indicating a progressive increase in local stability with
15 decreasing pH. These three regions form a continuous face of the extracellular
16 β-sandwich, implicated in modulation of GLIC [32] and in agonist binding to other
17 pentameric channels [23], [33]. Indeed, previous X-ray structures included residues
18 in the same three regions that were incomplete in the resting state [27], but resolved
19 under activating conditions, with a relative closure of the cleft between loops B and C
20 [22]. Apparent stabilization at low pH in both cryo-EM and X-ray structures thus
21 supports a coordinated contraction of this region associated with gating.

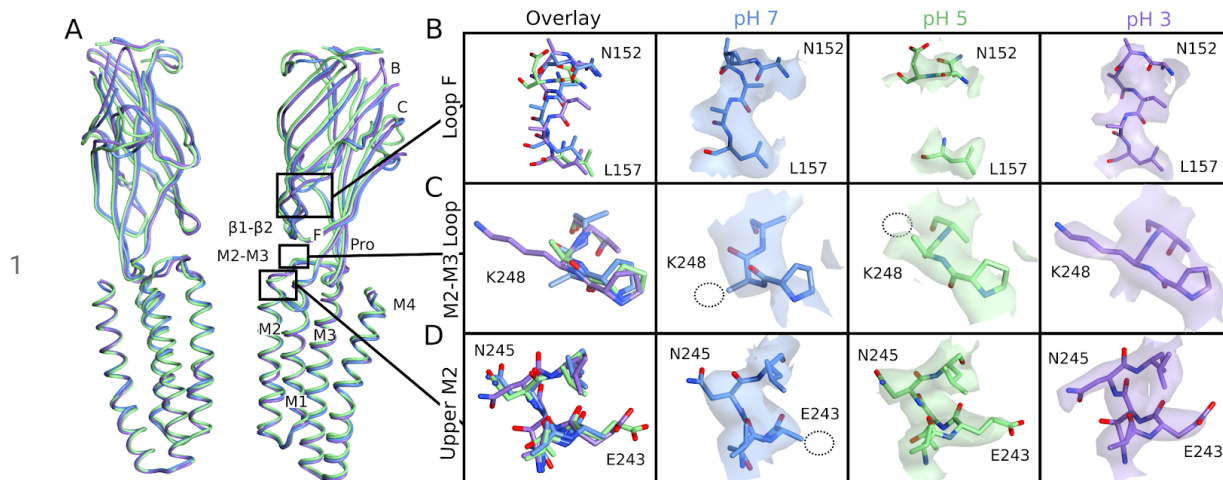
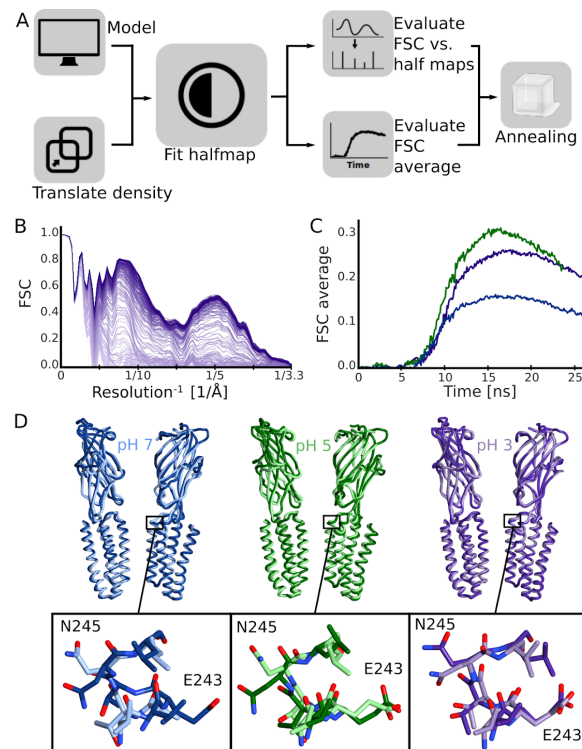


Figure 2: Lower-pH GLIC structures resolve increasing detail. (A) Overlaid side views of backbone ribbons representing two opposing subunits from GLIC cryo-EM models at pH 7 (blue), pH 5 (green), and pH 3 (purple). Functionally relevant features labeled in the righthand subunit include loops B, C, and F and the $\beta 1$ - $\beta 2$ and Pro loops in the ECD, and helices M1-M4 in the TMD. Remaining panels illustrate local model detail in (B) loop F (N152-L157), (C) the M2-M3 loop (P247-T249), and (D) the upper M2 helix (V242-L246). Leftmost boxes include all-atom overlays at pH 7, 5, and 3, colored by heteroatom with carbons colored as in panel A. Boxes at right show individual models and associated cryo-EM densities. Dotted circles indicate side chains of E243 and K248 that could not be confidently assigned in higher-pH densities; note also that backbone densities were poorly resolved in a portion of loop F (D154-F156) at pH 5.

Notably, backbone densities for three residues in $\beta 8$ - $\beta 9$ (loop F) were less defined in the pH 5 map compared to the otherwise lower-resolution pH 7 map (Fig. 2B). At pH 3, backbone traces and some side chains could again be built in these regions. Loop F has been extensively implicated in gating of pentameric channels [2] including GLIC [34]; it was recently shown by mutagenesis and covalent labeling to mediate key functional interactions with E35, the main proton sensor in GLIC pH gating [27], which could not itself be built in any of our cryo-EM maps. Thus, successive de- and re-stabilization of loop F under progressively lowered pH was consistent with sequential rearrangements involved in proton sensing.

Although the TMD was generally better resolved under all conditions than the ECD, resolution also improved in low-pH maps for the extracellular mouth of the pore, particularly residue E243 (Fig. 2D, Fig. 3D). This residue has been proposed to

1 stabilize the upper part of the M2 helix during channel opening [27]. In previous
2 X-ray structures, it displayed differential M2-hydrogen bonding patterns at low vs.
3 neutral pH, interacting with T244 of the neighboring subunit under resting conditions
4 [27] but with N245 of the same subunit under activating conditions [22]. Our cryo-EM
5 structures follow a parallel pattern, with E243 contacting T244 at pH 5, but N245 at
6 pH 3.



8 **Figure 3: Density-guided fitting enables automated modeling of GLIC structures.** (A) Density-guided
9 fitting workflow, starting from an approximate equilibrated model, and ending in a single structure that
10 can be used for comparison and evaluation. (B) Improvement in map-to-model FSC during
11 density-guided fitting to the pH-3 map, showing progress over simulation time (light to dark purple).
12 The FSC varies with resolution, dropping off at the highest overall resolution value for the map (3.4 Å).
13 (C) Average map-to-model FSC over simulation time for fitting to maps at pH 7 (blue), pH 5 (green),
14 and pH 3 (purple). Following steady increases in early fitting stages, each fit reached a maximum
15 similarity around 15 ns, after which the fitting force began to distort the structure. The best-fit frame
16 was chosen for simulated annealing and further analysis. (D) Models from density-guided fitting to
17 data collected at pH 7 (left, blue), pH 5 (center, green), and pH 3 (right, purple). For comparison,
18 manually built models are overlaid in lighter shades of the same color. Upper images show side views

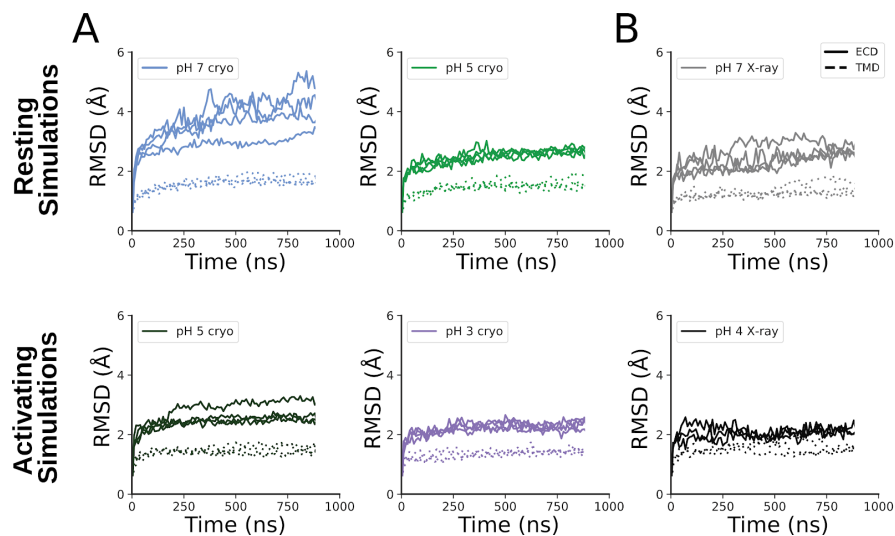
1 of backbone ribbons for two opposing subunits; lower images show detail for the upper M2 helix as in
2 Fig. 2D.

3 Whereas some residues in the M2–M3 (K248–T249) and M3–M4 (E282–S283)
4 loops remained ill-defined at pH 5 as well as pH 7, all TMD side chains could be built
5 at pH 3. We particularly noted the emergence of side chain density for K248 (Fig.
6 2D), which was previously reported to orient differently in resting and activating X-ray
7 structures [20], [23]: K248 faced the channel pore and the M2 helix of the same
8 submit at crystallographic pH 7, but reached across the intersubunit cleft to contact
9 N245 on the complementary M2 at pH 4. Similar to low-pH X-ray structures, K248 in
10 the pH 3 cryo-EM structure faced the subunit interface, although the closed
11 conformation of M2 precluded direct contact with N245. In automated density-guided
12 fitting models, K248 was oriented towards the pore in a majority of subunits fit to the
13 pH 7 map, but towards the subunit interface in a majority of subunits fit to pH 5 and 3
14 data. In several cases, lower-pH conditions produced an upward intersubunit
15 orientation, including electrostatic contacts with complementary ECD residues D32
16 or Y119. These interactions recall previous reports that ECD-crosslinking of K248
17 stabilizes a locally closed state [27], possibly representing a gating intermediate.

18 **Flexibility of the resting-state ECD revealed by molecular dynamics**

19 Given the differential local resolution and conformations observed upon decreasing
20 pH, we asked whether these properties might reflect a flexible resting state, which is
21 systematically stabilized through rearrangements upon protonation particularly in the
22 ECD. To this end, we ran quadruplicate 1- μ s all-atom MD simulations on each
23 cryo-EM model embedded in a lipid bilayer. The TMD was consistently stable under
24 all conditions, converging to ~ 2 Å C α -RMSD within 250 ns (Fig. 4A). Conversely, for
25 the ECD domain, deviation of C α atoms was substantially higher in simulations of
26 the pH 7 vs. pH 5 structures (<6 Å RMSD vs. <3 Å RMSD, Fig. 4A, upper row). To
27 assess the impact of presumed protonation at low pH, we also ran simulations of
28 lower-pH structures with a subset of acidic residues protonated according to
29 previously published accessibilities [32]. This adjustment had little effect on the
30 overall stability of the pH 5 structure, but indicated further ECD stabilization at pH 3

1 (Fig. 4A, lower row). In parallel, simulations of X-ray structures showed ECD
2 destabilization at pH 7 compared to pH 4 (Fig. 4B).



4 **Figure 4: Variable ECD flexibility revealed by MD simulations.** (A) RMSD over time for Ca-atoms of
5 the ECD (solid lines) and TMD (dotted lines) in four replicate 1- μ s MD simulations of cryo-EM
6 structures determined at pH 7 (blue), pH 5 (light and dark green), and pH 3 (purple). (B) RMSD plots,
7 shown as in panel A, for simulations of X-ray structures at pH 7 (light gray, PDB ID: 4NPQ) and pH 4
8 (dark gray, PDB ID: 4HFI). Upper images in both panels represent simulations in neutral-pH resting
9 conditions; lower images show simulations in which a subset of acidic residues were protonated to
10 approximate activating conditions.

11 We further investigated rearrangements in local geometry by monitoring key metrics
12 of gating across MD simulations. First, contraction of the ECD around the channel
13 axis—a reverse “blooming” motion—has been proposed as a key transition on the
14 pathway towards pentameric channel opening [3], [17], [35], [36]. Simulations of the
15 pH 7 cryo-EM structure showed a broad, irregular distribution of the ECD radius, with
16 no clear peak between 30 and 32 Å (Fig. 5A, blue). Conversely, simulations of
17 low-pH cryo-EM structures with either resting or activating protonation featured a
18 more stable and constricted ECD radius, with a single peak around 30 Å (Fig. 5A,
19 green, purple). Previous X-ray structures [22], [27] showed a parallel trend, shifting
20 to a smaller, narrower range in ECD radius values from pH 7 to 4 (Fig. 5A, gray).

21 Similarly, a counter-clockwise twist of the ECD with respect to the TMD has been
22 proposed as an initiating step in pentameric ion channel activation [37], [38], [39]. In

1 parallel to the ECD radius, domain twist fluctuated over a relatively wide range (from
2 -13° to -28°) in simulations of the pH 7 cryo-EM structure (Fig. 5B, blue). This
3 distribution was narrower in simulations of the pH 5 structure, with a single peak
4 around -21° under either resting or activating conditions (Fig. 5B, green). The pH 3
5 structure exhibited a similarly narrow range, with the peak shifted to around -19°
6 (Fig. 5B, purple). Simulations of previous X-ray structures also showed a relatively
7 broad distribution at pH 7, contracting and shifting to smaller (negative) twist angles
8 at pH 4 (Fig. 5B, gray).

9 The most prominent geometric change in pentameric channel gating that enables ion
10 flux is the expansion of the transmembrane pore, with the upper region of each M2
11 helix and the M2–M3 loop spreading outward from the conduction pathway [37].
12 Simulations of all our cryo-EM structures, as well as the previous pH 7 X-ray
13 structure, featured a relatively contracted upper M2 around 11 Å; in simulations of
14 the pH 4 X-ray structure, upper-M2 spread increased to 12 Å (Fig. 5C). Thus, all
15 major classes obtained by cryo-EM contained similarly closed pores, but exhibited
16 differences in local stability and geometry in the ECD.

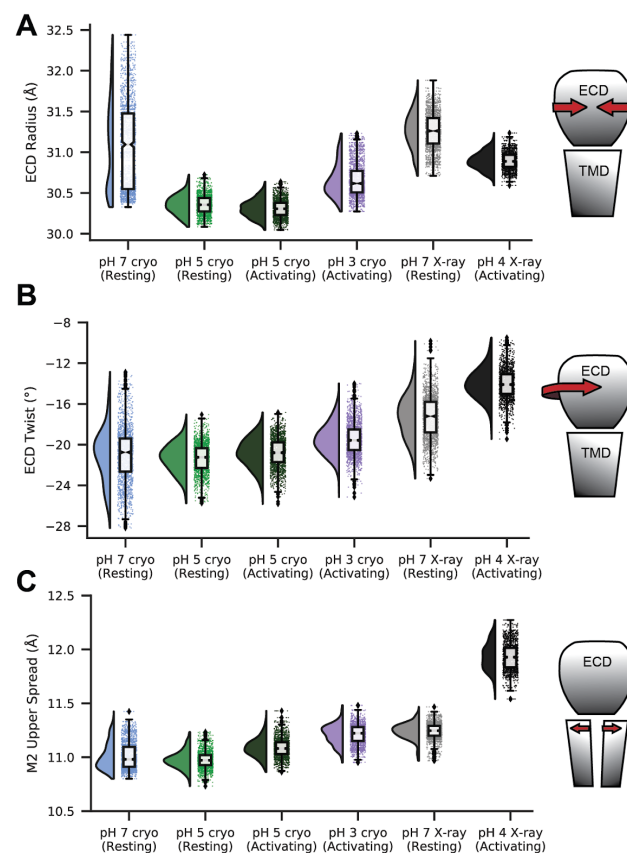


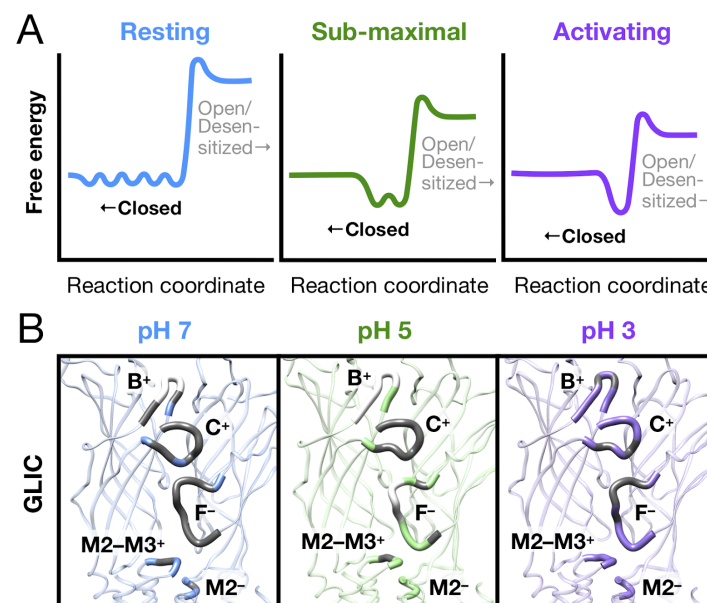
Figure 5: Trends in geometric properties associated with GLIC gating in MD simulations. All panels show raincloud plots [40] for quadruplicate 1- μ s MD simulations of cryo-EM structures determined at pH 7 (blue), pH 5 (green) and pH 3 (purple), as well as X-ray structures at pH 7 and 4 (light and dark gray). Simulations were run under deprotonated (resting) or protonated (activating) conditions as indicated, to approximate the experimental state. All plots show probability distributions at left, and raw data plus boxplots indicating sample median, interquartile range, minimum–maximum range, and outliers at right. (A) ECD radius, measured by the average distance from the Ca-atom center-of-mass (COM) of each subunit ECD to that of the full ECD, projected onto a plane perpendicular to the channel axis. (B) ECD twist, measured by the average dihedral angle defined by COM coordinates of 1) a single subunit-ECD, 2) the full ECD, 3) the full TMD, and 4) the same single-subunit TMD. (C) Upper-M2 spread, measured by the average distance from the COM of the upper M2 helix (residues L241–N245) to that of the full TMD.

Discussion

The subtle structural changes involved in gating of pentameric ligand-gated ion channels, and their sensitivity to pharmacologically relevant modulation by allosteric drugs, have driven substantial interest in solving structures in multiple key functional

1 states. However, few structures in this family have been reported in unliganded
2 resting states, the presumed “starting block” for gating and modulation. In this
3 context, cryo-EM structures and MD simulations in this work offer insight into the
4 dynamic as well as structural properties of resting and other closed states of GLIC.

5 Our cryo-EM reconstructions suggest that resting conditions (neutral pH) allow GLIC
6 to sample a rapidly exchanging collection of closed conformations, while activating
7 conditions produce a more discrete population, potentially in equilibrium with open
8 and desensitized states (Fig. 6A). Differential resolutions and orientations were
9 particularly observed in a series of loops spanning the subunit interface (Fig. 6B),
10 including ECD loops B and C on the principal subunit, ECD loop F on the
11 complementary subunit (Fig. 2B), the principal M2–M3 loop (e.g. residue K248; Fig.
12 2C), and complementary upper M2-helix (e.g. E243; Fig. 2D). Whereas all these
13 regions included incomplete residues at pH 7, backbone traces and most side chains
14 could be built in all these regions at pH 3 (Fig. 6B). Resolution improvements at
15 lower pH, even when controlled for particle number (Fig. 1—figure supplement 3),
16 suggested sampling of a more limited conformational space, including stabilization
17 at the subunit interface.



19 **Figure 6: A dynamic resting state of pentameric ligand-gated ion channels.** (A) Idealized free-energy
20 diagrams for GLIC, emphasizing closed states. Resting conditions (left, blue) are associated with a

1 rapidly exchanging population of closed channels. Introduction of sub-maximal (center, green) or fully
2 activating conditions (right, purple) condenses the closed free-energy well to a more stable, discrete
3 population, possibly primed for opening. The number of energy wells is meant to represent qualitative
4 progressive consolidation to a discrete population of closed channels upon protonation; details of
5 intermediate, open, and desensitized states to the right of the activation energy barrier are not
6 observed in this work. (B) Regions of the GLIC subunit-interface exhibiting differential resolution in
7 cryo-EM maps reconstructed at pH 7 (left, blue), pH 5 (center, green), or pH 3 (right, purple). Each
8 panel shows the interface between two representative subunits modeled by density-guided MD
9 simulations, viewed from the upper membrane plane. Ribbons for key subunit-interface regions,
10 including loop B, loop C, and the M2–M3 loop of the principal (+) subunit, and loop F and the upper
11 M2 helix of the complementary (–) subunit, are labeled and widened for emphasis. In these regions,
12 residues for which map resolution was insufficient to build complete side chains or backbone atoms
13 are shown in gray and white, respectively.

14 Transition from a broad, flexible population to a discrete equilibrium upon agonist
15 binding appears to be a consistent pattern in the growing catalog of pentameric
16 channel structures. To our knowledge, wherever such parallel structures have been
17 reported in this family, similarly broad and more flexible structures were observed in
18 resting compared to activating conditions. Within prokaryotes, X-ray structures of the
19 pathogen channel ELIC have been solved to 3.09 Å without ligands [41], but as high
20 as 2.59 Å bound to positive modulators [42]. Similarly, cryo-EM structures of an apo
21 form of ELIC were recently reported in lipid nanodiscs to reach 4.10 Å resolution,
22 while an agonist-bound form was resolved to 3.30 Å in the same work [43]. Although
23 the pore is closed in all reported ELIC structures, a decrease in crystallographic
24 temperature (B-)factors was consistent with extracellular stabilization upon ligand
25 binding, particularly in loops B, C, and F (Fig. 6 - supplement 1A). In the symbiote
26 channel DeCLIC, channel opening was recently shown to involve translocation of a
27 novel N-terminal domain (NTD) to contact the canonical ECD, specifically loops B
28 and C; comparison of DeCLIC structures in apparent open vs. closed states showed
29 a coordinated decrease in B-factors around loops B, C, and F upon NTD binding [44]
30 (Fig. 6 - supplement 1B). Eukaryotic channels have generally required extracellular
31 antibodies or other stabilizing ligands for structure determination in closed as well
32 as open/desensitized states, occluding such comparisons. However, for the
33 full-length mouse serotonin-3 receptor, the relatively low resolution (4.31 Å) of the

1 apo cryo-EM structure [11] was greatly improved by binding the inhibitor granisetron
 2 (2.92 Å) to the channel [45] without altering the functional state of the pore, as well
 3 as by the agonist serotonin (3.32 Å) with coordinated pore expansion [12].
 4 Interestingly, relatively loose packing of the ECD core has been proposed as a gating
 5 strategy specific to eukaryotic members of this channel family [46]; substantiation in
 6 eukaryotic receptors of the the dynamic patterns we report here for GLIC may prove
 7 a valuable extension of the present work.

8 Sampling ensembles of conformations in simulations restrained to cryo-EM map
 9 data is a potentially valuable approach to better understand the heterogeneity of
 10 functional states under different buffer or ligand conditions, provided that the
 11 heterogeneity is represented in the reconstruction. In this work, template structures
 12 were fitted to cryo-EM reconstructions by conducting an MDsimulation with
 13 additional forces to increase the model-to-map similarity. The critical challenge is to
 14 balance improved fit against retained structural integrity and sampling of the full
 15 ensemble of relevant conformations. We addressed this by adaptively changing the
 16 fitting strength according to the change in similarity between model and density
 17 during the fitting process. A subsequent analysis of average Fourier shell correlation
 18 (FSC) [29] across the fitting trajectory allowed assessment of model fit independent
 19 of the driving forces in the simulation, and selection of a model frame in each fitting
 20 simulation with minimal structural distortions. The local structure quality was then
 21 further improved by annealing. This approach enabled automatic fitting of structures
 22 into an experimental density without distorting protein geometry or restraining
 23 secondary structure, reflected in the Molprobit [47] (Tab. 2) scores. As an added
 24 benefit in this system, structural variability was inherently captured in the five GLIC
 25 subunits, which were free to sample conformations independently in density-guided
 26 simulations. To assess the flexibility and dynamics of the alternative functional
 27 states, we also ran non-restrained MD simulations of the final structures.

28 The apparent nonconducting conformation of the pore in the GLIC cryo-EM
 29 structures under activating conditions was initially surprising, but not inconsistent
 30 with previous data. Indeed, at least one previous X-ray structure at pH 4 contained

1 partially occupied closed and open states of the pore, consistent with a substantial
 2 population of closed channels even under activating conditions [27]. It is plausible
 3 that electrostatic conditions might be modified by interaction with the
 4 glow-discharged cryo-EM grid or air-water interface, masking the effect of
 5 protonation. Still, we consistently noted subtle shifts in stability and conformation,
 6 indicating that local effects of protonation were reflected in the major resolved class.
 7 We cannot exclude the possibility of nonconductive channels representing a
 8 desensitized state, which has been observed in some GLIC functional assays [48],
 9 albeit to a lesser degree than in eukaryotic homologs [16]. However, the cryo-EM
 10 structure at pH 3 does not resemble desensitized structures of other channel-family
 11 members, but rather hews closely to our other cryo-EM structures. A parsimonious
 12 explanation of our low-pH models is that a low open probability produces a
 13 predominant population of closed channels, even under maximally active conditions.
 14 Although the open probability of GLIC in particular pH conditions is not well
 15 established, other channels in this family are known to flicker between conducting
 16 and nonconducting states even in the presence of agonist [19], consistent with a
 17 large population of closed channels. Indeed, verifiable open states have proved
 18 difficult to capture in structural biology, possibly due to their relatively high free
 19 energy even under maximally activating conditions. Notably, this rationale is distinct
 20 from the apparent challenges to capturing resting structures as reported here, which
 21 more likely reflect intrinsic flexibility.

22 Although resolution and temperature factors can be influenced by multiple
 23 experimental and molecular properties aside from flexibility, this qualitative pattern
 24 presents a compelling case for a conserved free-energy landscape in this protein
 25 family where the resting “state” is rather a relative broad and dynamic ensemble of
 26 conformations with the unifying feature of a pore that is neither conducting nor
 27 desensitized. Activation of the channel (induced either by pH gating or ligand
 28 binding) would then correspond to a transition to a more specific/narrow-distribution
 29 state that for many channels would be quite short-lived. This model spotlights a
 30 fundamental challenge to capturing a channel’s resting/apo state by traditional
 31 structure determination methods, due to the low resolution of any large population of

1 heterogeneous molecules. Closed states captured by the addition of inhibitors,
2 antibodies, or other ligands are often found to be more stable, but may also
3 represent only a subset of the conformational ensemble accessible from the resting
4 state. Structural and dynamic data in this work thus shed light on fundamental
5 properties as well as technical challenges involved in dissecting the ligand-gated
6 channel gating landscape.

7 **Materials and Methods**

8 **GLIC expression and purification**

9 Expression and purification of GLIC-MBP was adapted from protocols published by
10 Nury and colleagues [32]. Briefly, C43(DE3) *E. coli* transformed with GLIC-MBP in
11 pET-20b were cultured overnight at 37° C. Cells were inoculated 1:50 into 2xYT
12 media with 100 µg/mL ampicillin, grown at 37° C to OD600 = 0.7, induced with 100
13 µM isopropyl-β-D-1-thiogalactopyranoside, and shaken overnight at 20° C.
14 Membranes were harvested from cell pellets by sonication and ultracentrifugation in
15 buffer A (300 mM NaCl, 20 mM Tris-HCl pH 7.4) supplemented with 1 mg/mL
16 lysozyme, 20 µg/mL DNase I, 5 mM MgCl₂, and protease inhibitors, then frozen or
17 immediately solubilized in 2 % n-dodecyl-β-D-maltoside (DDM). Fusion proteins were
18 purified in batch by amylose affinity (NEB), eluting in buffer B (buffer A with 0.02%
19 DDM) with 2–20 mM maltose, then further purified by size exclusion chromatography
20 in buffer B. After overnight thrombin digestion, GLIC was isolated from its fusion
21 partner by size exclusion, and concentrated to 3–5 mg/mL by centrifugation.

22 **Cryo-EM sample preparation and data acquisition**

23 For freezing, Quantifoil 1.2/1.3 Cu 300 mesh grids (Quantifoil Micro Tools) were
24 glow-discharged in methanol vapor prior to sample application. 3 µl sample was
25 applied to each grid, which was then blotted for 1.5 s and plunge-frozen into liquid
26 ethane using a FEI Vitrobot Mark IV. Micrographs were collected on an FEI Titan
27 Krios 300 kV microscope with a Gatan K2-Summit direct detector camera. Movies
28 were collected at 165,000x magnification, equivalent to a pixel spacing of nominally

1 0.82Å. A total dose of 40.8 e-/Å² was used to collect 40 frames over 6 sec, using a
2 nominal defocus range covering -2.0 to -3.8 µm.

3 **Image processing**

4 Motion correction was carried out with MotionCor2 [49]. All subsequent processing
5 was performed through the RELION 3.1 pipeline [50]. Defocus was estimated from
6 the motion corrected micrographs using CtfFind [51]. Following manual picking, initial
7 2D classification was performed to generate references for autopicking. Particles
8 were extracted after autopicking, binned and aligned to a 15Å density generated
9 from the GLIC crystal structure (PDB ID: 4HFI [22]) by 3D auto-refinement. The
10 acquired alignment parameters were used to identify and remove aberrant particles
11 and noise through multiple rounds of pre-aligned 2D- and 3D-classification. The
12 pruned set of particles was then refined, using the initially obtained reconstruction as
13 reference. Per-particle CTF parameters were estimated from the resulting
14 reconstruction. Global beam-tilt was estimated from the micrographs. Micelle density
15 was eventually subtracted and the final 3D auto-refinement was performed using a
16 soft mask covering the protein, followed by post-processing, utilizing the same mask.
17 Local resolution was estimated using the RELION implementation.

18 **Model building**

19 Models were built starting from a template using an X-ray structure determined at pH
20 7 (PDB ID: 4NPQ [27], chain A), fitted to each reconstructed density. PHENIX
21 1.15-3459 [52] real-space refinement was used to refine this model, imposing the
22 5-fold symmetry through NCS restraints detected from the reconstructed cryo-EM
23 map. The model was incrementally adjusted in COOT 0.8.9.1 EL [53] and re-refined
24 until conventionally used quality metrics were optimized under a maintained
25 agreement with the reconstruction. Model statistics are summarized in Tab. 1.

26 **MD simulations**

27 Manually built cryo-electron microscopy structures, as well as previously published
28 X-ray structures (resting, PDB ID 4NPQ [27]; activating, PDB ID 4HFI [22]), were
29 used as starting models for MD simulations. The Amber99sb-ildn force field [54] was

1 used to describe protein interactions. The protein was embedded in a bilayer of 520
 2 Berger [55] POPC molecules. Each system was solvated in a $14 \times 14 \times 15 \text{ nm}^3$ box
 3 using the TIP3P water model [56], and NaCl was added to bring the system to
 4 neutral charge and an ionic strength of 150 mM.
 5 All simulations were performed with GROMACS 2019.3 [30]. Systems were
 6 energy-minimized using the steepest descent algorithm, then relaxed for 100ps in
 7 the NVT ensemble at 300 K using the velocity rescaling thermostat [57]. Bond
 8 lengths were constrained [58], particle mesh Ewald long-range electrostatics used
 9 [59], and virtual interact sites for hydrogen atoms enabled a time step of 5 fs. Heavy
 10 atoms of the protein were restrained during relaxation, followed by another 45 ns of
 11 NPT relaxation at 1 bar using Parrinello-Rahman pressure coupling [60] and
 12 gradually releasing the restraints. Finally, the system was relaxed with all
 13 unresolvable residues unrestrained for an additional 150 ns. For each equilibrated
 14 system, four replicates of 1 μs unrestrained simulations were generated. Analyses
 15 were performed using VMD [61], CHAP [62], and MDTraj [63]. ECD radius, ECD
 16 twist, and upper-M2 spread were quantified as in previous studies [21], described in
 17 Fig. 5.

18 **Automated density-guided fitting**

19 Automated fitting into cryo-EM densities was performed by density-guided MD
 20 simulations using GROMACS 2020.1 [31]. The manually built pH 7 structure,
 21 embedded in a bilayer as described above, was used for fitting to all three data sets.
 22 Density-guided simulations were performed using one half-map as a bias. To check
 23 for overfitting, goodness-of-fit was evaluated using the other half-map. Alignment of
 24 the starting model to the density map was assessed and improved iteratively using
 25 VMD [61] and the GROMACS editconf functionality, rotating and translating the
 26 structure to ensure correct global alignment prior to starting the simulation.

27 The density-guided simulations were performed at 300K using adaptive force scaling
 28 starting at 10 kJ/mol with a feedback time-constant of 40 ps and a Gaussian
 29 transform spread-width of the model-generated density of 2 Å. Map similarities were
 30 calculated from their inner products after normalization. This normalization leads to

1 higher at-face force constants that however translate to much lower forces and
2 energies.

3 Evaluation of the overall fit of the model vs. experimental half-map was performed
4 using FSC average values with a threshold of 3.3 Å each 100 ps (equation 2 in [29]).
5 The model configuration at the largest FSC average was chosen as the starting
6 frame for subsequent simulated annealing, which was performed over 2 ns reducing
7 the temperature from 300K to 1K with a density-guided simulation force constant of
8 10^{24} kJ/mol, corresponding to the force-constant at the largest FSC average.
9 Molprobit scores [47] were calculated every 0.02 ns and the frame with lowest
10 score was used as the final model.

11

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16 data collection, modeling and supervision.

17 **Data Availability**

18 Three-dimensional cryo-EM density maps of the pentameric ligand-gated ion channel
19 GLIC in detergent micelles have been deposited in the Electron Microscopy Data
20 Bank under accession numbers EMD-11202 (pH 7), EMD-11208 (pH 5) and
21 EMD-11209 (pH 3), respectively. Each deposition includes the cryo-EM sharpened
22 and unsharpened maps, both half-maps and the mask used for final FSC calculation.
23 Coordinates of all models have been deposited in the Protein Data Bank. The
24 accession numbers for the three GLIC structures are 6ZGD (pH 7), 6ZGJ (pH 5) and
25 6ZGK (pH 3). Full input data, parameters, settings, commands and trajectory subsets
26 from MD simulations are archived at Zenodo.org under DOI
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11 Competing Interests

12 The authors declare that there are no competing interests.

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Data collection and processing	pH 7 data set	pH 5 data set	pH 3 data set
Microscope	FEI Titan Krios	FEI Titan Krios	FEI Titan Krios
Magnification	165,000	165,000	165,000
Voltage (kV)	300	300	300
Electron exposure (e ⁻ /Å ²)	~ 50	~ 50	~ 50
Defocus range (μm)	2.0 – 3.8	2.0 – 3.8	2.0 – 3.8
Pixel size (Å)	0.82	0.82	0.82
Symmetry imposed	C5	C5	C5
Number of images	~ 5300	~ 7000	~ 6400
Particles picked	~ 700,000	~ 1 million	~ 1 million
Particles refined	86,201	351,643	177,213
Refinement			
Initial model used	4NPQ monomer	4NPQ monomer	4NPQ monomer
Resolution (Å)	4.1	3.4	3.6
FSC threshold	0.143	0.143	0.143
Map sharpening B-factor	- 278	-223	- 246
Model composition			
Non-hydrogen protein atoms	9730	10,560	10,965
Protein residues	1410	1405	1450
Ligands	0	0	0
B-factor (Å ²)	172	35	133
RMSD			
Bond Lengths (Å)	0.005	0.004	0.007
Bond angles (°)	0.530	0.590	0.659
Validation			
Molprobity score	1.67	1.68	1.70
Clashscore	7.68	5.24	5.67
Poor rotamers (%)	0	0	0
Ramachandran plot			
Favored (%)	96.3	94.1	94.2
Allowed (%)	3.7	5.9	5.8
Outliers (%)	0	0	0

Table 1: Cryo-EM data collection and model refinement statistics

Density guided generated models	pH 7 data set	pH 5 data set	pH 3 data set
RMSD			
Bond Lengths (Å)	0.0151	0.0184	0.0156
Bond angles (°)	2.10	2.40	2.05
Validation			
Molprobity score	1.07	1.10	1.14
Clashscore	0.00	0.12	0.04
Poor rotamers (%)	1.59	1.59	2.06
Ramachandran plot			
Favored (%)	93.59	94.05	94.37
Allowed (%)	6.15	5.18	4.66
Outliers (%)	0.26	0.78	0.97
Fitted model vs. built model			
RMSD (Å)	1.72	1.75	1.38

Table 2: Model statistics and validation for models generated with density guided-guided simulations and an RMSD comparison of automatic and manually fitted model.

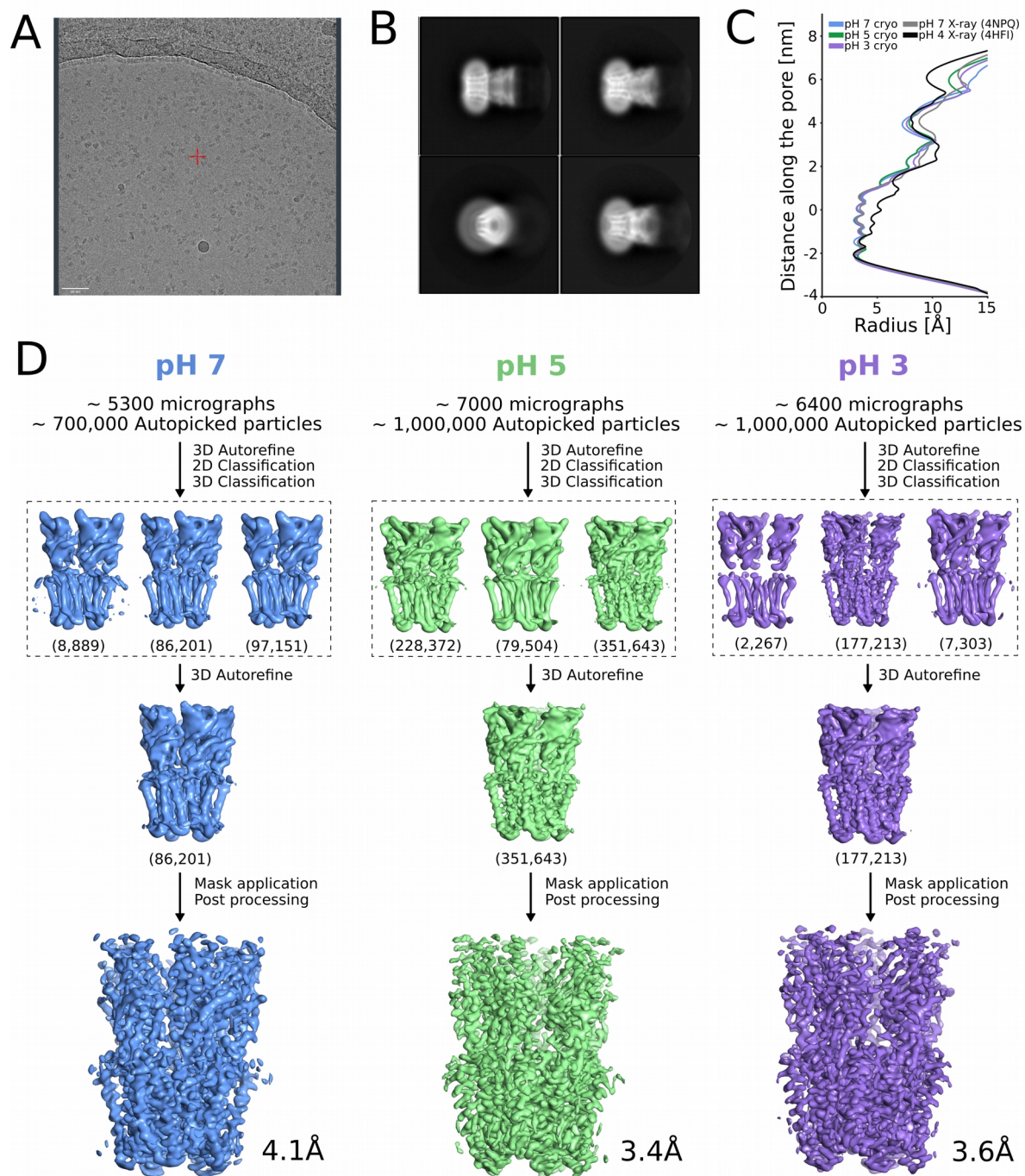


Figure 1 – supplement 1: Cryo-EM image-processing pipeline. (A) Representative micrograph from grid screening on a Falcon-3 detector (Talos-Artica), showing detergent-solubilized GLIC particles. (B) Representative 2D class averages at 0.82 Å/px in a 256 x 256 pixel box and a 180-Å mask. (C) Radius between C β atoms along the GLIC pore for cryo-EM datasets collected at pH 7 (blue), pH 5 (green), and pH 3 (purple), as well as X-ray structure at pH 7 and 4 (light and dark gray). (D) Overview of cryo-EM processing pipelines for data collected at pH 7 (blue), pH 5 (green), and pH 3 (purple) (see Methods).

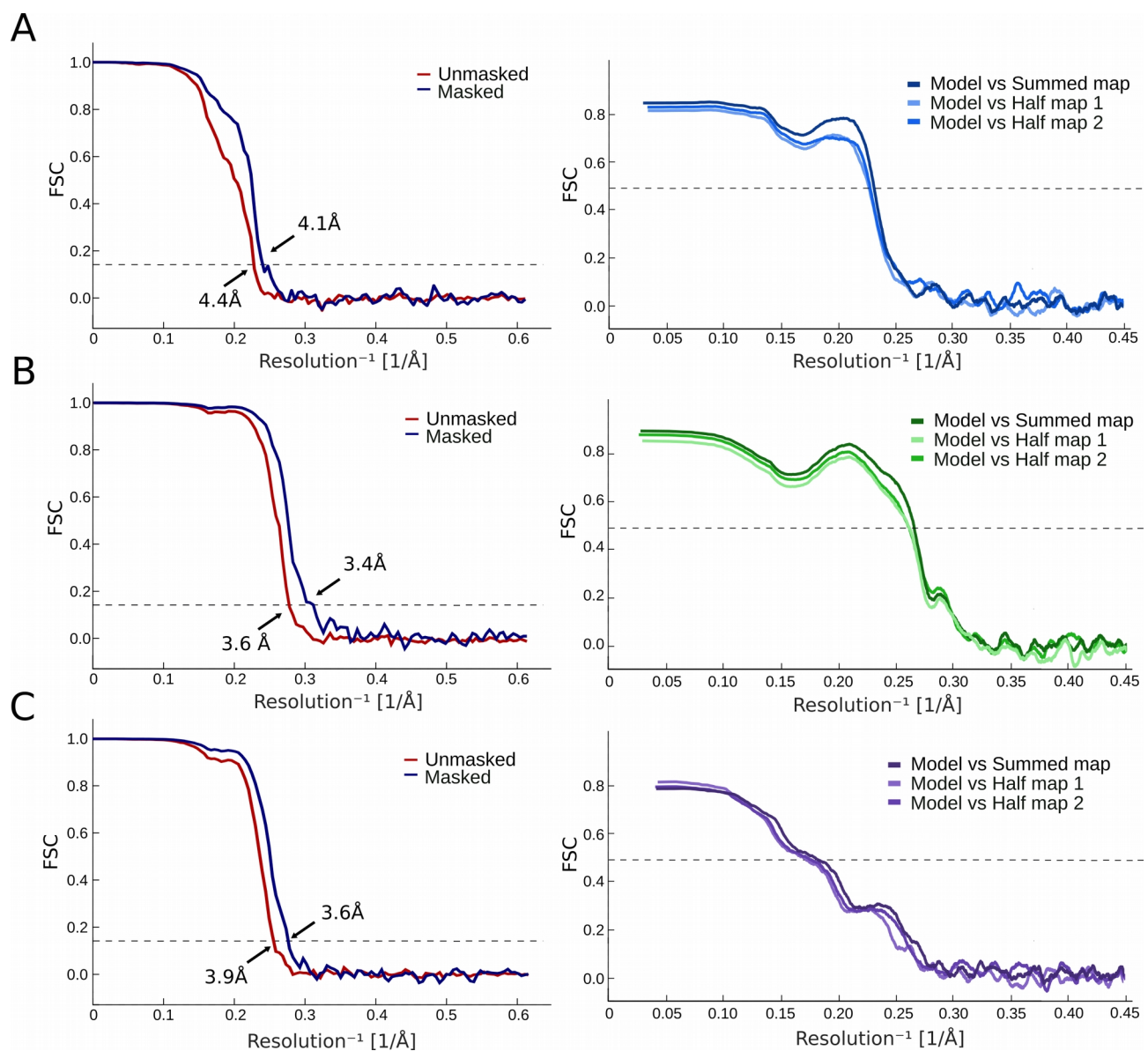


Figure 1 – supplement 2: FSC curves and map-to-model FSC for manually modeled cryo-EM reconstructions. *Left:* FSC curves for cryo-EM reconstructions at (A) pH 7, (B) pH 5, and (C) pH 3, before and after applying a soft mask. *Right:* FSC curves for cross-validation between model and half-map 1, model and half-map 2, and model and summed map at (A) pH 7, (B) pH 5, and (C) pH 3.

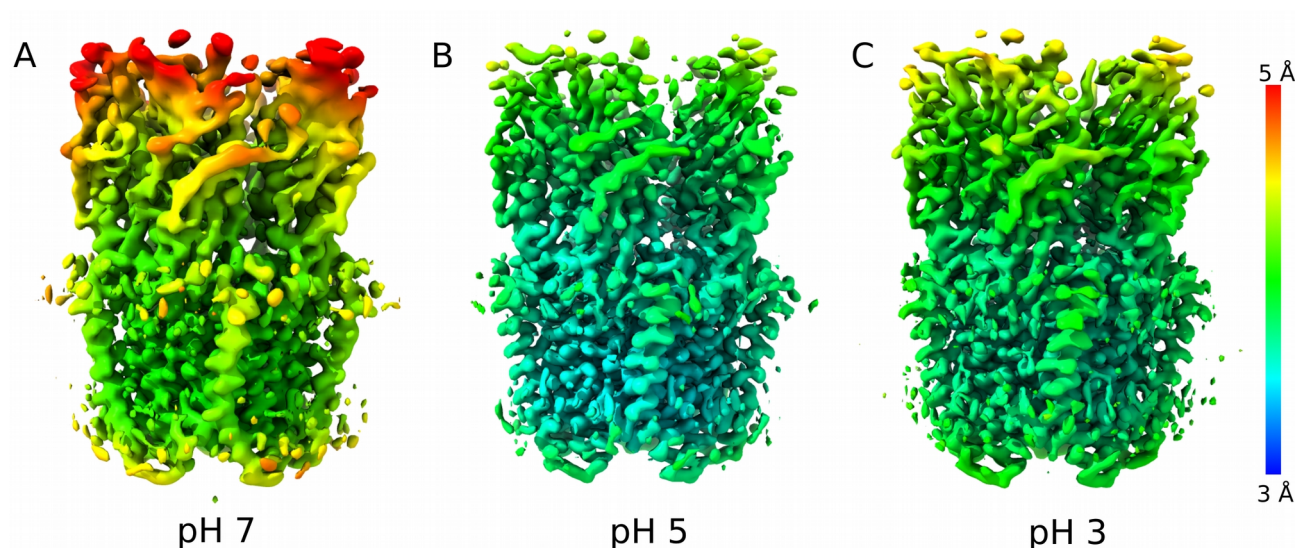


Figure 1 – supplement 3: Local resolution of cryo-EM data processed from equivalent particle subsets. Densities at equivalent contour colored by local resolution for reconstructions at (A) pH 7 and data subsets randomly selected to equalize particle number (86,000) at (B) pH 5 and (C) pH 3.

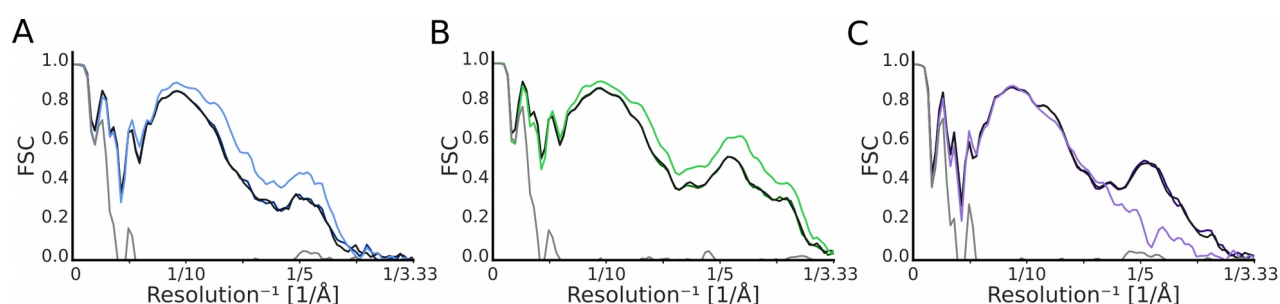


Figure 3 – supplement 1: Map-to-model FSC of fitted vs. manual models. Comparison of the map-to-model FSC fit between manually built model and model generated with the automated density-guided simulations. Each of the plots represents an increase of the fit during the course of the fitting process going from light gray to black. The FSC fit for the manually built model for (A) pH 7 is colored light blue and dark blue for the automated model. (B) For pH 5 the manually built model is colored light green and the automated dark green. (C) The FSC fit of the manually built model from the pH 3 dataset is colored light purple and the automated model is colored in dark purple. Note that the FSCs for the fitted are generated from the backbone rather than all atoms, and they are not masked.

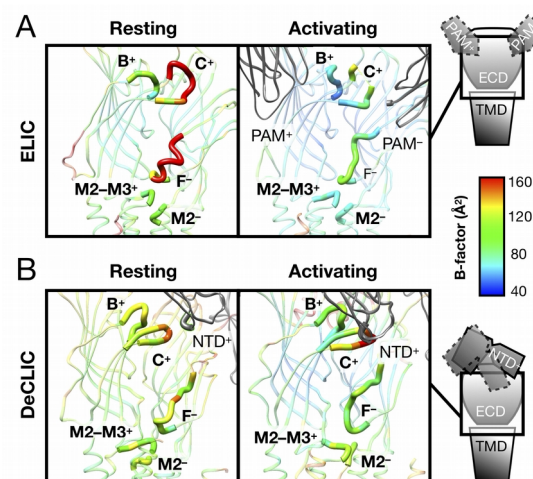


Figure 6 – supplement 1: B-factors in homologous channel structures support a conserved resting-state landscape. (A) B-factor distribution in ELIC indicates relative instability prior to ligand binding, particularly in ECD loops B, C, and F. Each panel shows a subunit interface viewed as in Fig. 6B, with residues colored by average B-factor. Comparison of the highest-resolution wild-type X-ray structures reported to date for ELIC in apo (left, PDB ID 3RQU) and liganded states (right, PDB ID 6SSI; complex with modulating nanobody, PAM) indicates lower stability prior to ligand binding, particularly in ECD loops B, C, and F. (B) B-factors in DeCLIC similarly indicate relative flexibility in the ECD under resting conditions. Each panel shows a subunit interface viewed and colored as in panel A. Under non activating conditions (left, PDB ID 6V4S; high calcium), the novel NTD (gray) makes relatively few contacts with ECD loops B and C. In contrast, despite moderately lower overall resolution, the channel under activating conditions (right, PDB ID 6V4S; no calcium) crystallized with generally lower B-factors in the ECD, including interfacial loops.