Structure of a force-conveying cadherin bond essential for inner-ear mechanotransduction

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Hearing and balance use hair cells in the inner ear to transform mechanical stimuli into electrical signals. Mechanical force from sound waves or head movements is conveyed to hair-cell transduction channels by tip links, fine filaments formed by two atypical cadherins known as protocadherin 15 and cadherin 23 (refs 4, 5). These two proteins are involved in inherited deafness and feature long extracellular domains that interact tip-to-tip in a Ca2+-dependent manner. However, the molecular architecture of this complex is unknown. Here we combine crystallography, molecular dynamics simulations and binding experiments to characterize the protocadherin 15–cadherin 23 bond. We find a unique cadherin interaction mechanism, in which the two most amino-terminal cadherin repeats (extracellular cadherin repeats 1 and 2) of each protein interact to form an overlapped, antiparallel heterodimer. Simulations predict that this tip-link bond is mechanically strong enough to resist forces in hair cells. In addition, the complex is shown to become unstable in response to Ca2+-removal owing to increased flux of Ca2+-free cadherin repeats. Finally, we use structures and biochemical measurements to study the molecular mechanisms by which deafness mutations disrupt tip-link function. Overall, our results shed light on the molecular mechanics of hair-cell sensory transduction and on new interaction mechanisms for cadherins, a large protein family implicated in tissue and organ morphogenesis, neural connectivity and cancer.

Hair-cell mechanotransduction occurs within each bundle of stereocilia (Fig. 1a) that is deflected by mechanical stimulation. Deflection results in tension applied to tip links, protein filaments linking the tip of each stereocilium to its tallest neighbour. The tip links, acting in series with an elastic ‘gating spring’, pull open transduction channels. Recently, protocadherin 15 (Pcdh15) and cadherin 23 (Cdh23), which feature exceptionally long extracellular domains containing 11 and 27 extracellular cadherin (EC) repeats, respectively (Fig. 1b), were shown to form the tip link. To elucidate the tip-link heterophilic molecular bond between Pcdh15 and Cdh23, we determined the crystallographic structure of their interacting N termini (Fig. 1c; results summary in Supplementary Fig. 1). Size-exclusion chromatography (SEC) of co-refolded protein fragments comprising the EC1 and EC2 repeats of both Pcdh15 and Cdh23 (hereafter termed Pcdh15-EC1+2 and Cdh23-EC1+2, respectively) showed a monodisperse peak with the two protein fragments interacting in solution (Supplementary Fig. 2a). The complex crystallized in two packing arrangements and two independent models were fully refined (S1a-S1b and S2, respectively; Supplementary Fig. 3 and Supplementary Table 1).

The structures show that Pcdh15-EC1+2 and Cdh23-EC1+2 form an overlapping and antiparallel heterodimer (Fig. 1c, f, g). The interaction resembles an ‘extended handshake’ and involves repeats EC1 and EC2 from both proteins. The overall fold of the Pcdh15 and Cdh23 fragments matched the well-known Greek key motif of classical cadherins (Supplementary Fig. 4). As expected, three Ca2+ ions are found in a canonical arrangement (sites 1, 2 and 3) at the linker region between repeats EC1 and EC2 of each protein (Fig. 1c, e). However,

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**Figure 1 | Structure of tip-link protocadherin 15 bound to cadherin 23.**

- **a.** Hair-cell stereocilia bundle. A tip–link filament extends from the tip of each stereocilium to the side of its tallest neighbour. The tip link formed by a Pcdh15 parallel dimer interacting tip-to-tip with a Cdh23 parallel dimer. These proteins feature 11 and 27 EC repeats, respectively. Inset shows possible arrangement at the junction. **b.** Ribbon diagram of Pcdh15-EC1+2 (purple) bound to Cdh23-EC1+2 (blue) with Ca2+ ions as green spheres. Arrowheads indicate the RGGP loop of Pcdh15-EC1+2 and the 310 helix of Cdh23-EC1+2. Residues Arg 113, Cys 11 and Cys 99 of Pcdh15 are shown in stick representations. **c.** Detail of disulphide bond Cys 11–Cys 99 and isoform-dependent residues Asp 4 to Tyr 8 at the Pcdh15 N terminus. **d.** Detail of disulphide bond Cys 11–Cys 99 and isoform-dependent residues Asp 4 to Tyr 8 at the Pcdh15 N terminus. **e.** Detail of Ca2+-binding sites 1, 2 and 3 at the Pcdh15-EC1+2 linker. Protein backbone and side chains are in stick representations for labelled residues. **f.** Surface representation of Pcdh15-EC1+2 (purple and pink) and Cdh23-EC1+2 (blue and cyan) as in **c.** Pcdh15-EC1+2 and Cdh23-EC1+2 interaction surfaces exposed with interfacing residues labelled. Residues labelled in red are involved in inherited deafness; underlined residues were mutated to probe the interface.
several novel structural features within Pcdh15-EC1 + 2 and Cdh23-EC1 + 2 enable the handshake interaction.

Pcdh15-EC1 + 2 has an elongated N terminus clamped by an intramolecular disulphide bond (Fig. 1d), which is followed by a conserved RXGPP (in which ‘X’ denotes glycine, threonine or serine) motif that forms a rigid and bulky loop (Supplementary Fig. 5a, b). This RXGPP loop, within strand A of Pcdh15-EC1, is tucked against the narrow wrist of the linker region of the adjacent Cdh23-EC1 + 2 (Fig. 1c). Similarly, Cdh23-EC1 + 2 has an elongated N terminus, stabilized at the tip by Ca$^{2+}$-binding site 0 (refs 16, 17), which is followed by a bulky $\beta_1$0 helix within strand A that sits at the narrow wrist of the linker in the adjacent Pcdh15-EC1 + 2 (Fig. 1c). Thus, the Pcdh15-EC1 + 2–Cdh23-EC1 + 2 interface exploits unique structural protrusions within strand A of each EC1 repeat, which in turn are stabilized by a disulphide bond and a Ca$^{2+}$-binding site, and lead to the two main areas of interaction described below.

The Pcdh15-EC1 + 2–Cdh23-EC1 + 2 heterotetrameric interface differs from the strand-exchanged or X-dimer homophilic interfaces of classical cadherins16–20. Furthermore, this interface is not directly mediated by Ca$^{2+}$ as previously speculated16–17. Several factors indicate that this is a robust interface. The buried surface area is ~1,000 Å$^2$ per protomer (see Supplementary Tables 1 and 2), similar to that of classical cadherin interfaces (850 Å$^2$ and 1,270 Å$^2$ for type I and type II, respectively). The interface is amphiphilic (Supplementary Fig. 6); all its residues are highly conserved in mouse, human and chicken homologues, and none are predicted to be glycosylated (Fig. 1g and Supplementary Fig. 7). Finally, the same interface was observed in two different crystal lattices, so it is unlikely to represent unphysiological crystal-packing interactions.

To further validate the Pcdh15-EC1 + 2–Cdh23-EC1 + 2 interface we used isothermal titration calorimetry (ITC) and site-directed mutagenesis. The stoichiometry of the wild-type complex was determined to be $N = 0.88 \pm 0.1$, consistent with the one-to-one crystallographic arrangement (Fig. 1). The measured dissociation constant was $K_d = 2.9 \pm 0.4$ μM (temperature $T = 10 \, ^\circ\text{C}$, enthalpy change $\Delta H = 7.1 \pm 0.2$ kcal mol$^{-1}$, entropy change $\Delta S = 50.4 \pm 1.1$ cal mol$^{-1}$ deg$^{-1}$, two trials; Fig. 2a, b and Supplementary Discussion). The tip link is thought to be a heterotetramer of parallel Pcdh15 and Cdh23 dimers6 (Fig. 1b). However, our biochemical and crystallographic data do not show homophilic binding of Cdh23-EC1 + 2 or Pcdh15-EC1 + 2, suggesting that parallel dimerization is mediated by repeats other than EC1 and EC2. If so, the binding affinity for the heterotetramer is expected to be higher21.

The ‘extended handshake’ features two main areas of interaction. The first is located at and above the RXGPP loop (Fig. 1d), which is followed by a bulky $\beta_1$0-helical loop, within strand A of Pcdh15-EC1, is tucked against the narrow wrist of the linker region of the adjacent Cdh23-EC1 + 2 (Fig. 1c). Thus, the Pcdh15-EC1 + 2–Cdh23-EC1 + 2 interface exploits unique structural protrusions within strand A of each EC1 repeat, which in turn are stabilized by a disulphide bond and a Ca$^{2+}$-binding site, and lead to the two main areas of interaction described below.

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The ‘extended handshake’ features two main areas of interaction. The first is located at and above the RXGPP loop of Pcdh15 and centres on Tyr8, Pro 19 and Ile 108 in Pcdh15, and Leu 145 and Gln 187 in Cdh23.

**Figure 2 | Pcdh15-EC1 + 2–Cdh23-EC1 + 2 complex formation probed using ITC and site-directed mutagenesis.** a, Raw power versus time data for Pcdh15-EC1 + 2 (111 μM) titrated with Cdh23-EC1 + 2 (1.1 mM) at 10 °C (black, wild-type wild type (WT–WT)). Inset shows raw data (blue) for Pcdh15-EC1 + 2 (I22A) (114 μM) titrated with Cdh23-EC1 + 2 (L145G) (1.2 mM). b, c, Change in molar enthalpy for Pcdh15-EC1 + 2 titrated with Cdh23-EC1 + 2 (black, WT–WT); Pcdh15-EC1 + 2 (I22A) with Cdh23-EC1 + 2 (light green); Pcdh15-EC1 + 2 with Cdh23-EC1 + 2 (L145G) (dark green); and Pcdh15-EC1 + 2 (I22A) with Cdh23-EC1 + 2 (L145G) (blue) (b), and Pcdh15-EC1 + 2 with Cdh23-EC1 + 2 (S47P) (violet); Pcdh15-EC1 + 2 (R113G) with Cdh23-EC1 + 2 (magenta); and Pcdh15-EC1 + 2 with Cdh23-EC1 + 2 (D101G) (indigo) (concentrations in Supplementary Fig. 8) (c). Sigmoidal isothermals were observed only for Pcdh15-EC1 + 2–Cdh23-EC1 + 2 and Pcdh15-EC1 + 2–Cdh23-EC1 + 2 (S47P). d–h, Details of the Pcdh15-EC1 + 2–Cdh23-EC1 + 2 interface, highlighting residue Leu 145 (e), the RGGPP loop (f) and residues Ile 22 (g) and Arg 113 (h). I is a 180°-rotated version of g. Protein backbone and interfacing residues (as identified by the Protein Interfaces, Surfaces and Assemblies (PISA) server) are in purple/pink for Pcdh15 and blue/cyan for Cdh23.

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Cdh23 (Figs 1g and 2d–f). The second, located between the RXGPP loop and the $3_{10}$ helix of Cdh23, involves Ile 22, Arg 113 and Val 115 in Pcdh15 along with Tyr 16 and Gln 98 in Cdh23 (Figs 1g and 2d, g). To test the two interaction areas we introduced mutations predicted to disrupt them: I22A in Pcdh15 (Pcdh15-EC1 + 2(I22A)) and L145G in Cdh23 (Cdh23-EC1 + 2(L145G)); Fig 2d, e, g. SEC confirmed proper folding and structural integrity of the mutant proteins. ITC experiments, testing binding with either one or both mutant partners, showed decreased affinity for each single-mutant complex (Pcdh15-L145G; Fig. 2a, b, d, e, g and Supplementary Figs 2, 8 and 9). Likewise, SEC of Pcdh15-EC1 + 2(I22A) and Cdh23-EC1 + 2(I22A) did not show complex formation (Supplementary Figs 2, 8 and 9). Likewise, SEC of Pcdh15-EC1 + 2 and Cdh23-EC1 repeats alone did not show complex formation (Supplementary Fig. 2b). Taken together, these results indicate that the interface observed in the crystals is consistent with the interface observed in solution.

Next we wanted to know whether the interface has the properties expected for a tip-link bond. Tip links are regularly subjected to (and must withstand) forces ranging from 10 to 100 pN, both in vivo and in physiological experiments. Although SEC and ITC experiments provide a characterization of the bond in thermodynamic equilibrium, they do not probe its response to mechanical force. To determine whether the Pcdh15-EC1 + 2–Cdh23-EC1 + 2 interface is mechanically strong we used steered molecular dynamics (SMD) simulations (Methods and Supplementary Table 3). Force was applied to the carboxy terminus of each protomer to induce complex dissociation (Fig. 3a). In all SMD simulations of Pcdh15-EC1 + 2–Cdh23-EC1 + 2 with Ca$^{2+}$, unbinding was observed without unfolding of repeats. Partial rupture of the binding interface at contacts formed by residues Pcdh15(T106)–Cdh23(L145) and Pcdh15(R84)–Cdh23(N96) was followed by sliding of the $3_{10}$ helix in strand A of Cdh23-EC1 over the Pcdh15 RXGPP loop, and simultaneous rupture of a salt bridge between Pcdh15 Arg 113 and Cdh23 Glu 77 (Fig. 3b, Supplementary Discussion, Supplementary Figs 10 and 11 and Supplementary Videos 1 and 2). Simulations performed using different stretching speeds, initial conditions, and thermodynamic ensembles revealed a similar scenario, with at least one force peak of >400 pN associated with complex unbinding (Fig. 3c, d and Supplementary Figs 10 and 11).

Unbinding forces followed the well-known dependence on stretching speed, with less force required when the stretch was slower. The slowest speed used in our simulations matched the measured velocity of the basilar membrane induced by loud sound as well as speeds of mechanical stimulators used in ex vivo electrophysiological experiments (see Supplementary Discussion). In all our simulations the Pcdh15-EC1 + 2–Cdh23-EC1 + 2 interface was stronger than that of the classical C-cadherin interface pulled under identical conditions (Supplementary Fig. 12). Furthermore, the predicted force required to unbind parallel complexes was almost double that required to unbind a single Pcdh15-EC1 + 2–Cdh23-EC1 + 2 complex (Supplementary Figs 12b, c), which may correspond to the actual force that heterotetrameric tip links can withstand in vivo before rupture due to large mechanical stimuli, such as loud sound.

Figure 3 | Mechanical strength of the Pcdh15-EC1 + 2–Cdh23-EC1 + 2 complex probed by SMD simulations. a, Snapshots of Pcdh15-EC1 + 2 (purple) and Cdh23-EC1 + 2 (blue) unbinding during simulation SNA7 (Supplementary Table 3). The complex is shown in both cartoon and surface representations at the beginning, and in surface representation at indicated time points. Force ($F$) was applied to the C termini of both protomers (Supplementary Videos 1 and 2). Green arrows point to broken interfaces. b, Region of grey box in panel a, showing interacting residues during unbinding. c, Force applied to one C terminus versus distance between C-termini ends of Pcdh15-EC1 + 2 and Cdh23-EC1 + 2. Different traces correspond to independent simulations performed at stretching speeds of 10 (blue and black), 1 (light and dark green), 0.1 (cyan, 1-ns running average shown in black) and 0.02 nm s$^{-1}$ (magenta, 1-ns running average). Snapshots in a are indicated by arrowheads. d, Maximum-force peak values versus stretching speed for unbinding simulations of Pcdh15-EC1 + 2–Cdh23-EC1 + 2 started after a 1-ns or 1-µs equilibration (light green, SN2–SN6; dark green, SN2–SNA7; cyan, SN10–SN13). Simulations SN2–SN6 and SNA2–SNA7 used the Sib structure and SN10–SN13 used S1a; unbinding forces for all three sets were equivalent.
Inherited deafness (PCDH15(D157G), CDH23(D101G) and PCDH15(R113G)), and one mouse mutation that accelerates progressive hearing loss (Cdhr23(S47P)), are located within the crystallized Pcdh15-EC1 + 2-Cdh23-EC1 + 2 complex (Supplementary Figs 1c and 15). Our data provide a structural context to interpret their effect on tip-link function. We also constructed all four mutants to test formation of heterophilic complexes in vitro with ITC and SEC, finding that they each affect the Pcdh15-EC1 + 2-Cdh23-EC1 + 2 complex in different ways. Cdh23-EC1 + 2(D101I), Cdh23-EC1 + 2(S47P) and Pcdh15-EC1 + 2(R113G) refolded well, as assessed by SEC, whereas Pcdh15EC1 + 2(D157G) did not and its analysis was not possible. The D101G and S47P Cdh23-EC1 + 2 fragments crystallized in complex with Pcdh15-EC1 + 2 and show only minor changes in the interface and in binding (Fig. 2c, Supplementary Discussion and Supplementary Fig. 16). On the other hand, Pcdh15-EC1 + 2(R113G) showed impaired binding to Cdh23-EC1 + 2.

Residue Arg 113 of Pcdh15 is of particular interest because it is at the interface between Pcdh15-EC1 + 2 and Cdh23-EC1 + 2 (Fig. 2h). Mutation R113G, causing human non-syndromic deafness DFNB23 (ref. 27), eliminates the long arginine side chain that flanks the hydrophobic core of this interface and disrupts its integrated hydrogen-bond network. SEC of either co-refolded or independently refolded proteins showed no evidence of Pcdh15-EC1 + 2 (R113G)–Cdh23-EC1 + 2 complex formation (Fig. 4b and Supplementary Fig. 2). Furthermore, ITC experiments show impaired binding and indicate an estimated Kd at least an order of magnitude larger than that measured for wild-type Pcdh15-EC1 + 2–Cdh23-EC1 + 2 (>20 μM, Fig. 2c and Supplementary Fig. 8). In other work, R113G impaired binding of full-length Pcdh15 and Cdh23 in vitro, as well as binding of protein fragments to hair-cell tip links ex vivo11. Together, these observations help to validate the interface observed in our crystal structure and indicate that this mutation causes deafness by directly interfering with binding between Pcdh15 and Cdh23. Residual interactions detected in ITC experiments may explain why vestibular function is not affected in human subjects carrying this mutation.

In summary (Supplementary Fig. 1), the Pcdh15-EC1 + 2–Cdh23-EC1 + 2 structure provides the first view of a heterophilic cadherin complex, revealing a novel extended-handshake interface that simulations predict to be mechanically stronger than required to resist forces produced by moderate sound. The structure helps explain the Ca2+ sensitivity of the tip link, and the aetiology of certain inherited deafness conditions. For other cadherins, both the existence of heterophilic cadherin bonds and the possibility of interdigitation have been debated. Although protocadherin 15 and cadherin 23 are rather specialized members of the cadherin family, the overlapping heterophilic complex formed by these molecules suggests structural determinants that could also favour these types of interactions in related members of the cadherin family, such as the Fat3 and Fat4 cadherins that control neuronal morphology and morphogenesis (Supplementary Discussion and Supplementary Figs 18 and 19).

**METHODS SUMMARY**

Wild-type and mutant Cdh23 EC1 and EC1 + EC2 repeats and Pcdh15 EC1 + EC2 repeats were subcloned into a pet21a plasmid, expressed independently in BL21-CodonPlus (DE3)-RIPL Escherichia coli cells, purified under denaturing conditions with nickel-sepharose beads, and then mixed and co-refolded in six steps at 4°C. Refolded proteins were further purified by SEC. Crystals were grown by vapour diffusion, cryoprotected and cryo-cooled in N2. X-ray diffraction data were collected as indicated in Supplementary Tables 1 and 2. Structures were determined by molecular replacement. ITC experiments were carried out using a MicroCal iTC200 calorimeter with buffer-matched samples at 10°C. Analytical SEC was performed on a Superdex200 PC 3.2/30 column on an AKTAmicro. Systems for molecular dynamics simulations were prepared with Visual Molecular Dynamics (VMD). Molecular dynamics simulations were performed using NAMD 2.7 or 2.8 with the CHARMM27 force field, the CMAP correction and the TIP3P water model. Interfaces of complexes were analysed with VMD and the Protein Interfaces, Surfaces and Assemblies (PIA) server.
Full Methods and any associated references are available in the online version of the paper.

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Author Contributions All authors participated in all parts of this study. M.S. did all experiments and simulations.

Author Information Atomic coordinates and structure factors for the reported crystal structures have been deposited with the Protein Data Bank under accession codes 4axp (S1a), 4axw (S1b), 4aq8 (S2), 4aq4 (S3) and 4aqe (S4). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to D.P.C. (dcorey@hms.harvard.edu) or R.G. (gaudet@mcb.harvard.edu).
METHODS

Cloning, expression and purification of Pcdh15 and Cdh23 repeats. Clones of mouse Cdh23 repeats EC1 and EC2 were previously described in ref. 16. Numbering corresponds to mouse cadherin-23 and protocadherin-15 without their signal sequences. Mouse protocadherin-15 EC1+2 comprising residues Q1–D233 (Q27–D259 in mouse NCBI reference sequence NP_001136218.1) was subcloned into the Ndel and XhoI sites of the vector pET21a (C-terminal His-tag). The signal sequence was replaced by a methionine at position 0. The R113G, D157G and J22A mutations in Pcdh15-EC1+2, as well as the L145M and S47P mutations in Cdh23-EC1+2 were generated using the QuikChange Lightning mutagenesis kit (Stratagene). All constructs were verified by DNA sequencing. Pcdh15-EC1 +2, Pcdh15-EC1 +2(R113G), Pcdh15-EC1 +2(D157G), Pcdh15-EC1 +2(I22A), Cdh23-EC1 +2, Cdh23-EC1 +2(D101G), Cdh23-EC1 +2(I22A), and Cdh23-EC1 +2(D101G) and Cdh23-EC1 +2(D157G) were expressed independently in BL21 CodonPlus (DE3)-RIPL (Stratagene) cultured in Luria-Bertani medium and induced at OD600 = 0.6 with 100 µM IPTG at 20 °C for 16 h. Cells were lysed by sonication in denaturing buffer (20 mM HEPES at pH 7.5, 6 M guanidine hydrochloride (GuHCl), 10 mM CaCl2, 20 mM imidazole at pH 7.0). The cleared lysates were loaded onto nickel- sepharose (GE Healthcare) and eluted with denaturing buffer supplemented with 500 mM imidazole. Wild-type and mutant Pcdh15-EC1 +2 protein fragments were reduced by adding 1 mM dithiothreitol and incubating at 37 °C for 30 min. Reduced and denatured samples were mixed (Pcdh15-EC1 +2 and Cdh23-EC1 +2, Pcdh15-EC1 +2(R113G) and Cdh23-EC1 +2, Pcdh15-EC1 +2(I22A) and Cdh23-EC1 +2, Pcdh15-EC1 +2(D101G) and Cdh23-EC1 +2(D101G), Pcdh15-EC1 +2 and Cdh23-EC1 +2(S47P), Pcdh15-EC1 +2 and Cdh23-EC1, Pcdh15-EC1 +2(R113G) and Cdh23-EC1, and Pcdh15-EC1 +2(I22A) and Cdh23-EC1) and co-refolded in six steps at 4 °C using membranes with a molecular weight cutoff of 2,000 (protocol adapted from ref. 31). First, the mixture was dialysed for 24 h against D buffer (20 mM Tris HCl, pH 8.0, 10 mM CaCl2) with a weight cutoff of 2,000 (protocol adapted from ref. 31). First, the mixture was

Analytical SEC. SEC of co-refolded proteins (Pcdh15-EC1 +2–Cdh23-EC1+2, Pcdh15-EC1 +2(R113G)–Cdh23-EC1+2, Pcdh15-EC1 +2(D157G)–Cdh23-EC1+2, Pcdh15-EC1 +2(I22A)–Cdh23-EC1+2, Cdh23-EC1 +2(D101G)–Cdh23-EC1+2, Pcdh15-EC1 +2–Cdh23-EC1+2(S47P), Pcdh15-EC1 +2 and Cdh23-EC1+2, and Pcdh15-EC1 +2(R113G)–Cdh23-EC1+2) was performed on a Superdex75 160/20 column with 20 mM Tris HCl pH 8.0, 300 mM NaCl and 1 mM CaCl2 (Supplementary Fig. 2). Fractions with pure Cdh23-EC1 +2 (excess from Pcdh15-EC1 +2–Cdh23-EC1+2, Pcdh15-EC1 +2–Cdh23-EC1+2(S47P), and Pcdh15-EC1 +2(R113G)–Cdh23-EC1+2) were collected, concentrated (0.5–2 mg/ml), and used for subsequent analytical SEC (Fig. 4a, b) on a Superdex 200 PC 3.2/3.0 column equilibrated with the same buffer (plus 5 mM EGTA when indicated). Experiments were performed at 4 °C using a 10-µl loop and a 50 µl/min flow rate on an AKTAmicro system equipped with a fused silica capillary tubing for collection of 40-µl fractions. EGTA was added to individual or pre-mixed concentrated samples followed by a 1 h mild shaking before SEC.

Simulated systems. The psfgen, solvate, and autoize Visual Molecular Dynamics (VMD)40 plugins were used to build all systems (Supplementary Table 3) as previously reported in ref. 16. Most of the Pcdh15-EC1 +2–Cdh23-EC1 +2 complex simulations used structure S1b (which we determined first), whereas S9 to S13 (which used higher resolution structure S1a; Supplementary Tables 1 and 3) were previously described in ref. 16. Most of the Pcdh15-EC1 +2–Cdh23-EC1 +2 complex simulations used structure S1b (which we determined first), whereas S9 to S13 (which used higher resolution structure S1a; Supplementary Tables 1 and 3). Structures with non-native N- and C-terminal tails were modified back to native sequences. Systems without bound Ca2+ were prepared by replacing Ca2+ atoms with K+.

Molecular dynamics simulations using NAMD. Molecular dynamics simulations were performed using NAMD 2.7 (ref. 38), the CHARMM27 force field for proteins with CMAP correction41,42, and the TIP3P model for water. Simulation parameters were as in ref. 16, except for simulations SNA7 and SNC6, in which a multiple-step scheme was used with electrostatic interactions evaluated every other time step. Parameters for Ca2+ were from ref. 41. Each system was energy-minimized, then equilibrated in the constant number, pressure and temperature (NpT) ensemble, and the resulting state used to perform subsequent Anton or SMD simulations43. All simulations used T = 310 K. Coordinates of all atoms were saved for analysis every picosecond. Constant-velocity stretching simulations used the SMD method and NAMD Tcl Forces interface44,45.

Molecular dynamics simulations using Anton. Anton is a massively parallel special-purpose machine for molecular dynamics simulations46. Systems pre-equilibrated in NAMD (1.1 ns, T = 310 K) were converted to the Anton-compatible Maestro format using the convertNAMDtoMaestro.py script provided by NRBS/PSC. Anton and NAMD simulations used the same force field. Hydrogen atoms were preserved with SHAKE. Restraints were applied to Cα of residues 121, 173 and 205 of Cdh23-EC1+2 to avoid rotation of the complex and contact between periodic images. A multiple-step scheme was used with interactions evaluated every 2.5 fs, except for non-bonded interactions computed every other time step. A set of cutoff radii and parameters for evaluation of electrostatic forces was automatically generated for each simulated system using the guess_anton config script. Simulations were performed in either the constant number, volume, and temperature (NVT) ensemble using the Nose-Hoover thermostat, or the NpT ensemble using the Berendsen thermostat/barostat. Centre of mass motion was removed. Coordinates of all atoms were saved for analysis every 50 (NVT) or 200 (NVT) picoseconds. Anton simulation results are restricted in size (≤120,000 atoms) and cannot incorporate SMD-like forces, hence the use of complementary NAMD simulations.

Analysis tools. The PISA server was used to analyse complex interfaces47 and identify residues shown in Figs 1g and 2d. The VMD ‘measure SASA’ command was used to determine interface area throughout simulations with a probe radius of 1.4 Å. Interface area was defined as the difference in total solvent-accessible surface areas for each isolated protomer and for the complex divided by two. Glycosylation sites were predicted using OGPET (http://ogpet.utep.edu/OGPET/)48. SMD simulations were performed using the NAMD software43. Centre of mass was rigidly restrained in position and mobile in orientation. SMD simulations were analysed using DynDom40. Regression fits to data points of maximum force peaks versus stretching speeds were performed using a logarithmic expression of the form y = a + b log x. Plots and curve fits were prepared using xmgrace. Molecular images in this paper were created with the molecular

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graphics program VMD\textsuperscript{37}, except for Supplementary Fig. 5 which used PyMOL (Schrodinger, LLC).

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