Structure of native lens connexin 46/50 intercellular channels by cryo-EM

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Gap junctions establish direct pathways for cell-to-cell communication through the assembly of twelve connexin subunits that form intercellular channels connecting neighbouring cells. Co-assembly of different connexin isoforms produces channels with unique properties and enables communication across cell types. Here we used single-particle cryo-electron microscopy to investigate the structural basis of connexin co-assembly in native lens gap junction channels composed of connexin 46 and connexin 50 (Cx46/50). We provide the first comparative analysis to connexin 26 (Cx26), which— together with computational studies— elucidates key energetic features governing gap junction permselectivity. Cx46/50 adopts an open-state conformation that is distinct from the Cx26 crystal structure, yet it appears to be stabilized by a conserved set of hydrophobic anchoring residues. ‘Hot spots’ of genetic mutations linked to hereditary cataract formation map to the core structural–functional elements identified in Cx46/50, suggesting explanations for many of the disease-causing effects.

Cell-to-cell communication directed by gap junctions is essential to neuronal function and cardiac coupling, and for coordinating intercellular signalling and metabolic activity in most tissues (for example, heart, skin, liver and eye lens)1. Genetic mutation or aberrant regulation of gap junctions is linked to a variety of pathological conditions, including cardiac arrhythmia, stroke, blindness, deafness, skin disease and cancers2–4.

Intercellular channel formation occurs through an assembly of twelve connexin subunits5. Within the plasma membrane, six connexins are organized into a hemichannel structure. Hemichannels from neighbouring cells dock together to form complete cell-to-cell channels, which cluster to form large gap junction plaques. A remarkably large channel pore provides passage to diverse chemical messages: these include ions, metabolites, hormones and other small signalling molecules less than about 1 kDa in size (for example, K+, cyclic AMP (cAMP), inositol triphosphate (Ins(1,4,5)P3) and glucose). In this way, interconnected cells can exchange electrical and chemical information across an entire tissue or organ.

Humans express 21 connexin isoforms in a cell-type-specific fashion6. Most cells express multiple isoforms, and certain connexins display an ability to co-assemble, either by docking two hemichannels composed of different isoforms (heterotypic) or through mixed isoform assembly within the same hemichannel (heteromeric). This complexity is thought to allow cells to fine-tune the conductance of chemical messages and support coupling across different cell types7. However, our understanding of the physical basis of connexin isoform compatibility, conductance, substrate selectivity and channel gating remains limited8,9, as high-resolution structural information obtained by crystallographic analysis has so far been restricted to just a single model system, Cx2610,11.

To gain further insight into the mechanistic effects of gap junction isoform diversity and heteromeric assembly, we applied single-particle imaging methods by cryo-electron microscopy (cryo-EM) to elucidate the structure of native channels made up of Cx46 and Cx50 (Cx46/50), isolated from the eye lens, in which connexin-mediated communication is required for growth, differentiation and maintenance of lens transparency to support vision12. Comparative molecular dynamics simulations reveal key features of ion permeation and selectivity, and suggest that Cx46/50 adopts a more stable open-state conformation compared to the previously described Cx26 crystal structure10.

Structural overview of Cx46/50

Cx46 and Cx50 form intercellular channels in the mammalian lens, which are potentially heteromeric or heterotypic13,14. We isolated native Cx46/50 intercellular channels from core lens tissue (sheep Cx44/49), and verified heteromeric co-assembly by biochemical analysis and chemical cross-linking mass spectrometry (Extended Data Fig. 1). The structure of these Cx46/50 intercellular channels was resolved by single-particle cryo-EM to near-atomic resolution (3.4 and 3.5 Å, from two independent datasets) (Fig. 1a, b, Extended Data Figs. 1–3). The resulting density maps revealed a 15-nm-long dodecameric (12-mer) channel with a girdled waist (about 6–9-nm wide). There is a large unobstructed pore, approximately 1.4 nm in diameter, along the channel axis, consistent with the proposed open-state conformation (Fig. 1b).

We were unable to resolve a specific pattern of Cx46/50 heteromeric or heterotypic co-assembly using 3D classification or refinement strategies (Methods, Extended Data Figs. 4, 5). Nevertheless, high-resolution features corresponding to side-chain densities are observed throughout the reconstructions following 12-fold symmetry refinement (that is, by averaging signal contributed by both Cx46 and Cx50). Therefore, these two isoforms, which share about 80% core-sequence identity (88% similarity), also share a highly similar 3D structure (Fig. 1b, c, Extended Data Figs. 4–6), consistent with the ability of Cx46/50 to co-assemble in a variety of heteromeric and/or heterotypic states.

Atomic models for Cx46 and Cx50 were built into the averaged cryo-EM density map, and various heteromeric and heterotypic channels were constructed for comparative analysis. Whereas the structures display excellent validation statistics (Extended Data Fig. 2), local resolution assessment of the atomic models and experimental

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density identified features of both models that were less well-defined by the density map, in particular at sites at which the two isoforms differ in sequence (Extended Data Figs. 3–5, Supplementary Tables 2, 3). Analysis of the presented models should be approached with caution owing to intrinsic limitations of our heterogeneous dataset, which may extend beyond local differences in primary sequence; for example, owing to the possibility of one isoform being more well-ordered and potentially biasing interpretation.

The refined Cx46/50 structures comprise four alternating transmembrane α-helices (TM1–TM4), two extracellular domains (EC1 and EC2) connecting TM1–TM2 and TM3–TM4, respectively, and an N-terminal helix (NTH) domain that folds into the channel vestibule and is connected to the pore-lining TM1 helix via a short linker (Fig. 1c, d). Density for Met1 is not observed in the cryo-EM maps and was shown, by tandem mass spectrometry (MS/MS), to be removed in both Cx46 and Cx50. The resulting N-terminal glycine (G2) is partially acetylated (Extended Data Fig. 1), as shown for the bovine isoforms.15,16. The intracellular loop (ICL) connecting TM2–TM3 and the cytoplasmic C-terminal domain (CTD) containing the native cleavage sites of Cx46 and Cx50 are also not resolved.16 The ICL and CTD were also not observed in the crystallographic structures of Cx26.10,11, presumably owing to intrinsic disorder in these regulatory domains.

The close structural similarity between Cx46 and Cx50 results in high similar interfacial interactions that include conserved regions of hydrophobic packing over the transmembrane region, and a highly similar hydrogen-bond–ion-pair network between adjacent (heteromeric) domains; Fig. 2a, b, Extended Data Fig. 6) and opposed subunits (heterotypic interface; Fig. 2c, d, Extended Data Fig. 6). Most of these stabilizing interactions are present in Cx26.10,17, including the EC1 Q/N motif (Fig. 2c) and the EC2 pairing involving the K/R–N–D motif (Fig. 2d), a conserved element among group I heterotypic compatible isoforms.18 Although Cx46/50 and Cx26 are not classified as heteromeric compatible channels, the conserved features at the heteromeric interface are congruent with the current understanding that heteromeric co-assembly of connexins is established during biogenesis in the ER–Golgi network.19

Overall, despite significant sequence differences, Cx46 and Cx50 (family connexins) display core structural features that are very similar to the β-family member, Cx26.10 (pairwise Cα root mean squared deviation (r.m.s.d.) = 2.18 Å and 2.14 Å versus Cx46 and Cx50, respectively). These different connexin family members thus share a conserved connexin fold and gap junction channel architecture, as presented in Fig. 1d, and our structures are consistent with early low-resolution electron diffraction studies on Cx43 obtained in a lipid bilayer.20,21 Despite these general similarities, however, we uncovered substantial differences between Cx46/50 and Cx26 localized to key functional sites, which we expect to contribute to isomeric-specific permeation and selectivity properties and provide insight into the interactions responsible for fully stabilizing the open-state conformation of these channels, detailed below.

**Energetics of ion permeation and selectivity**

Comparisons between Cx50, Cx46 and Cx26 intercellular channels reveal distinct electrostatic pore pathways, with shared regions of negative charge potential and sterically constricted sites formed by the NTH domains that narrow the cytoplasmic vestibule to around 10–12 Å at both ends of the channel (Fig. 3a, b). The pore diameters are within the range determined for other connexin channels,22 and fitting with the general ability of gap junctions to enable a variety of molecules of less than about 1 kDa in size (such as those in Fig. 3b) to cross between cells. However, these channels can display a substantial level of isoform-specific selectivity for molecules below this size cut-off, including discrimination between small charged ions.23

To validate our structural models and gain insight into the mechanism of ion selectivity, we conducted comparative all-atom molecular dynamics simulations and potential-of-mean-force (PMF) calculations to define the free-energy landscape of potassium (K+) and chloride (Cl-) permeation for Cx50, Cx46 and Cx26 (Fig. 3c, d, Extended Data Figs. 7, 8). PMFs obtained for Cx26 should be interpreted cautiously owing to significant dynamical behaviour observed for the NTH domain during molecular dynamics simulations (Extended Data Fig. 7), described in detail in the following section.

Cx50, Cx46 and Cx26 form high-conductance ion channels, with preference for conductance of cations over anions. For molecular dynamics simulation, the N terminus of each of the models was...
Fig. 3 | Comparative pore profile and energetics of ion permeation. a, Cut-away surface representation of Cx50 (left), Cx46 (centre) and Cx26 (right) (PDB 2ZW319; residues 2–96 and 132–217), coloured by coulombic potential (red, negative; white, neutral; blue, positive). T, temperature; k, Boltzmann constant; e, charge of an electron; *, location of N terminus. b, Pore radius determined for experimental structures of Cx50 (red), Cx46 (blue) and Cx26 (grey, PDB 2ZW319). Locations of constriction sites (CS) and ECV are indicated. Structures of representative substrates are displayed to scale: K+ ion (1), glucose (2), Ins(1,4,5)P3 (3), cAMP (4) and Lucifer yellow (5). c, Snapshot of the Cx50 molecular dynamics simulation, showing the membrane channel (white) embedded in two lipid bilayers and solvated in the presence of intracellular K+, and extracellular Na+ and Cl− ions. Water molecules not shown. d, PMF describing the free-energy landscape (ΔG) experienced by K+ ions (blue trace) and Cl− ions (red trace) permeating the channel pore. Symmetrized values are shown for acetylated models of Cx50ac (left), Cx46ac (centre) and Cx26ac (right), with non-symmetrized values in lighter shading. Amino acid positions are presented for correlation purposes, and do not represent deconvolution of the free-energy components.

Free-energy minima for K+ ions are localized within the extracellular vestibule (ECV; z ≈ 10 Å and z ≈ 30 Å) of all three isoforms (Fig. 3d), supporting the role of EC1 in establishing charge selectivity and conduction31−33. In Cx46 and Cx50, several negatively charged residues (for example, E48, D51 and E62) localize with regions of high K+ ion density (Fig. 3c, d, Extended Data Fig. 9). Notably, charge substituitions at D51 resulted in decreased unitary conductance in Cx46 hemichannels34. E48 and D51 are conserved in Cx26 (equivalent to E47 and D50) (Fig. 3d), and establish transient binding interactions with K+ ions during molecular dynamics simulation. These sites have also been implicated in Ca2+ regulation in Cx26 by X-ray crystallography11, molecular dynamics studies35,36 and by functional mutation studies of Cx4636. Therefore, competitive K+ binding at these sites may contribute to the mechanism of Ca2+ regulation or sensitivity. E62 (in Cx46/50) appears to form an additional cation-binding site, through coordination between the carboxylate side chain and nearby backbone-carbonyl oxygens (Extended Data Fig. 9). E62 is not conserved in other human connexin isoforms (with the exception of Cx43), and may therefore constitute an isoform-specific regulatory site. Extracellular Ca2+ is involved in the mechanism of closing (or gating) connexin hemichannels37, and competition by K+ binding at this putative site may contribute to the mechanism of potentiation of Cx50 and Cx46 hemichannels by extracellular K+ ions38.

Cx50, Cx46 and Cx26 display an appreciable level of selectivity towards positively charged small ions, with permeability ratios of K+ to Cl− (P/K+/P/Cl−) ranging between around 2.5 to 10 (refs. 28,32,39−41). Hydrated K+ and Cl− ions, with a diameter of about 7 Å, would pass unobstructed through a 10–12 Å steric constriction site; yet for all three isoforms, the peak energy barriers to Cl− are considerably larger than for K+ (Fig. 3d, Extended Data Fig. 8). Peak Cl− barriers localize within the constriction-site region of Cx46ac and Cx50ac acetylated (Cx50ac, Cx46ac and Cx26ac), as this form is expected to represent the predominant species in vivo24, and previous molecular dynamics studies suggest this co-translational modification is required to obtain physiologically relevant charge selectivity of Cx2622. PMFs for K+—the major permeant ion—reveal peak energetic barriers within the constriction site, ranging from 1.4 kcal mol−1 for Cx50ac to 2.1 kcal mol−1 in Cx46ac and 2.5 kcal mol−1 in Cx26ac (Fig. 3d). These relatively low barriers are similar to the peak energetic barrier determined for other high-conductance Na- and K-channels (~2–3 kcal mol−1)25−27, and are consistent with the range of experimental unitary conductance values of these channels (around 220 pS for Cx50ac29 versus 140–135 pS for Cx4630 and Cx2630, in 130–140 mM CsCl).

The differences in K+ PMF correlate with isoform-specific differences in both steric and electrostatic environments. The constriction site of Cx50ac displays the lowest barrier and is characterized by a nearly completely electronegative coulombic potential, owing in part to neutralization of the N terminus by acetylation (shown by the asterisk in Fig. 3a, Extended Data Fig. 8). The major K+ energy barrier of Cx46ac correlates with the position of the positively charged residue R9 (z ≈ 50 Å, in which z is distance along the pore axis) (Fig. 3d, Extended Data Fig. 8), which also limits the constriction site of Cx46 to about 10 Å in our model (versus about 12 Å for Cx50) (Fig. 3b). However, the cryo-EM density map is not well-defined at this site (Supplementary Table 2), probably because (at least in part) of the conformational flexibility of this residue, as dynamical behaviour is observed during molecular dynamics simulation. These dynamics of R9 effectively modulate the steric barrier of Cx46 (between about 10 and 12 Å). The constriction-site K+ energy barrier of Cx26ac correlates with the location of the basic residue K41 (z ≈ 50 Å) (Fig. 3d), located on TM1 just below the NTH domain, as previously reported22,31.
(4.8 kcal mol$^{-1}$, $z \approx 40 \AA$; and 4.6 kcal mol$^{-1}$, $z \approx 50 \AA$, respectively), and slightly deeper into the channel pore for Cx26ac, near the constriction-site–ECV border (4.1 kcal mol$^{-1}$, $z \approx 20 \AA$). As a proxy for degree of $P_{K^+}/P_{Cl^-}$ selectivity, we assessed the difference in peak $K^+$ and $Cl^-$ PMF barriers ($\Delta \Delta G = 3.2$ kcal mol$^{-1}$ for Cx50ac, 2.7 kcal mol$^{-1}$ for Cx46ac and 1.6 kcal mol$^{-1}$ for Cx26ac). These relatively small differences in free energy are consistent with their moderate $P_{K^+}/P_{Cl^-}$ selectivity ratios, and on the order of those defined for bacterial sodium channels (around 3.0–3.5 kcal mol$^{-1}$)\cite{26,27}, which display only modest selectivity for $Na^+$ over $K^+$ ($P_{Na^+}/P_{K^+} \approx 10–30$). By contrast, voltage-gated $K^+$ channels display almost ideal selectivity for $K^+$ over $Na^+$ ($P_{K^+}/P_{Na^+} \approx 1,000$), with energetic barrier differences to these ions reported to be about 6.6 kcal mol$^{-1}$ for KCa\textsc{ii}A\textsc{v}.

Diffusion of $Cl^-$ ions across the constriction-site energy barriers was relatively rare on the timescale of our equilibrium molecular dynamics simulations, which necessitated enhanced sampling methods to construct robust $Cl^-$ PMF calculations (see Methods, Extended Data Fig. 8). Nevertheless, a few $Cl^-$ entry events were observed in our simulation data for Cx50 and Cx46, and in these cases, $Cl^-$ ions appear to co-migrate across the high energy barrier of the constriction site alongside a $K^+$ counter ion. It is possible that similar mechanisms involving ionic-charge neutralization enable cation-preferring gap junction channels to permit passage of negatively charged signalling molecules (for example, cAMP and Ins(1,4,5)P$_3$). However, it is difficult to provide a general mechanism for selectivity, as conductance properties for ions do not always correlate well with conductance properties for larger molecules\cite{41,42}.

The analysis above supports models that propose that substrate selectivity and conductance properties of gap junctions are established by complex mechanisms involving both steric aperture and the unique pattern of electrostatic features contributed by isoform-specific amino acid composition\cite{43,44}. In this way, Cx46/50 heteromeric or heterotrophic channels confer distinct conductance properties of potential functional significance. For example, rectification observed in Cx46/50 heterotypic channels can be explained by the resulting asymmetric free-energy landscape (Extended Data Fig. 8) induced by the uneven distribution of fixed charges\cite{39,40}. Cx46/50 heteromeric assemblies also produced unique $K^+–Cl^-$ PMF profiles, with peak barriers that were intermediate to their homeric counterparts (Extended Data Fig. 8), supporting observations made from single-channel measurements\cite{29,44}.

Additional fine tuning of gap junction permeation properties may be achieved through co-translational and/or post-translational modification of pore-lining residues\cite{42}. In our studies, N-terminal acetylation was found to enhance the cation-to-anion specificity of Cx50, Cx46 and Cx26 intercellular channels (Extended Data Fig. 8). Although N-terminal acetylation is irreversible, the effect of this co-translational modification indicates how other dynamic and reversible charge-modifying post-translational modifications may serve to spatially and temporally modulate the behaviour of intercellular communication.

Open–state stabilization of the NTH domain

Despite general similarities, substantial differences between the Cx46/50 cryo-EM structures and the Cx26 crystal structure are localized to the NTH domain (Fig. 4, Extended Data Fig. 7). The connexin NTH domain contributes to ion selectivity and ‘fast’ trans-junctional voltage gating that is common to all connexin isoforms\cite{45}. The NTH domain folds into the cytoplasmic vestibule, where it forms the constriction site, and is well-positioned to function as a selectivity filter or gating domain (Figs. 1d, 4a).

In the proposed open-state conformation of Cx46/50 described here, the NTH domain adopts a regular amphipathic $\alpha$-helix and ordered loop connecting to TM1 (Fig. 4a–c). The hydrophobic face is established by a set of aromatic and hydrophobic residues that are conserved across various connexin isoforms (W4, L7, I10, L11 and V14 in Cx46 and Cx50) (Fig. 4b). These anchoring sites pack against the pore-lining helices (TM1–TM2), and along the interface of neighbouring subunits. Despite sequence conservation at these sites, the NTH domain modelled in the crystal structure of Cx26 is in a distinctively different conformation and overall arrangement with respect to the transmembrane domains compared to Cx46/50 (C$_r$, r.m.s.d.$= 5.2 \AA$, after alignment of the transmembrane and extracellular domains) (Fig. 4d, Extended Data Fig. 7e). In Cx26, the NTH domain and loop connecting TM1 is less regular, and with the exception of W3 (Cx26 numbering) the conserved hydrophobic residues are modelled towards the solvent\cite{49}.

We propose that the network of hydrophobic anchoring observed in the cryo-EM structure of Cx46/50 supports a stabilized open-state conformation. Accordingly, analyses of our molecular dynamics simulations show that the NTH domains of Cx50 and Cx46 are conformationally stable in both acetylated and non-acetylated states, with only small-amplitude backbone fluctuations (root mean square fluctuation (r.m.s.f.) $\approx 1.0–1.2 \AA$) (Fig. 4e, Extended Data Fig. 7f). By contrast, the NTH domain of Cx26 (and Cx26ac) becomes rapidly disorder (that is, unfolded), and remains conformationally dynamic throughout the production phase of our molecular dynamics simulations (using Protein Data Bank (PDB) code 2ZW3\cite{26}), after super-positioning of TM1–TM4, EC1 and EC2 domains.

The functional significance of the differences in NTH domain structure and dynamic stability is currently unclear. Indeed, instability of the Cx26 NTH domain is consistent with previous molecular dynamics studies\cite{35,46,47}. The functional significance of the differences in NTH domain structure and dynamic stability is currently unclear. Indeed, instability of the Cx26 NTH domain may be an intrinsic feature. In a more recent X-ray crystallographic study of Cx26, the NTH domain was completely unresolved, presumably owing to local disorder\cite{11}; however, potential effects of the conditions required for crystallization cannot be ruled out.

The amphipathic nature of the Cx46/50 NTH positions hydrophilic residues implicated in voltage sensing and ion selectivity at the solvent-exposed face, forming the cytoplasmic vestibule\cite{45}. A network of hydrogen-bond interactions appears to contribute to the precise localization of some of these key residues, including the site of N-terminal acetylation. The carbonyl group at the acetylated-G2 site appears to be oriented—at least transiently—through hydrogen bonding to the indole ring of W4 in the same subunit, whereas in the non-acetylated state, G2 forms a transient intermolecular ion pair with D3 of a neighbouring subunit (Extended Data Fig. 10). The side chain of D3 is oriented by a relatively stable intramolecular hydrogen...
Fig. 5 | Mutational hot spots in Cx46 and Cx50 linked to congenital cataracts. a, Histogram of genetic variants of Cx46 and Cx50 linked to hereditary (congenital) cataracts. Sites of Cx46 (orange) and Cx50 (blue) mutations are overlaid using Cx50 amino acid numbering, with secondary structure and domain elements indicated. Hot spots, regions of high genetic variation, within the NTH, TM1, TM2 and EC2 domains are boxed. b, c, Exterior (b) and interior (c) view of the Cx46/50 gap junction channel with cataract mutation sites mapped for Cx46 (orange) and Cx50 (blue), with magnified views of the EC2 domain (b, inset), NTH domain (c, top inset) and TM1–TM2 pore-lining helices (c, bottom inset), with representative mutation sites labelled.

Mutational hot spots linked to hereditary cataracts

Cx46/50 gap junctions have a critical role in maintaining the transparency of the eye lens by establishing a pathway for water, ion and nutrient circulation and removal of metabolic waste in this avascular organ. Consequently, a variety of human genetic variants in Cx50 and Cx46 have been linked to hereditary cataract formation. Age-related cataracts are currently incurable (except by surgery) and remain the leading cause of blindness in the world. The rarer congenital forms of this disease have been linked to genetic mutation of various lens proteins, including Cx46/50—offering critical insight into the mechanisms of maintaining lens transparency throughout life. We mapped 46 mutation sites in Cx46/50, currently reported on the CatMap database, that are linked to congenital cataracts (Fig. 5a). This analysis suggests explanations for many of the disease-causing effects induced by these polymorphisms, as the mutational hot spots localize to functionally important regions of the Cx46/50 gap junction structure. These include a cluster of residues localized within the EC2 docking site—for example, Cx46(N188T/I) and Cx50(R189Q/W)—in regions that deviate markedly from the Cx26 structure, such as the NTH gating or selectivity domain—such as Cx46(G2D) and Cx46(D3Y/H), and Cx50(L7P)—and several sites localized to the TM1–TM2 pore-lining helix that form an interaction network with the NTH domain, of which mutation is expected to affect the permeation pathway or folding and stability within these regions (Fig. 5b, c). The localization of disease-causing mutations underscores the functional significance of these core structural–functional elements, and the importance of proper cell-to-cell communication through Cx46/50 gap junctions for the maintenance of lens transparency. The ability of cryo-EM to provide high-resolution structural information on gap junctions may finally enable detailed mechanistic investigation of these disease-causing mutations in Cx46/50 and in other isoforms responsible for a diverse range of connexinopathies.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated access codes are available at https://doi.org/10.1038/s41586-018-0786-7.

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METHODS

Cx46/50 purification and amphipol reconstitution. Lamb eyes were obtained from the Wolverine Packers slaughterhouse (Detroit), and the lenses were removed using a surgical blade and stored at –86 °C. Gap junction intercellular channels were isolated from the core lens fibre tissue, containing C-terminal truncation variants of Cx46 and Cx50 (also known as MP38)54,55 (Extended Data Fig. 1). Details of the purification procedure are provided below.

Core lens fibre tissue was dissected from cortical tissue using a surgical blade, and stripped membranes were prepared as described21–24. Total protein concentration was determined by BCA (Pierce) and membranes were stored at –86 °C suspended in storage buffer containing 10 mM Tris pH8.0, 2 mM EDTA, 2 mM EGTA, at a total protein concentration of ~2 mg ml⁻¹. Stripped membranes were thawed from –86 °C and solubilized in 10 mM Tris pH8.0, 2 mM EDTA, 2 mM EGTA, 1% (v/v) n-decyl-3-β-maltoside (DM) for 30 min at 37 °C. Unsolubilized debris was cleared by ultracentrifugation at 150,000g for 30 min at 4 °C. The solubilized fraction was applied to an anion–exchange chromatography column (UnoQ, BioRad) equilibrated with buffer A (10 mM Tris pH 8.0, 2 mM EDTA, 2 mM EGTA, 0.3% DM (v/v)). Protein was eluted with buffer B, which additionally contained 500 mM NaCl. Elution peaks containing Cx46/50, as determined by SDS–PAGE, were pooled and applied to a size–exclusion chromatography (SEC) column (ENC560, BioRad) equilibrated with SEC buffer (20 mM HEPES, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA and 0.3% DM (v/v)). Peak fractions containing purified Cx46/50 were pooled and protein concentration was determined by UV absorbance. All chromatography steps were performed at 4 °C. The presence of both Cx46 and Cx50 was confirmed by western blot analysis using polyclonal antibodies directed against the N-terminal domain of Cx46 (AP15170P, N. Acera, and C-terminal domain of Cx50 (sc-50432, Santa Cruz) (Extended Data Fig. 1a) and by mass spectrometry analysis, described below.

Purified Cx46/50 was exchanged from DM to amphipol A8–35 (Anatrace), as follows. Amphipol was added to freshly purified protein in a 5:1 amphipol:protein (w/w) ratio using a stock solution prepared at 5% (w/v) in water. This mixture was incubated for 2.5 h at 4 °C with rotation. Detergent was then removed by application of SM-2 Biobeads (BioRad) at a ratio of 30:1 (w/v) beads:detergent. Biobeads were incubated overnight at 4 °C with rotation. Biobeads were then removed by running samples over a Polyprop column (BioRad) that had been washed with detergent-free SEC buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA). Samples were further cleared by ultra-centrifugation at 150,000g for 20 min at 4 °C. The clarified sample was then applied to an SEC column (ENC560, BioRad) equilibrated with detergent-free SEC buffer to remove excess amphipol. Peak fractions corresponding to amphipol-stabilized Cx46/50 were pooled and concentrated for single-particle electron microscopy studies (Extended Data Fig. 1b). Final protein concentration was determined by UV absorbance at 280 nm.

Chemical cross-linking and mass spectrometry. Cx46/50 was prepared for chemical cross-linking and mass spectrometry analysis to confirm heteromeric assembly of the two lens isoforms (Extended Data Fig. 1d–f). Cross-linking was carried out directly into a QExactive mass spectrometer (Thermo Fisher Scientific) through an Ultimate 3000 UHPLC system (Thermo Fisher Scientific) and electrosprayed in 38% to 58% solvent B (0.1% formic acid in acetonitrile, flow rate: 200 nl/min) for 15 min. Cross-linked protein bands were excised and digested with trypsin (Promega) by adding Tris pH7.4 at a final concentration of 100 mM for 15 min at room temperature. An initial dataset of 1,104 micrographs (dataset 1) was obtained by automated picking of 66,480 good particles was obtained after five rounds of 2D classification and an initial model was generated de novo in EMAN2 using a subset of 12 class averages as input. This model was refined against the good particle image dataset in EMAN2 with applied D6 symmetry to a final resolution of ~20 Å (Extended Data Fig. 2).

Cryo-EM specimen grids were imaged on a Titan Krios (FEI) operated at 300 kV. Image stacks were recorded using a K2 summit direct electron detector (Gatan) in counting mode with a super-resolution pixel size of 0.665 Å/pixel. The dose rate was 3.2 electrons pixel⁻¹ s⁻¹, with 4 frames s⁻¹ collected for a total exposure time of 10 s. A Gatan energy filter with a slit width of 30 eV was used during data collection. Drift correction and 3D reconstruction were performed using cisEMTV59, with a nominal magnification of 49,000 × at the specimen level (Extended Data Fig. 1c).}

Cryo-EM data collection, image processing and 3D reconstruction. Samples were prepared for cryo-EM by applying 5 μl of amphipol-stabilized Cx46/50 (2.35 mM −1) to a glow-discharged holey carbon grid (Quantifoil R 1.2/1.3) for 10 s. The grid was blotted for 4.0 s and plunge-frozen in liquid ethane using a Vitrobot (FEI) at 100% humidity and stored under liquid nitrogen.

Cryo-EM specimen grids were imaged on a Titan Krios (FEI) operated at 300 kV. Image stacks were recorded using a K2 summit direct electron detector (Gatan) in counting mode with a super-resolution pixel size of 0.665 Å/pixel. The dose rate was 3.2 electrons pixel⁻¹ s⁻¹, with 4 frames s⁻¹ collected for a total exposure time of 10 s. A Gatan energy filter with a slit width of 30 eV was used during data collection. Drift correction and 3D reconstruction were performed using cisEMTV59, with a nominal magnification of 49,000 × at the specimen level (Extended Data Fig. 1c). An additional 1,093-micrograph dataset was collected and processed as above. Particles were picked from dataset 1 using DoGPicker66. Particles were extracted with 2 × binning (resulting in a pixel size of 1.3 Å/pixel). Five rounds of 2D classification in Relion 2.065 left 53,791 good particles. These particles were then subjected to 3D classification in Relion with four classes and no imposed symmetry. The most populated class contained 33,967 particles. These particles were unbinned and another round of 3D classification was performed, reducing the population of particles to 30,128. 3D auto-refinement was then performed on this set of particles with D6 symmetry imposed. After making and post-processing in Relion, the final map had a resolution of 3.4 Å by gold-standard Fourier shell correlation (FSC) (Extended Data Figs. 2, 3a).

After the mass spectrometry scans, the 10 most intense peaks were selected for HCD fragmentation at 30% of normalized collision energy. HCD spectra were also acquired in the Orbitrap (resolution 17500, AGC target 5 × 10⁶, maximum injection time 120 ms) with first fixed mass at 180 m/z. Charge exclusion was selected for 1+ and 2+ ions. The dynamic exclusion set to 5 s. Cross-linking identification and analysis was done using PLink67 and Xcalibur 2.2 (Thermo Scientific). All peptides were manually validated.

The 3D model was defined and post-translational modifications, protein bands were excised from the gel and processed as described above. Mass spectrometry analysis was carried out similarly with a gradient of 15–38% for 30 min and the Orbitrap set to 350–1,500 m/z. Charge exclusion was selected for 1+ and unassigned ions, dynamic exclusion was set to 5 s. PTM identification was done using the Mascot Daemon client program.

Negative-stain electron microscopy. Amphipol-stabilized Cx46/50 was prepared for negative-stain electron microscopy as described54–56. In brief, 3 μl sample (~0.02 mg ml⁻¹) was applied to a glow-discharged continuous carbon coated electron microscopy specimen grid (Ted Pella), blotted with filter paper and washed two times with detergent-free SEC buffer. The specimen was then stained with freshly prepared 0.75% (w/v) uranyl formate (SPI-Chem). Negative-stained specimens were visualized on a 120 kV TEM (C,orr, FEI) at a nominal magnification of 49,000 × at the specimen level (Extended Data Fig. 1c). Digital micrographs were recorded on a 2 k × 2 k CCD camera (FEI Eagle) with a calibrated pixel size of 4.37 Å. A total of 75 micrographs were collected. Contrast transfer function (CTF) parameters were determined in EMAN2 and micrographs free of significant astigmatism and drift were selected based on Thou rings in the power spectra. A total of 5,330 particles were hand-selected in EMAN2 and extracted with a box size of 84 × 84 pixels. Reference-free 2D class averages were generated using CTF-correction (phase-flipped) images without applied symmetry (Extended Data Fig. 1c). A subset of 3,952 ‘good’ particles was selected following multiple rounds of 2D classification, and an initial model was generated de novo in EMAN2 using a subset of 12 class averages as input. This model was refined against the good particle image dataset in EMAN2 with applied D6 symmetry to a final resolution of ~20 Å (Extended Data Fig. 2).

The grid was blotted for 4.0 s and plunge-frozen in liquid ethane using a Vitrobot (FEI) at 100% humidity and stored under liquid nitrogen.

Cryo-EM specimen grids were imaged on a Titan Krios (FEI) operated at 300 kV. Image stacks were recorded using a K2 summit direct electron detector (Gatan) in counting mode with a super-resolution pixel size of 0.665 Å/pixel. The dose rate was 3.2 electrons pixel⁻¹ s⁻¹, with 4 frames s⁻¹ collected for a total exposure time of 10 s. A Gatan energy filter with a slit width of 30 eV was used during data collection. Drift correction and 3D reconstruction were performed using cisEMTV59, with a nominal magnification of 49,000 × at the specimen level (Extended Data Fig. 1c).
Cx46/50 symmetry analysis. In an attempt to uncover a specific pattern(s) of Cx46/50 heteromeric/heterotypic co-assembly, 3D auto-refinement was also pursued in Relion using C1, C3, C6 and D3 symmetries, using the final 30,128-particle (dataset 1) and 3.4 Å map (filtered to 15 Å) as input. These refinements converged to 4.1 Å (C1), 3.9 Å (C3) and 3.7 Å (D3 and C6). Examination of the resulting maps provided no indication that the Cx46 and Cx50 subunits were being separately resolved (Extended Data Figs. 4, 5). Further attempts were performed using 3D classification in Relion with C3, C6 and D3 symmetry, using the larger 55,475 particle set (which had already been subjected to one round of 3D classification with no imposed symmetry). The initial model was the 3.4 Å map filtered to 25 Å. No resolution limit was enforced, and classification was attempted with and without image alignment. Some classifications converged to a single class, whereas others maintained a more even distribution of particles throughout 3D classification. 3D auto-refine was attempted with the most populated class from each attempted symmetry group. C3 symmetry refined to 3.9 Å from a set of 47,074 particles; C6 symmetry refined to 3.8 Å from a set of 38,404 particles; D3 symmetry refined to 4.2 Å from a set of 16,520 particles. Inspection of the resulting maps provided no indication that isoform-specific features were being separately resolved into any specific symmetric arrangements (not shown). Finally, focused refinement strategies with signal subtraction were also explored using Relion, by masking a single hemisphere or just a single subunit. However, these procedures did not produce isoform-specific features, or improved results compared to the D6-symmetrized maps.

As we were unable to identify a specific pattern of co-assembly for the Cx46/50 dodecameric channel, all further analysis and model building was performed using the 3D maps generated with imposed D6 symmetry. Both pre-processed and post-processed maps and associated masks generated from datasets 1 and 2 have been deposited in the Electron Microscopy Data Bank under accession code EMD-9116.

Atomic modelling, refinement and validation. The post-processed maps obtained with D6 symmetry were used to build and stereochemically refine atomic models for both Cx46 and Cx50, following similar procedures. An initial Cα model was generated using the available crystal structure of Cx46 (PDB ZW237) and placed into the post-processed 3.4 Å density map using rigid-body fitting. Starting from this template, all atom models of Cx46 and Cx50 were built separately into the cryo-EM density using COOT71. Disulfide bonds were modelled for Cx50 (C54–C201, C61–C195 and C65–C190) and Cx46 (C54–C189, C61–C183 and C65–C178). Models were subjected to real-space refinement in Phenix72 with non-crystallographic symmetry (D6-symmetry) and secondary structure restraints imposed. Successive rounds of modelling and refinement were conducted until refinement statistics converged, as judged by Molprobity73 (Extended Data Fig. 2). The FSC of the model versus map dropped below 0.5 Å at 3.4 Å (dataset 1) and 3.5 Å (dataset 2) for both Cx46 and Cx50, judged by the output of Phenix real-space refine (Extended Data Fig. 3a, b). The NTH domain of Cx46 and Cx50 (residues 2–20) were further refined using the post-processed 3.5 Å density map (dataset 2), as this region of the map was more well-defined compared to the original 3.4 Å map. Over areas of the density maps where the sequence of Cx46 and Cx50 are identical or similar (80% identical and 8% similar) both models fit well into the D6-symmetrized map, and these regions tend to display well-resolved side-chain density. Over regions in which the sequence of Cx46 and Cx50 differs, side-chain density is sometimes weaker. This observation is possibly due to the imposed D6 symmetry averaging the density of two different side chains in these areas, or relative flexibility as many of these residues contain solvent-exposed side chains. In these areas of difference, where electron microscopy density is observed, both Cx46 and Cx50 can be fit into the density equally well (Extended Data Figs. 4, 5). Fit of the models to the cryo-EM density maps were assessed quantitatively by local resolution analysis using BlookRes70, comparing the calculated maps of Cx50 and Cx46 atomic models to the 3.4 Å experimental cryo-EM map (Extended Data Fig. 3). This analysis was tabulated by assigning each residue a range of resolution values corresponding to the output of this analysis, including the alpha carbon and extending to the end of the side chain (Supplementary Tables 2, 3).

Completed models of the dodecameric structures—corresponding to residues 2–97, 142–222 (Cx46), and 2–97, 154–234 (Cx50)—have been deposited in the Protein Data Bank with accession numbers 6MHQ and 6MHY, respectively. The Protein Data Bank with accession numbers 6MHQ and 6MHY, respectively. The Protein Data Bank with accession numbers 6MHQ and 6MHY, respectively. The Protein Data Bank with accession numbers 6MHQ and 6MHY, respectively. The Protein Data Bank with accession numbers 6MHQ and 6MHY, respectively. The Protein Data Bank with accession numbers 6MHQ and 6MHY, respectively. The Protein Data Bank with accession numbers 6MHQ and 6MHY, respectively. The Protein Data Bank with accession numbers 6MHQ and 6MHY, respectively. The Protein Data Bank with accession numbers 6MHQ and 6MHY, respectively. The Protein Data Bank with accession numbers 6MHQ and 6MHY, respectively.

Molecular dynamics simulations. Visual Molecular Dynamics (VMD) v1.9.375 was used to build systems for the Cx50, Cx46, Cx46/50 heteromeric and heterotypic models, and for Cx26 (PDB ZW2310). Representative Cx46/50 heteromeric models (heteromeric models I and II, with C3 or D3 point group symmetry, respectively) were constructed by applying the appropriate symmetry operations to the coordinates of the individual subunits. The Cx46/50 heteromeric and heterotypic channels were run through a steepest descent minimization routine using Phenix72 to ensure no clashes were introduced in the preparation of these models. Each system comprised the full dodecameric gap junction, and was prepared in explicit solvent and embedded in two lipid bilayers composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), mimicking a cell-cell junction. The Cx26 crystal structure was prepared for molecular dynamics by completing the side chains at residues Lys15, Ser17 and Ser19 and missing protons were added to all amino acids at standard positions. Side chains were protonated according to neutral conditions, and the HSD model was used for all histidine residues. To facilitate comparison to the Cx46/50 models, the Cx26 model was constructed with a Met1 residue added, which was missing in the published crystal structure, but was expected to be present in the protein based on proteomic analysis26, as previously described27. Disulfide bonds identified in the experimental structures were enforced for Cx50 and Cx46 (as described above), and for Cx26 (C53–C180, C60–C174 and C64–C169). Amino acids corresponding to the intracellular loop (ICL) connecting TM2–TM3, and the C-terminal domain (CTD) of Cx50, Cx46 and Cx26 and were not included for molecular dynamics simulation, as experimental data describing the structure of these large domains (~50 residue ICL and ~200 residue CTD in Cx46/50) are missing. The introduced N- and C-terminal residues resulting from the missing ICL segment (Cx46 L197 and L142; Cx50 V97 and L154; and Cx26 G109 and K125) were neutralized. N-terminal acetylation sites were introduced in VMD through an all-atom acetylation patch in the automated PSF-Builder. A complete list of modelled residues for each system is provided in Supplementary Tables 1.

The prepared protein structures were submerged in a hydration shell using Solvate v1.0.117. Water was removed from sections of the channel corresponding to transmembrane domains, based on hydrophobic character and localization of amphiphil observed in the experimental cryo-EM data (~20–50 Å from the centre of the channel). The VMD membrane-builder plugin was used to add two POPC bilayers, with dimensions of 152 × 152 Å for Cx46, Cx50 and Cx46/50 models, and 155 × 155 Å for Cx26, and lipids overlapping with protein were removed. The entire system was then placed in a water box with dimensions 150 × 150 × 180 Å for Cx46, Cx50 and Cx46/50 models, and 150 × 150 × 183 Å for Cx26, using VMD’s Solvate plugin. The system was neutralized using the Autoionize plugin, then 150 mM KCl and 150 mM NaCl were added to the solvent areas corresponding to intracellular and extracellular regions of the simulation box, respectively (see Fig. 3c). A summary of atoms counts for each system is provided in Supplementary Table 1.

GPU-accelerated nanoscale molecular dynamics v2.122 was used for all classical molecular dynamics simulations, using the CHARMM36 force field28 for the protein and the AMBER99SB-ILDN force field29 for lipid. Each system was following the same minimization and equilibration protocol as follows. An initial minimization of the lipid tails, with all other atoms fixed, was performed for 1 ns with a 1-fs time step, allowing the tails to ‘melt’. Next, the system—including lipids, solvent and ions—was allowed to minimize around the protein, with the protein harmonically constrained for 1 ns. For the Cx46/50 heteromeric/heterotypic and acetylated models, a second minimization step was applied, in which the system was free to minimize with a harmonic constraint on the protein backbone to ensure stable quaternary structure. The entire system was then released from restraints and subjected to all-atom equilibration runs using Langevin thermostat, with a constant temperature of 310 K and constant pressure of 1 atm, with 1 or 2-fs time steps and allowed to proceed for 30 ns (see Supplementary Table 1). Periodic boundary conditions were used to allow for the particle mesh Ewald calculation of electrostatics. Finally, all of the models were continued for a minimum of 50 ns of production. Root mean squared deviations (r.m.s.d.) and root mean squared fluctuations (r.m.s.f.) were calculated using VMD. All three gap junctions approached a steady r.m.s.d. within 20 ns of the equilibration phase (Extended Data Fig. 7a, b). All of these systems maintained an electro-chemical seal to extracellular sodium ions (Na+) during molecular dynamics simulation (for example, Fig. 3c), validating the stability of intermembrane docking-site interactions and the various heteromeric/heterotypic models generated for analysis.

Calculation of the PMF with respect to K+ and Cl− was performed using the fundamental principle of detailed balance via a one-dimensional Markov state model (MSM). Configuration space was subdivided based on a natural coordinate, the channel pore (z axis), and segmented into bins of 4 Å in length. Using a lag-time of 2 ps, a transition matrix was calculated from the trajectories of individual ions within the simulation. The → transition probability kij is computed using equation (1):

\[
k_{ij} = \frac{N_{ij}}{N_i} \]

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in which $N_i$ is the count of transitions during the lag interval and $N_i$ is the count of ions in bin $i$ at the beginning of each lag interval. PMFs were constructed using the principle of detailed balance:

$$P_{i}^{\text{eq}} k_{i+1,i} = P_{i}^{\text{eq}} k_{i,i+1},$$

(2)

$$\Delta C_{i,j} = \text{the free-energy difference from bin } i \text{ to bin } j,$$

(3)

$$\Delta PMFs \text{ obtained by this approach would therefore be strongly influenced by the high mobility of ions within the channel pore, and the timescales used for analysis.}$$

Here, $P_{i}$ is the equilibrium probabilities for an ion to occupy the respective bin (equation (2)), $\Delta C_{i,j}$ is the gas constant (1.986 cal mol$^{-1}$ K$^{-1}$), and $T$ is the temperature (310 K) (equations (3), (4)). Final PMF values were adjusted so that the values of the bulk solvent were zero. PMF curves in Fig 3 and Extended Data Fig. 7 were derived by mapping $z$ values to the corresponding bin index $i$ and subsequently smoothed using Microsoft Excel. The detailed-balance (rates-based) approach is justified by the grand canonical Monte-Carlo Brownian dynamics (GCMC/BD)-based approach for modelling ion conductance using a model of the Cx26 hemichannel (by extracting a single hexamer of the Cx26 intercellular channel). However, a limitation of GCMC/BD method is that the protein structure is held static, and the resulting PMFs obtained by this approach would therefore be strongly influenced by the selected conformational state of the Cx26 NTH domain. These caveats should be considered when interpreting results presented in this work.

**Statistical analysis.** 95% confidence intervals for comparison of $C_i$, r.m.s.f. values were calculated using a two-tailed Student’s $t$-test. No statistical methods were used to predetermine sample size for the cryo-EM datasets. The experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability**

Cryo-EM density maps have been deposited in the Electron Microscopy Data Bank under accession number EMD-9116. Coordinates for Cx46 and Cx50 atomic models have been deposited in the Protein Data Bank under accession codes 6MHQ and 6MHY. The original multi-frame micrographs have been deposited in the Electron Microscopy Public Image Archive under accession code EMPIAR-10212.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Preliminary structural analysis of native lens Cx46/50 gap junction intercellular channels. a, Denaturing protein gel and western blot analysis of purified Cx46/50 (also known as MP38) isolated from lens core tissue. Protein bands corresponding to Cx46 and Cx50 co-migrate at a molecular weight of ~38 kDa, consistent with age-dependent proteolytic cleavage within the C-terminal domain of both isoforms16. Experiment performed 3 times with similar results. b, SEC elution profile of Cx46/50 gap junctions reconstituted in decyl-maltoside (DM, grey trace) or amphipol (A-835, blue trace), monitored by UV absorbance. Experiment performed more than 3 times with similar results. Inset, calibration curve (n = 3 runs) demonstrating that Cx46/50 elutes at an apparent molecular weight of ~560 kDa, consistent with the size of a dodecameric protein complex (12 × ~38 kDa) and two micelles (2 × ~50 kDa). c, Electron micrograph of negatively stained Cx46/50 gap junctions reconstituted into amphipol. Scale bar, 100 nm. Inset, representative 2D class averages of negatively stained particles (selected from 25 classes). Scale bar, 20 nm. d–g, Chemical cross-linking and mass spectrometry. d, Representative MS/MS m/z spectrum, identifying inter-subunit cross-linking at the N-terminal Gly2 positions of Cx50 and Cx46. Identified peaks in the m/z spectrum and amino acid identities are indicated (Cx50 b-ions, green; Cx50 y-ions, yellow; Cx46 y-ions, blue). MS/MS data represent the consensus of 3 independent runs. e, Structural analysis of cross-linking results, showing inter-subunit distances between the symmetrically related N-terminal Gly2 positions within the connexin hemichannel, ranging from 9.1 Å (i to i ± 1), 15.8 Å (i to i ± 2) and 18.2 Å (i to i ± 3). The cross-linker spacer length is 11.4 Å, indicating a probable (i to i ± 1) arrangement of Cx50 and Cx46 within the same hemichannel, although other arrangements cannot be ruled out. f, Overview of identified inter-subunit cross-links between Cx50 and Cx46 assembled gap junctions. Residues in red indicate the site of primary amines involved in the cross-linking reaction using either DSS or BS3. All detected inter-subunit cross-links are between cytoplasmic domains. g, Schematic showing sites of inter-subunit cross-linking between Cx46 and Cx50 (black lines) and post-translational modifications identified during proteomics analysis (yellow, phosphorylation; purple, N-terminal acetylation). Met1 was determined to be removed in both Cx46 and Cx50 and the resulting N-terminal Gly2 position was identified in both acetylated and non-acetylated forms of Cx46 and Cx50, consistent with the specificity of the NatA acetylation complex24. The predicted CTD cleavage sites in Cx46 and Cx50 (orange), based on previous analysis of bovine Cx46/50 isolated from lens core tissue16, are also shown. Secondary structure and domain labels are indicated for the NTH, TM1–TM4, EC1, EC2, ICL and CTD.
Extended Data Fig. 2 | Overview of cryo-EM image processing and 3D reconstruction. a, A total of 1,104 micrographs were collected in an automated fashion using SerialEM on a 300-kV Titan Krios (dataset 1). Movie stacks were recorded using a K2 summit-direct electron detector operated in super-resolution mode and acquired with an effective pixel size of 0.665 Å. Movie stacks were corrected for drift and CTF using MotionCor2 and GCTF, respectively. An initial dataset of 261,206 raw particles was obtained using unbiased autopicking procedures in DoG Picker. A refined dataset of 53,791 good particles was obtained following several rounds of 2D classification and removal of ‘bad’ particles (or ice contamination) was done in Relion. 3D classification was seeded using an initial model obtained by negative-stain electron microscopy, filtered to 60 Å. A majority of particles fell into a single 3D class (~62.5% of the good particles). These 30,128 particles were used for final 3D auto-refinement and post processing, yielding a final map at 3.4 Å resolution by gold-standard FSC (dataset 1). Dataset 2 was processed in a similar fashion from a total of 2,197 micrographs and 44,547 good particles, resulting in a final map at 3.5 Å resolution by gold-standard FSC. b, Summary of cryo-EM data collection, refinement and model validation statistics. Dataset 1 was used to obtain the 3.4 Å resolution reconstruction (map 1). Dataset 2 was used to obtain the 3.5 Å resolution reconstruction (map 2). Pre-processed and post-processed maps and associated masks from both datasets have been deposited in the Electron Microscopy Databank (EMD-9116). The original multi-frame micrographs have been deposited to EMPIAR (EMPIAR-10212). Coordinates for Cx50 and Cx46 atomic models have been deposited in the Protein Data Bank (6MHY and 6MHQ, respectively).
Extended Data Fig. 3 | Global and local resolution analysis. a, b, FSC analysis obtained from dataset 1 (a) and dataset 2 (b). Gold-standard FSC curves following auto-refinement (light grey), post-processing (grey), and masking (dark grey). The final masked maps display an overall resolution of ~3.4 Å (dataset 1) and ~3.5 Å (dataset 2), using a 0.143 cut-off. FSC curves comparing atomic models of Cx46 (orange) and Cx50 (blue) fit to the cryo-EM maps display correlation at 0.5 cut-off to a resolution of 3.4 Å (dataset 1) and 3.5 Å (dataset 2). c, d, Local resolution analysis using BlocRes, obtained for the half-maps for dataset 1 (c) and 2 (d). e, f, Local resolution analysis comparing the experimental density map (dataset 1) to the calculated maps of Cx46 (e) and Cx50 (f). Local resolution ranges in c–f are indicated by colour (2.5–4.0 Å, blue–cyan; 4.0–5.0 Å, white; 5.0–6.5 Å, yellow–orange). Values obtained for local resolution of Cx46 and Cx50 models compared to the experimental density map are shown in Supplementary Tables 2, 3. Local resolution assessment comparing the density map to the two models indicates that the sites at which the two isoforms differ in sequence were generally less well-resolved, as compared to equivalently exposed residues at which Cx46 and Cx50 share conserved sequence.
Extended Data Fig. 4  |  Cx46 and Cx50 atomic models fit to the cryo-EM density maps. Segmented cryo-EM map with atomic models for sheep Cx46 and Cx50 fit to the experimental densities derived from dataset 1 (3.4 Å, D6 symmetry), including regions for TM1–TM4, EC1 and EC2. The NTH domain is fit into the map from dataset 2 (3.5 Å, D6 symmetry), which was more well-defined in this region. Cx46 (top) and Cx50 (bottom) models are coloured according to their pairwise sequence homology, as being identical (grey, 80%), similar (blue, 8%) and different (orange, 12%). Windows show magnified views corresponding to boxed regions of the segmented maps, highlighting representative side-chain densities and fit to the atomic models. Regions of identical or similar amino acids are fit equally well by both models (for example, Cx46 L172 versus Cx50 I184, blue labels). Over regions in which the sequence of Cx46 and Cx50 differs, side-chain density is typically weaker (see also Extended Data Fig. 5). This is possibly due to the imposed averaging of two different side chains in these areas, or relative flexibility as many of these residues correspond to solvent-exposed side chains. In these areas of difference, and where electron microscopy density is present, both Cx46 and Cx50 models were typically fit equally well into the density map (for example, Cx46 T53 versus Cx50 V53 and Cx46 Q171 versus Cx50 R183, orange labels).
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Analysis of different symmetry refinements on the ability to resolve differences between Cx46 and Cx50. Eight sites of sequence differences involving bulky amino acids (labelled, and indicated by arrowhead) were selected as potential fiducial markers for resolving the two different isoforms following 3D refinement with various applied symmetries (C1, 4.1 Å resolution; C3, 3.9 Å resolution; C6, 3.7 Å resolution; D3, 3.7 Å resolution; D6, 3.4 Å resolution). For the applied symmetries, views are presented for each unique asymmetric subunit (boxed). Despite the modest resolution of the asymmetric (C1) reconstruction, side-chain density for bulky amino acids is typically observed at sites at which the two isoforms are conserved (asterisk). However, at the selected sites of sequence variation (arrow head) the side-chain densities are either not well-resolved, or there was no systematic variation that indicated an ability to distinguish the two isoforms. The most resolved features at these sites of variation were obtained with D6 symmetry, and typically corresponded to regions where these different amino acids share similar structure (such as Cβ positions). Although the cryo-EM density at these sites of variation were typically weak, the resolvable side-chain features throughout the rest of the map were generally enhanced when higher symmetry was applied during map refinement (indicated by asterisk), suggesting that regions of sequence similarity between Cx46 and Cx50 also share a high level of structural similarity.
Extended Data Fig. 6 | Sequence and structure conservation of Cx46 and Cx50 heteromeric/heterotypic interfaces. a, Multiple sequence alignment of mammalian Cx46 and Cx50 isoforms with residues contributing to heteromeric and heterotypic interfaces annotated. Circle, heteromeric interface; square, heterotypic interface; filled, ≥70% buried; half-filled, 20–70% buried. Colouring corresponds to amino acid type (grey, hydrophobic; dark grey, aromatic; red, acidic; blue, basic; orange, hydrophilic; yellow, cysteine). Regions of sequence homology are indicated by the level of shading. Secondary structure and domain labels are indicated for the NTH domain, TM1–TM4, EC1 and EC2. Regions lacking defined structure and with poor sequence homology within the intracellular loop (ICL) and C-terminal domain (CTD) have been omitted for clarity. Sheep and human Cx46 and Cx50 orthologues contain ~95% sequence identity (~98% similarity) over the structured regions of the protein. Numbering corresponds to the amino acid sequence of sheep Cx44 and Cx49 used in the main text. b, Illustration of homomeric and heteromeric interface interactions involving the three sites lacking conservation between Cx44 and Cx49 at this interface (positions 39, 43 and 53). Despite these sequence differences, the interactions involving these residues are generally similar (hydrophobic, grey; hydrogen bonding, orange; ion-pairing, blue).
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Analysis of protein backbone dynamics during molecular dynamics equilibration and production. a, Cα r.m.s.d. analysis of equilibrium (0–30 ns) and production phases (30–80 ns) of the molecular dynamics simulations, calculated with respect to the experimental starting structure for non-acetylated models of Cx50 (red traces), Cx46 (blue traces) and Cx26 (with Met1 added; grey traces). Separate analysis for the NTH domains are shown in lighter shades.

b, Same analysis as in a, for models with N-terminal acetylation added. The NTH domain of Cx26 (light grey traces) shows significantly higher r.m.s.d. values, for both non-acetylated and acetylated models.

c, Plot of average Cα r.m.s.f. during the production phase of the molecular dynamics simulations for Cx50 (left, red traces), Cx46 (centre, blue traces) and Cx26 (right, grey traces). Data obtained for the N-terminal acetylated models are shown in lighter shades. Averages are determined for the 12 subunits composing the intercellular channel. Error bars represent 95% confidence intervals (n = 12 subunits). Secondary structure and domain labels are indicated for the NTH, TM1–4, EC1 and EC2, and ICL (not modelled).

d, e, Average r.m.s.f. values of the acetylated models mapped to the experimental starting structures of Cx50 (left), Cx46 (centre) and Cx26 (right). Colours correspond to r.m.s.f. amplitudes: 0–1.0 Å (cyan); 1.0–2.0 Å (yellow–orange), >2.0 Å (red). In e, a single NTH domain is circled and the average r.m.s.f. values and 95% confidence intervals (Student t-test) calculated over the NTH domain of each isoform are displayed (n = 12 subunits). The NTH domain of Cx26 shows significantly higher r.m.s.f. values, for both acetylated and non-acetylated models (P < 0.0001).
Extended Data Fig. 8 | Modulation of coulombic surface potential and K⁺/Cl⁻ PMFs resulting from N-terminal acetylation and Cx46/50 heterotypic/heteromeric assembly. a, Coulombic surface potential maps (top) and PMF (bottom) obtained for a set of non-acetylated and acetylated Cx50–Cx50ac and Cx46–Cx46ac models characterized by molecular dynamics simulation. Acetylated Cx50ac and Cx46ac monomers were used to construct a heterotypic channel and two different heteromeric channels (labelled I and II). Icons at the top of each structure show the relative configurations of Cx50 (black) and Cx46 (white). The resulting coulombic surface potentials are coloured as in Fig. 3a (negative, red; neutral, white; positive, blue). Only eight subunits are shown to portray both the channel pore and subunit interfaces. An asterisk (shown in a and b) indicates the site of N-terminal acetylation, which neutralizes the positively charged N terminus. PMFs obtained for K⁺ (blue traces) and Cl⁻ ions (red traces) are displayed directly beneath each model displayed in a. Free-energy maxima are labelled and pore axis (z axis) is indicated. Traces indicate symmetrized values, with unsymmetrized values in lighter shading. In the case of the asymmetric Cx46/50 heterotypic model (middle left), PMFs represent the average from the first and last ~60 ns of simulation. b, Coulombic surface potential (top) and PMFs (bottom) obtained for non-acetylated Cx26 (left) and Cx26ac (right) are displayed as in a. c, Table of peak free-energy barriers for K⁺ (ΔΔG钾) and Cl⁻ (ΔΔG氯) and corresponding ΔΔG, reported as a proxy for charge selectivity. Asterisk indicates models with acetylated N terminus. d, e, Validation of methods used to construct the PMFs. d, Comparison of K⁺ PMFs obtained for Cx50ac and Cx46ac using population states (left) or transition rates (right) (see Methods). Both methods yielded similar PMF profiles. All other PMFs were constructed using the distributed seeding approach to enhance sampling (see Methods).
Extended Data Fig. 9 | Analysis of K⁺ trajectories and putative binding site observed during molecular dynamics simulation. a–d, K⁺ ion trajectories obtained for Cx50 and Cx46 along the channel pore (z axis). a, b, Representative traces of mobile ions transiting and exiting or entering the channel pore in both acetylated (a) and non-acetylated (b) models of Cx46 and Cx50. c, d, K⁺ ions displaying long dwell times (≈10–20 ns) localized at one or more putative binding sites within the channel pore (asterisk at z = ~14 Å) in both models of Cx46 and Cx50. In a–d, similar results were observed from 6 independent runs using non-acetylated models (1 × 80-ns and 2 × 10-ns runs for both Cx50 and Cx46) and 13 independent runs using the acetylated models (1 × 80-ns and 6 × 10-ns runs for Cx50; and 1 × 80-ns and 7 × 10-ns runs for Cx46). e, Representative snapshot showing an enlarged view of the putative K⁺ binding site identified for Cx50 and Cx46, corresponding to the region indicated by the asterisk in c and d. A single K⁺ ion is bound by a conserved set of amino acids (among Cx46/50 orthologues), coordinated by the side-chain carboxylate of Glu62 and backbone carbonyls of Gln49, Ser50 and Phe52 (identical in Cx46 and Cx50). Two transient water molecules observed coordinating the bond K⁺ ion are shown. Twelve binding sites are present within the dodecameric channel. Similar behaviour was observed from simulations using both non-acetylated and acetylated models (19 independent simulations). A functional role for this putative binding site is not yet clear, but may represent a physiologically relevant cation–binding site similar to the recently proposed Ca²⁺–binding site in Cx2611.
Extended Data Fig. 10 | Dynamic hydrogen-bond network within the NTH domain observed by molecular dynamics simulation.

a–e, Analysis of hydrogen-bond interactions for non-acetylated models of Cx46 and Cx50 observed during molecular dynamics simulation. a, Inset, magnified view of D3 pairing with the positively charged N-terminal G2 position from a neighbouring subunit (intermolecular) and with the hydroxyl of S5 within the same subunit (intramolecular). The D3–G2 interactions are dynamically formed and broken during molecular dynamics simulation, whereas the intramolecular D3–S5 hydrogen bond is relatively stable (as shown in b–e). b, c, Population statistics of inter-atomic distances involving D3 (C\(\gamma\)) and G (N) of the neighbouring chain (b) and Ser5 (H\(\gamma\)) of the same chain (c), extracted from molecular dynamics simulation production runs of Cx46 (blue histogram) and Cx50 (red histogram). For D3 and G2, heavy atoms were chosen as proxies to monitor hydrogen-bonding interactions involving equivalent rotameric donor-acceptor configurations. The population centred at \(~3.6\ \text{Å}\) (b) and \(~2.8\ \text{Å}\) (c) are considered to be within hydrogen-bond distance.
d, e, Trajectories extracted from molecular dynamics simulation of Cx46 (blue traces) and Cx50 (red traces) showing the dynamical behaviour of the D3–G2 intermolecular charge paring (d) and D3–S5 intramolecular hydrogen bonding (e). The dwell times showing hydrogen-bond pairing (\(~3.6\ \text{Å}\) in d; and \(~2.8\ \text{Å}\) in e) are indicated with transparent grey shading. In the Cx26 crystal structure, the equivalent D2 site is modelled in hydrogen-bond distance to a neighbouring T5 site (Cx26 numbering)\(^{10}\), but this intermolecular interaction is rapidly broken during molecular dynamics simulations and does not appear to reform within the timescale of our molecular dynamics experiments, and instead forms a stable intramolecular interaction with T5, as previously described\(^{15}\) (data not shown). f–j, Analysis of hydrogen-bond interactions observed during molecular dynamics simulation for Cx46ac and Cx50ac modelled with the N-terminal G2 position acetylated. f, Inset, magnified view of acetylated G2ac position hydrogen bonded to the indole ring of W4 from the same subunit (intramolecular) and the same intramolecular D3–S5 hydrogen-bond interaction observed in the non-acetylated channel. g, h, Population statistics of inter-atomic distances involving W4 (N\(\epsilon\)) and G2ac (acyetyl carbonyl) (g) and D3 (C\(\gamma\)) distance to Ser5 (H\(\gamma\)) of the same chain (h), extracted from molecular dynamics simulation production runs of Cx46ac (blue histogram) and Cx50ac (red histogram). i, j, Trajectories extracted from molecular dynamics simulation of Cx46ac (blue traces) and Cx50ac (red traces) showing the dynamical behaviour of the W4–G2ac hydrogen-bond pairing (i) and D3–S5 intramolecular hydrogen bonding (j). The dwell times showing hydrogen-bond pairing (\(~2.4\ \text{Å}\) in i, and \(~2.5\ \text{Å}\) in j) are indicated with transparent grey shading. For clarity, only the first 15 ns of the production period is shown (d, e, i, j). Similar results were observed from 6 independent runs using non-acetylated models (1 \times 80-ns and 2 \times 10-ns runs for both Cx50 and Cx46) and 13 independent runs using the acetylated models (1 \times 80-ns and 6 \times 10-ns runs for Cx50; and 1 \times 80-ns and 7 \times 10-ns runs for Cx46).
Reporting Summary

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Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

CryoEM: SerialEM(v3.6)

Data analysis

CryoEM Image Analysis Software: MotionCor2(v1.1.0), GCTF(v0.5), DoGPicker(v1.0), Relion(v2.0), EMAN2(v2.12), BlocRes(v2.0); Atomic Modeling and Visualization: COOT(v0.8.6), Phenix(v1.13), Molprobity(v4.4), Chimera(v1.11); Molecular Dynamics: VMD(v1.9.3), NAMD(v2.12), Solvate(v1.0.1); Mass Spectrometry: pLink(v1.07), Xcalibur(v2.2), MASCOT Daemon(v2.5)

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Data

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
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CryoEM density maps (pre-processed and post-processed) and associated masks have been deposited to the Electron Microscopy Data Bank (EMD-9116).
Coordinates for Cx46 and Cx50 atomic models have been deposited to the Protein Data Bank (6MHQ, 6MHY). The original multi-frame micrographs have been deposited to EMPIAR (EMPIAR-10212).

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

CryoEM sample size were not predetermined. An initial dataset of 261,206 particles (Dataset 1) was collected, and an additional dataset of 398,066 particles (Dataset 2) was further used to assess the effects of particle number on the achievable resolution and/or resolvability of heterogeneous features during 3D classification. The initial size of dataset 1 was estimated based on the particle density observed in test images. The increased size of dataset 2 vs. dataset 1 did not improve the global resolution of the resulting CryoEM maps, indicating the size of the particle datasets were sufficiently sampled.

Data exclusions

Single particle image data was excluded based on the absence of high-resolution features (e.g. alpha-helical transmembrane domains), which were conditions that had been pre-established based on expected structural homology to connexin-26.

Replication

All attempts at replication were successful. This included processing two independent datasets obtained from unique particles in dataset 1 and dataset 2, and by processing with alternative CryoEM image analysis software (CisTEM(v1.0)).

Randomization

Single particle image data was split randomly into two groups and processed in the same way to calculate Fourier-shell correlation coefficients, in accordance to Gold Standard Methods. Samples were not further allocated into groups, outside of what is performed by the computational image analysis programs used in this work.

Blinding

Investigators were not blinded during data acquisition or analysis. Blinded studies in this case were not possible because the investigator performing the experiments and analysis also contributed to isolation of the specimen being analyzed.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a Involved in the study
☐ Unique biological materials
☐ Antibodies
☐ Eukaryotic cell lines
☐ Palaeontology
☐ Animals and other organisms
☐ Human research participants

Methods

n/a Involved in the study
☐ ChIP-seq
☐ Flow cytometry
☐ MRI-based neuroimaging

Antibodies

Antibodies were purchased from outside vendors. Primary antibodies directed against the n-terminal domain of Cx46 (rabbit, poly-clonal) where purchased from Acris (AP11570PU-N, lot SH021017A) and used for western blot analysis at a dilution of 1:500. Antibodies directed against the proximal c-terminal domain of Cx50 (mouse, poly-clonal) where purchased from Santa Cruz (sc-50432, lot C0107), and used for western blot analysis at a dilution of 1:2,000.

Validation

The Cx46 antibody was validated by the manufacturer against the human isoform using ELISA and Immunohistochemistry. The Cx50 antibody was validated by the manufacturer by western blot analysis of connexin-50 expression in 293T and TK-1 cells, and whole cell lysates from rat brain tissue. Both antibodies were further validated by in-house western blot analysis using lens fiber cell lysates (sheep).