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3 Molecular structure and conformation of stereocilia tip-links elucidated by cryo-electron  
4 tomography

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17     **Abstract**

18           Mechanosensory transduction (MT), the conversion of mechanical stimuli into electrical  
19   signals, underpins hearing and balance and is carried out within hair cells in the inner ear. Hair  
20   cells harbor actin-filled stereocilia, arranged in rows of descending heights, where the tips of  
21   stereocilia are connected to their taller neighbors by a filament composed of protocadherin 15  
22   (PCDH15) and cadherin 23 (CDH23), deemed the ‘tip-link’. Tension exerted on the tip-link  
23   opens an ion channel at the tip of the shorter stereocilia, thus converting mechanical force into an  
24   electrical signal. While biochemical and structural studies have provided insights into the  
25   molecular composition and structure of isolated portions of the tip-link, the architecture, location  
26   and conformational states of intact tip-links, on stereocilia, remains unknown. Here we report *in*  
27   *situ* cryo-electron microscopy imaging of the tip-link in mouse stereocilia. We observe  
28   individual PCDH15 molecules at the tip and shaft of stereocilia and determine their  
29   stoichiometry, conformational heterogeneity, and their complexes with CDH23. The  
30   PCDH15/CDH23 complexes occur in clusters, frequently with more than one copy of PCDH15  
31   at the tip of stereocilia, suggesting that tip-links might consist of more than one copy of the  
32   PCDH15/CDH23 heterotetramer and by extension, might include multiple MT complexes.

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### 35      **Introduction**

36            Vertebrates sense sound, movement and balance using specialized sensory neurons,  
37            called hair cells (McPherson, 2018). In mammals, hair cells are found in the inner ear and are  
38            organized in several specialized organs. Examples include the cochlea, which senses sound, and  
39            the utricle, which contributes to balance sensation (Ekdale, 2016). Hair cells harbor sensory  
40            microvilli at their apical surface, which are called stereocilia. Stereocilia are rigid, due to being  
41            filled with crosslinked actin filaments (Tilney et al., 1992), and assemble in a staircase pattern in  
42            rows of descending height (Pickles et al., 1984). Upon deflection of the stereocilia staircase,  
43            tension is exerted on a filament connecting the tip of stereocilia with their taller neighbor, called  
44            the tip-link (Pickles et al., 1984). This tension then causes the opening of a channel, called the  
45            mechanosensory transduction (MT) channel, at the base of the tip-link (Zheng and Holt, 2021).  
46            The molecular mechanism of MT channel function, the conversion from mechanical to electrical  
47            signal in the inner ear, remains poorly understood.

48            The molecular nature of components of the MT machinery have been recently elucidated.  
49            The tip-link consists of the two-noncanonical cadherins protocadherin 15 (PCDH15) and  
50            cadherin 23 (CDH23) (Zheng and Holt, 2021). PCDH15 is situated at the tip of the shorter  
51            stereocilia and its two N-terminal cadherin domains bind to the two N-terminal cadherin domains  
52            of CDH23 (Kazmierczak et al., 2007; Sotomayor et al., 2012). The MT channel is likely formed  
53            by the transmembrane-channel like (TMC) protein 1 or 2 and the transmembrane protein of the  
54            inner ear (TMIE) (Zheng and Holt, 2021). PCDH15 assembles in the membrane together with  
55            lipoma HMGIC Fusion Partner-Like 5 (LHFPL5,TMHS) (Ge et al., 2018, p. 5) and pull-down  
56            studies using protein fragments suggest that PCDH15 may also interact with TMC-1 (Maeda et  
57            al., 2014). PCDH15 is also theorized to interact with the cytoskeleton via whirlin (Michel et al.,

58 2020), while TMC may be coupled to the cytoskeleton via CIB2 and ankyrin repeats (Tang et al.,  
59 2020). However, the molecular composition of the MT machinery and the 3D arrangements of  
60 each component remain unknown.

61 The fundamental transduction activity of the MT machinery is the conversion of force  
62 into an electrical signal (Hudspeth, 1989). Indeed, the mechanical displacement of the stereocilia  
63 can be modeled using Hooke's Law, suggesting the presence of an elastic element, or a 'gating  
64 spring', that couples displacement to the ion channel opening (Howard and Hudspeth, 1987). On  
65 the one hand, elasticity measurements of PCDH15 suggest that the tip-link itself acts as the  
66 gating-spring (Bartsch et al., 2019), while on the other hand the plasma membrane at the tip of  
67 stereocilia, as well as elements that couple the tip-link to the cytoskeleton, are other candidates to  
68 act as 'elastic elements' (Powers et al., 2012). Therefore, insights into the molecular structure  
69 and dynamics of the tip-link are essential for understanding mechanosensory transduction.

70 The molecular unit of the tip-link is a heterotetramer of PCDH15 and CDH23, where  
71 both cadherins form a parallel dimer (Dionne et al., 2018) and come together as a dimer-of-  
72 dimers using an anti-parallel 'handshake' of EC-1 and 2 of both cadherins (Sotomayor et al.,  
73 2012). The extracellular domains of both PCDH15 and CDH23 are a chain of cadherin domains  
74 with 11 and 27 repeats, respectively. Canonical cadherin domains form a 'stiff' dimer in the  
75 presence of calcium, due to stabilizing calcium binding sites in the linker regions between  
76 cadherin domains (Marquis and Hudspeth, 1997). Both PCDH15 and CDH23 have non-  
77 canonical linkers devoid of canonical calcium binding sites, thus likely promoting  
78 conformational mobility (Araya-Secchi et al., 2016; Jaiganesh et al., 2018; Powers et al., 2017).  
79 Notably, the linker between EC9 and EC10 of PCDH15 is flexible *in vitro*, allowing a bend of up  
80 to 90° (Araya-Secchi et al., 2016; Ge et al., 2018). Bending and extension along this linker has

81 been suggested as underlying the elasticity of the gating spring (Araya-Secchi et al., 2016), yet  
82 atomic force microscopy measurements and molecular dynamics simulations of PCDH15 also  
83 have suggested that individual cadherin domains can unfold to give rise to tip-link extension  
84 (De-la-Torre et al., 2018; Oroz et al., 2019). However, it is unclear which of these mechanisms  
85 occur *in situ*.

86 The majority of our current knowledge about the assembly of the tip-link *in situ* is based  
87 on fixed and stained scanning-electron microscopy (SEM) or freeze-fracture transmission  
88 electron microscopy (TEM) of stereocilia (Kachar et al., 2000; Michel et al., 2005). These  
89 images reveal a 120-170 nm long, helically coiled, filament with a diameter of 5 nm and a repeat  
90 of 40 nm (Kachar et al., 2000). Furthermore, these links appear to bifurcate at the upper and  
91 lower insertion sites into 2-3 individual strands, an observation that is difficult to reconcile in  
92 light of high resolution structural data showing that PCDH15 and CDH23 are parallel dimers and  
93 that both proteins also harbor membrane-proximal ‘dimerization domains’ (De-la-Torre et al.,  
94 2018; Dionne et al., 2018; Ge et al., 2018). This may be because the imaging methods relied on  
95 fixation and staining procedures, which are manipulations that can introduce artifacts or  
96 distortions.

97 More recently, cryo-electron microscopy imaging has been used to image stereocilia in  
98 their native state, avoiding these artifacts (Metlagel et al., 2019; Song et al., 2020). However, due  
99 to rapid damage of the specimen by high-energy electrons, the contrast of this imaging modality  
100 is low, making it challenging to visually identify relatively small features, such as the tip-link.  
101 Therefore, labeling approaches are required to unambiguously identify components of the MT  
102 machinery. In this study, we combine a stereocilia preparation technique for cryo-EM imaging  
103 (Metlagel et al., 2019; Song et al., 2020) with a highly specific, immuno-affinity labeling

104 approach using anti-PCDH15 antibody-coupled gold nanoparticles (AuNP) (Azubel et al., 2019)  
105 to elucidate the molecular structures and conformational states of tip-links, *in situ*, under native  
106 conditions.

107 **Results**

108 **Cryo-CLEM imaging of immunolabeled stereocilia**

109 To develop tools to identify PCDH15 in cryo-electron tomograms, we raised polyclonal  
110 antibodies (pAbs) against the entire extracellular region of mouse PCDH15. Immunostaining of  
111 mouse cochlea using these anti PCDH15 pAbs produces punctate features at the tips of  
112 stereocilia in hair cells derived from wild-type (WT) mice, features that are entirely absent from  
113 the stereocilia of PCDH15 knock-out mice, thus demonstrating the specificity and utility of the  
114 anti-PCDH15 pAbs (Figure 1A). We observed similar staining of stereocilia derived from mouse  
115 utricles, both in the presence of calcium and after chelation of calcium using BAPTA, indicating  
116 that the binding was independent of calcium and thus, also independent of the PCDH15-CDH23  
117 ‘handshake’ (Figure 1B). To facilitate the collection of a large dataset of tomograms focused on  
118 the tip-region of stereocilia, we adopted a stereocilia preparation technique in which the sensory  
119 epithelium is touched to holey carbon support film cryo-EM grids treated with poly-lysine, thus  
120 enabling the deposition of stereocilia onto the grid (Metlagel et al., 2019). We subsequently  
121 identified stereocilia by staining the sample with an actin dye and imaging with cryo-light  
122 fluorescence microscopy (Figure 1C), allowing us to estimate the number of squares with a  
123 favorable number of stereocilia and appropriate ice thickness. We then collected tomograms in  
124 these squares, focusing on positions where the tips of stereocilia coincided with holes in the  
125 holey carbon film (Figure 1C).

126 In initial experiments, we labeled stereocilia with the anti-PCDH15 pAbs using  
127 secondary antibodies conjugated to 5 nm gold particles. In the resulting images, we clearly  
128 visualized clusters of 5 nm gold particles in the images of some of the tips (Figure 1D). While  
129 this validated our immuno-labeling approach, we were unable to determine the number of  
130 PCDH15 molecules present due to the undefined stoichiometry of both the primary and  
131 secondary reagent. Furthermore, the gold particles obstructed the direct observation of the  
132 PCDH15 electron density.

133 **Preparation of a PCDH15 gold-labeling reagent with 1:1 stoichiometry**

134 To specifically label PCDH15 subunits with single gold particles, we developed a high  
135 affinity, slow off-rate ( $k_{off} = 4.6 \times 10^{-5} \text{ 1/s}$ ), anti-PCDH15 monoclonal antibody (mAb), deemed  
136 39G7. By examining the binding of 39G7 to a series of PCDH15 truncation constructs, we  
137 determined that the mAb binds to the EC3 cadherin domain, near the amino terminus (Figure  
138 2A). Similar to the anti-PCDH15 pAbs, the 39G7 mAb stained PCDH15 on the surface of  
139 vestibular stereocilia, either in the presence or absence of calcium (Figure 2B).

140 To create a reagent to label PCDH15 subunits with a single gold particle, we created a  
141 39G7 Fab construct with a single free cysteine residue at the C terminus of the heavy chain, thus  
142 enabling conjugation of the Fab to 3 nm AuNPs (Azubel et al., 2019, p. 2). Purification of the  
143 39G7 Fab – AuNP complex via polyacrylamide electrophoresis and size exclusion  
144 chromatography (SEC) yielded a homogeneous species in which the Fab was a labeled with a  
145 single AuNP (Figure 2C+D), devoid of non AuNP-labeled Fabs. Using this reagent, we reasoned  
146 that we could directly count PCDH15 subunits because only a single Fab will bind to each  
147 PCDH15 subunit and each Fab is labeled by a single AuNP. We confirmed this prediction by  
148 forming a complex between the 39G7 Fab – AuNP and recombinant PCDH15, isolating the

149 complex by SEC (Figure 2E) and imaging the complex by single particle cryo-EM (Figure 2F).

150 On micrographs and tomographic reconstructions (Movie S1), we clearly identified pairs of

151 AuNPs bound to the PCDH15 extracellular domain, consistent with a dimeric model of the

152 PCDH15 extracellular domain, the 39G7 Fab binding to EC3 and the ability of the Fab – AuNP

153 complex to allow us to identify and count PCDH15 subunits.

154 **39G7-AuNP labeling of stereocilia shows that PCDH15 is a dimer**

155 We prepared cryo-EM grids using utricles stained with 39G7 Fab-AuNP and collected

156 tomograms, primarily of stereocilia tips (Figure 3E). On approximately half of the tomograms

157 we identified 3 nm gold particles within ~38 nm of the stereocilia membrane, a distance that is

158 consistent with the binding of 39G7 to ECD3 of PCDH15. We also identified AuNP labels in

159 tomograms of the stereocilia shaft region, but only in about a third of the tomograms that

160 featured only stereocilia shaft regions. Close inspection of the electron density around the AuNPs

161 frequently revealed ~50 nm long filamentous density consistent with the extracellular domain of

162 PCDH15 (Figure 3A-B, Movie S2-3). In tomograms with relatively thin ice (<200 nm) and an

163 appropriate orientation of the filament to the tilt-axis, dimeric features of the PCDH15 domain

164 are evident that, together with the presence of the AuNP pairs with a distance of 20 nm,

165 demonstrates that PCDH15 forms a dimer *in situ*. In some cases, we observed individual gold

166 particles instead of a pair of AuNPs (Figure 3C-D, Movie S4-5) yet also dimeric features

167 associated with PCDH15 chain, thus indicating that the visualization of individual AuNPs was

168 likely due to incomplete labeling of PCDH15 and not the presence of PCDH15 monomers. We

169 quantified the occurrence of AuNP dimers and monomers (Figure 3F) and found a ratio of 3:1.

170 Under the assumption that bindings of the 39G7 Fab to either protomer are independent events,

171 we can use this ratio to estimate the fraction of PCDH15 protomers labeled with 39G7-AuNP

172 (0.86) and the fraction of PCDH15 dimers that are completely unlabeled (0.02). While this may  
173 be an underestimation of the number of unlabeled PCDH15 molecules, either due to  
174 cooperativity of 39G7 binding or due to air-water interface effects, it nevertheless demonstrates  
175 that the 39G7 Fab-AuNP robustly labels PCDH15 *in situ* and therefore allows precise  
176 quantification of the number of PCDH15 molecules at the tips of stereocilia.

177 **Stereocilia tips harbor multiple copies of PCDH15**

178 We carefully quantified the number of PCDH15 molecules in all imaged stereocilia tips  
179 (n=396) (Figure 4F). Slightly more than half of the stereocilia tips (58%) did not contain a  
180 PCDH15 label. While this could be due to an underestimate of the labeling efficiency or due to  
181 preferential selection of stereocilia in the tallest row, we believe the most likely explanation is  
182 damage to stereocilia tips during the blotting of stereocilia on the cryo-EM grids. In some cases  
183 (5.8%), we found PCDH15 molecules in the shaft region just ‘below’ the stereocilia tips (Figure  
184 4A, Movie S6). It is possible that these molecules were initially located on the tip, but diffused  
185 away in the time between applying tissue to the grid and plunge-freezing. In 13% of tips we  
186 found a single molecule of PCDH15 at the tip (Figure 4B+C, Movie S7-8), similar to commonly  
187 depicted models of the MT complex. However, in almost twice the number of stereocilia tips  
188 (23%) we found multiple copies of PCDH15 at the tip, either clustered at the tip (Figure 4D,  
189 Movie S9) or spread across its surface (Figure 4E, Movie S10). Because we cannot determine  
190 that maturity of the hair cells from which the stereocilia are derived, multiple PCDH15  
191 molecules at the tips may be due to stereocilia derived from immature hair cells. Indeed, many  
192 molecular models of the MT machinery posit the presence of one copy of the PCDH15 dimer  
193 bound to a single MT-channel. Our data, however, suggests that many tips might harbor multiple

194 copies of the MT machinery, which in turn might underly variation in the ion channel  
195 conductance at individual tips (Beurg et al., 2018).

196 **Tomographic reconstructions of PCDH15-CDH23 heterotetramers**

197 In a few tomograms, we found electron-density extending beyond the tip of PCDH15,  
198 consistent with a 120 nm long filament inserted into two distinct membranes (Figure 5). While  
199 we cannot determine the molecular identity of this density unambiguously, the dimensions of this  
200 molecule suggest that the density corresponds to CDH23. In one example, we found a stereocilia  
201 tip with 5 copies of a PCDH15 dimer, 3 of which were connected to a putative CDH23 density,  
202 which in turn was inserted into a small spherical liposome (Figure 5A, Movie S11). Our  
203 interpretation of this structure is that this was a tip-link assembly, disrupted during sample  
204 preparation, where a portion of the membrane surrounding CDH23 was ‘torn off’ of the  
205 neighboring stereocilia, yielding CDH23 bound to a liposome. In another case, a cluster of five  
206 PCDH15 molecules close to a stereocilium tip is connected to four CDH23 densities inserted into  
207 a lipid membrane fragment (Figure 5B, Movie S12).

208 We also observed structures in which it appears as though PCDH15, together with a fraction of  
209 surrounding membrane, were extracted from the shorter stereocilium (Figure 5C+D, Movie S13-  
210 14). In both cases we found 3-5 copies of PCDH15 and CDH23. The fact that we observed most  
211 PCDH15/CDH23 complexes in structures that appear to be the result of partial damage of the  
212 tip-link, is consistent with the earlier interpretation of the missing PCDH15 label is due to  
213 damaged tip-links. It is also possible that clusters of PCDH15/CDH23 complexes are more stable  
214 than isolated PCDH15/CDH23 complexes, and thus more frequently observed.

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217 **An intact tip-link surrounded by non CDH23-bound PCDH15 molecules**

218 In one tomogram, we imaged the tip of a stereocilium situated next to a longer  
219 stereocilium (Figure 6A, Movie S15). While we observed 17 AuNPs at the tip of the shorter  
220 stereocilium, we only unambiguously identified the density for four PCDH15 molecules. On one  
221 of these PCDH15 molecules we observed a 120 nm long filament, connecting PCDH15 to the  
222 longer stereocilium (Figure 6B). We hypothesize that this is most likely an intact tip-link. Upon  
223 closer inspection (Figure 6C), we can clearly identify dimeric features on this PCDH15  
224 molecule. Other adjacent PCDH15 molecules are also clearly dimeric entities (Figure 6D), yet  
225 are not bound to CDH23. Further down the two stereocilia we identified another copy of  
226 PCDH15, apparently connected via CDH23 to the other stereocilium (Figure 6E). In this case  
227 PCDH15 appears to be bent, by about 90°, at juncture of the EC9-EC10 cadherin domains, a  
228 ‘joint’ between cadherin domains that has previously been identified as flexible in cryo-EM and  
229 crystallography structures of PCDH15 extracellular domain constructs (Araya-Secchi et al.,  
230 2016; Ge et al., 2018). We speculate that this link may be in the process of being trafficked to the  
231 tip and that the EC9-EC10 bend helps to accommodate the long filament within the relatively  
232 narrow space between stereocilia.

233 **Lateral links consisting of PCDH15/CDH23**

234 While in the majority of tomograms we identified 10 or fewer copies of PCDH15, several  
235 tomograms exhibit numbers of labels consistent with hundreds of copies of PCDH15. In the most  
236 striking example (Figure 7A, Movie S16), we observed these labels between stereocilia that  
237 appear thinner and less densely packed with actin than other stereocilia. In this tomogram we  
238 counted 309 AuNPs and modeled 56 PCDH15 molecules in cases where we observed the  
239 corresponding electron density. Many of the PCDH15 molecules are involved in links to

240 neighboring stereocilia, via a 120 nm filament, which again is likely CDH23. We observed  
241 density for 20 putative CDH23 molecules, molecules which likely participate in the ‘lateral  
242 links’ which form early in development of stereocilia bundles and are composed of PCDH15 and  
243 CDH23 (Michel et al., 2005). Inspection of the densities shows that multiple copies of CDH23  
244 cluster together, primarily via the N-terminal half of CDH23, so as to obscure identification of  
245 individual stands (Figure 7B+C). We speculate that these clusters might have given rise to the  
246 appearance of single tip-links with multiple upper and lower insertion points in stained EM  
247 images. Furthermore, we find examples of CDH23 that appear bent half-way between the N- and  
248 C-termini, indicating that CDH23 might also have a flexible cadherin domain ‘joint’, similar to  
249 the EC9-EC10 interface of PCDH15 (Figure 7D). A potential candidate for this joint is the non-  
250 canonical linker between EC12 and EC13 of CDH23 (Jaiganesh et al., 2018).

251 **Discussion**

252 The combination of cryo-EM and AuNP labeling provides a molecular resolution view of  
253 the structure, stoichiometry, and organization of tip-links in their native environment. This work  
254 is the first look at native tip-links *in situ*, as previous studies relied on microscopy of fixed and  
255 stained specimens, which have an inherently lower resolution and are prone to artifacts  
256 (Indzhykulian et al., 2013; Kachar et al., 2000; Michel et al., 2005).

257 We observe clusters of PCDH15-CDH23 complexes on the stereocilia tip and shaft, as  
258 well as multiple copies of PCDH15 at stereocilia tips, ranging from two to more than five.  
259 Because the majority of the PCDH15 molecules we observed were not bound to CDH23 to form  
260 an intact tip-link, the mechanistic underpinnings of multiple PCDH15 molecules, if any, are  
261 unclear. One possibility is that each PCDH15 molecule was coupled to a functional MT channel  
262 and the tip-link was ruptured during cryo-EM grid preparation. This would imply that there are

263 multiple MT complexes per stereocilia tip, as suggested previously (Beurg et al., 2018).  
264 Confocal microscopy images of stereocilia expressing mCherry-tagged TMC1 indicate that there  
265 are an average of 7.1 TMC1 channels per stereocilia tip derived from the inner hair cells of P4  
266 aged mice. The number of TMC1 per stereocilia tip varies from four to twenty depending on the  
267 cochlear localization, mirroring the high degree of variability we observed for PCDH15.

268 It is also possible that a large number of PCDH15 molecules may be necessary for  
269 rebinding to CDH23 in the event of tip-link breakage. Atomic force microscopy experiments  
270 indicate that the lifetime of the PCDH15-CDH23 bond is only ~8 seconds at resting tension,  
271 suggesting that the tip-link is a highly dynamic connection (Mulhall et al., 2021). A pool of  
272 nearby PCDH15 molecules may enable fast recovery after tip-links are broken. It has also been  
273 suggested that intermediate PCDH15-PCDH15 tips form first during tip-link regeneration,  
274 followed by mature PCDH15-CDH23 tip-links, necessitating the presence of multiple PCDH15  
275 molecules (Indzhykulian et al., 2013).

276 Our tomograms also revealed distinct structural features of PCDH15 and CDH23. Most  
277 intact tip-links were approximately straight, ~120 nm length PCDH15-CDH23 heterotetramers  
278 consisting of a PCDH15 dimer joined to a CDH23 dimer. However, in several tip-links, we  
279 observed a ~90° bend in PCDH15 or CDH23. PCDH15 is bent at approximately the EC9-EC10  
280 interface, which has been identified as flexible in cryo-EM and crystal structures of recombinant  
281 PCDH15, while CDH23 appears to be bent near EC13. Crystal structures of CDH23 identified  
282 non-canonical linker regions and altered  $\text{Ca}^{2+}$  binding sites within this region, motifs that could  
283 confer increased flexibility (Jaiganesh et al., 2018). Furthermore, we often observe a ‘splitting’  
284 of the CDH23 dimer at various points, most frequently at the C-terminal end. This type of  
285 structural heterogeneity is not observed for PCDH15. Crystallography studies of CDH23 have

286 noted a curious lack of multimerization interfaces between CDH23 protomers, consistent with  
287 our observations, yet a rationale for minimal interchain CDH23 interactions remains to be  
288 determined (Jaiganesh et al., 2018), other than to reduce likelihood of CDH23 aggregation.

289 In selected stereocilia, we note that the high densities of lateral PCDH15-CDH23 links  
290 are consistent with our capture of an immature hair bundle. While the utricles employed in our  
291 study were isolated from P6-9 mice, lateral links are still present until approximately P9  
292 (Goodyear et al., 2005). These extensive lateral links stabilize the stereocilia during the early  
293 stages of development by acting as a cohesive tethers, gradually being pruned until only the tip-  
294 link remains in a mature hair bundle (Boëda et al., 2002; Michel et al., 2005).

295 Our work highlights the power of gold immunolabeling and cryo-EM to study rare  
296 protein complexes. The MT complex is notorious for its low abundance, conspiring to make  
297 studies of the mechanism of MT channel gating by tip-link tension challenging. The images  
298 presented here reveal structural features of the MT machinery and demonstrate that the employed  
299 techniques can be used to visualize single molecules in their native environment. It will be  
300 exciting to use this technique to explore additional components of the MT complex, including  
301 TMC1 and TMIE, in order to define their locations, stoichiometries and structures within the  
302 architecture of the MT machinery.

303 **Methods**

304 **PCDH15 EC1-EL expression and purification**

305 A gene encoding the amino acid sequence of the mouse PCDH15 extracellular region  
306 (Uniprot entry Q99PJ1), from the first cadherin domain (EC1) to the membrane-proximal ‘EL’  
307 domain (PCDH15 EC1-EL), was synthesized and cloned into a pBacMam vector (Goehring et  
308 al., 2014), and included a C-terminal yellow fluorescent protein (YFP) fluorophore followed by a

309 polyhistidine tag. This construct was used to generate baculovirus, which was then employed to  
310 infect HEK293 cells as previously described (Goehring et al., 2014). Approximately 96 hours  
311 after viral transduction, the cell medium was harvested and the secreted PCDH15 EC1-EL  
312 protein was isolated by metal ion affinity chromatography, and further purified by size exclusion  
313 chromatography (SEC) in TBS Buffer (20 mM Tris pH8, 150 mM NaCl). The final material was  
314 concentrated to ~ 2mg/ml, aliquoted and stored at -80 °C.

315 **Antibody generation**

316 Rabbit polyclonal and monoclonal antibodies were generated using standard techniques  
317 by Genscript using the soluble PCDH15 EC1-EL extracellular region as the antigen. Polyclonal  
318 serum was used to isolate antibodies using affinity-purification with the PCDH15 EC1-EL  
319 extracellular domain. Hybridoma supernatants were screened against the PCDH15 EC1-EL  
320 antigen in the presence and absence of 1 mM calcium to identify clones that recognize the  
321 calcium-bound and apo forms of the protein. Supernatants that tested positive by ELISA were  
322 further screened by fluorescence detection chromatography (FSEC) and Western blot and the  
323 clone 39G7 was sequenced and monoclonal antibody was produced recombinantly.

324 **Immunofluorescence**

325 Cochlea and utricles were dissected from mice at ages P6-9 in DMEM/F12 media  
326 (Gibco). The tissue was incubated for 30 minutes in 10 ug/ml of indicated antibody in  
327 DMEM/F12 media, followed by washing, 3 times for 10 minutes each, in the same media.  
328 Where indicated, 5 mM BAPTA was included in staining and washing media to chelate calcium.  
329 The tissue was next fixed with in a buffer composed of 4% paraformaldehyde in phosphate-  
330 buffered saline (PBS) for 10 minutes. After 3 washes in PBS, the tissue was permeabilized and  
331 blocked using PBS with 0.1% Triton X-100, 5% bovine serum albumin (BSA), and 10%

332 standard goat serum. Subsequently, the tissue was stained with goat-anti-rabbit antibodies fused  
333 to Alexa-594 and phalloidin fused to Alexa-405. After 3 washes with PBS, the tissue was  
334 mounted with Vectashield mounting media and imaged using a Zeiss LSM 980 confocal  
335 microscope using a 63x/1.49 NA objective.

336 **AuNP generation**

337 A solution of 84 mM 3-mercaptopbenzoic acid (3-MBA) in methanol was mixed with a 28  
338 mM solution of HAuCl<sub>4</sub> in methanol at 7:1 molar ratio, followed by 2.5 volumes of water. The  
339 pH was adjusted by adding concentrated aqueous NaOH to a final concentration of 100 mM.  
340 This solution was mixed by end-over-end rotation for at least 16 hours. Afterwards, the solution  
341 was diluted with 27% methanol to achieve a final concentration of 2.5 mM 3-MBA. NaBH<sub>4</sub> was  
342 added to a final concentration of 2 mM using a fresh 150 mM stock solution prepared in 10 mM  
343 NaOH. After mixing for 4.5 hours, the gold particles were precipitated by adjusting the NaCl  
344 concentration to 100 mM and by adding methanol to a final concentration of 70%. Gold AuNPs  
345 were pelleted by centrifugation at 5,000 rpm for 20 minutes and washed with 70% methanol. The  
346 pellet was dried overnight in a desiccator and re-suspended in water.

347 An expression construct for a Fab' fragment of 39G7 was designed using a dual-promoter  
348 Sf9-expression plasmid (Vectorbuilder). For the 39G7 light chain, we replaced the native signal  
349 peptide with the GP64 signal peptide and inserted the coding sequence downstream of the pH  
350 promoter. For the 39G7 heavy chain, we replaced the native signal peptide with the GP64 signal  
351 peptide and truncated the coding sequence after G256, thereby removing the Fc fragment but  
352 retaining C243 and C248 at the C-terminus. The coding sequenced was then inserted after the  
353 P10 promoter and fused with a histidine tag at the C-terminus. The 39G7 Fab' was expressed in  
354 Sf9 cells for 96 hours at 27 °C. The media was adjusted to pH 8, cleared by centrifugation and

355 then concentrated to about 100 ml using a tangential-flow concentrator. The concentrated media  
356 was pumped over a 5 ml metal ion affinity column, equilibrated with PBS. The column was  
357 washed extensively with PBS supplemented with 30 mM imidazole and Fab' was eluted with  
358 PBS supplemented with 500 mM imidazole. The yield was 2 mg of Fab' per liter of culture. The  
359 pooled fractions were concentrated to 5 mg/ml and aliquots were plunge-frozen in liquid  
360 nitrogen and stored at -80 °C.

361 To conjugate the Fab to the AuNPs, an aliquot of 39G7 Fab' was thawed and incubated  
362 with 2 mM tris (2-carboxyethyl) phosphine hydrochloride (TCEP) for 1 hour at 37 °C. After  
363 clarification by ultracentrifugation, the 39G7 Fab was applied to a SEC column equilibrated with  
364 TBE buffer (100 mM Tris, 100mM boric acid, 2mM EDTA). The peak fractions were  
365 concentrated immediately to 2 mg/ml and a test conjugation was set up at different molar ratios  
366 of Fab' and AuNP (4:1,2:1,1:1,1:2,1:4). After a 30-minute incubation at 37 °C, the reactions were  
367 analyzed on a 12.5% PAGE gel made with TBE and 10% glycerol. The condition with the  
368 highest yield of the 1:1 AuNP/Fab complex was chosen for large scale conjugation. To coat the  
369 AuNPs with PEG, the conjugate was immediately separated on a PAGE gel following and 1:1  
370 39G7:AuNP recovered in TB buffer. After concentration, 1 mM of mPEG550-SH was added and  
371 incubated for 60 minutes at 37 °C. The conjugate was then purified by SEC using a Superose 6  
372 column equilibrated with PBS. Fractions corresponding to 39G7-AuNP conjugate were pooled,  
373 concentrated to 1  $\mu$ M (assuming an extinction coefficient of  $3 \times 10^6$  ( $M^{-1} cm^{-1}$ ) at 500 nm) and  
374 stored at 4 °C.

375 **Grid preparation**

376 Utricles were dissected from P8-P11 mice in DMEM/F12 buffer. Otoconia were removed using  
377 an eyelash. Utricles were incubated for 30 minutes in DME M/F12 containing 100 nM 39G7-  
378 AuNP and 500 nM SirActin and then washed three times for 5 minutes in DMEM/F12. Utricles  
379 were then placed stereocilia-side down on C-Flat 200 mesh copper grids with a 2/1 spacing  
380 carbon film that were pretreated with 0.1 mg/ml poly-D-lysine and suspended in 20 ul drops of  
381 DMEM/F12. After 2-3 seconds, the utricle was removed and placed on another region of the grid  
382 for up to three times. The grid was removed ‘edge-first’ from the drop and excess liquid was  
383 removed by touching the edge to a piece of Whatman No. 40 filter paper. A 2.5 ul drop of  
384 DMEM/F12 containing 0.05% fluorinated octyl-maltoside and 10 nm highly uniform gold  
385 fiducials at an OD<sub>500</sub> of roughly 5.0 were added to the grid. The grid was then placed on a  
386 manual blotting apparatus and excess solution was removed by placing a 595 filter paper (TED-  
387 Pella) for 6-10 seconds to the side of the grid without sample. Afterwards, the grid was rapidly  
388 plunged into a mixture of ethane and propane cooled to liquid nitrogen temperature. Grids were  
389 imaged in a CMS196 cryostage (Linkam) on a LSM880 confocal microscope (Zeiss) using the  
390 AiryScan detector. Grids that did not exhibit SirActin fluorescence in multiple squares, or had  
391 excessive damage to the carbon support, were discarded.**Tilt-series acquisition**

392 Tilt-series of the recombinant PCDH15 ECD1-EL extracellular region labeled with the  
393 9G7-AuNP was acquired using a Thermo Fisher Arctica microscope operated at 200 keV,  
394 without an energy filter, on a Gatan K3 detector. Tilt-series of stereocilia were obtained on a  
395 Thermo Fisher Krios microscope operated at 300 keV with an energy filter on a Gatan K3  
396 detector. A subset of tomograms were obtained from a Krios microscope also equipped with a  
397 spherical aberration corrector.

398 In all cases, tilt-series were obtained from -60 to 60 degrees using a 3-degree interval  
399 using SerialEM. Data was either acquired by sweeping from -30 to 60 degrees and then from -30  
400 to -60 degrees or by using a grouped dose symmetric scheme. In all cases the total electron dose  
401 was 80-120 e-/Å<sup>2</sup>. Defocus was varied between -2.5 and -4.0 um.

402 **Tomography processing**

403 Tomograms were reconstructed using the IMOD program (Mastronarde and Held, 2017),  
404 employing the 10 nm gold fiducials for alignment. In some cases, the 3 nm AuNP label was also  
405 included as a fiducial. The tilt series was binned by 4 to a final pixel size of 6.6 Å, CTF  
406 corrected, the 10 nm gold particles were subtracted, and the tilt-series was dose-weighted.  
407 Tomograms were reconstructed using a SIRT-like filter with 8 iterations. In cases where a  
408 substantial amount of sample deformation was observed, tomograms were reconstructed using  
409 the tomoalign program (Fernandez et al., 2019), using polynomials and the ‘thick’ preset. If  
410 indicated, tomograms were denoised using the denoise3d model of Topaz (Bepler et al., 2020).

411 **Tomogram annotation**

412 Membranes were annotated manually in XY slices using the 3dmod program (Kremer et  
413 al., 1996). Actin filaments were manually annotated in 26.4 nm thick slices perpendicular to the  
414 stereocilium length. AuNP nanoparticles were either manually annotated or annotated using the  
415 findbeads3d program of the IMOD suite. PCDH15 was positioned by identifying the membrane  
416 insertion point and the PCDH15 tip in the ‘Slicer’ windows of the 3dmod program. The density  
417 of the PCDH15 model was then aligned along these two points and, in some cases, was rotated  
418 until the projection matched the density visible in the slicer window. Actin filaments were  
419 manually annotated in slices perpendicular to the stereocilia axis using 3dmod. A low-pass  
420 filtered electron-density of actin was placed along the annotated filaments using the

421 clonevolumes program. The final render of each model was performed using UCSF ChimeraX  
422 (Pettersen et al., 2021).

423 **Data availability**

424 Tomograms depicted in the figures have been deposited in the EMDB under accession  
425 codes EMD-25046, EMD-25047, EMD-25048, EMD-25049, EMD-25050, EMD-25051, EMD-  
426 25052, EMD-25053, EMD-25054, EMD-25055, EMD-25056, EMD-25057, EMD-25058, EMD-  
427 25059, EMD-25060, and EMD-25061 (in order of appearance). The corresponding tilt-series  
428 have been deposited in EMPIAR under accession code EMPIAR-10820. Other raw data is  
429 available from the authors upon request.

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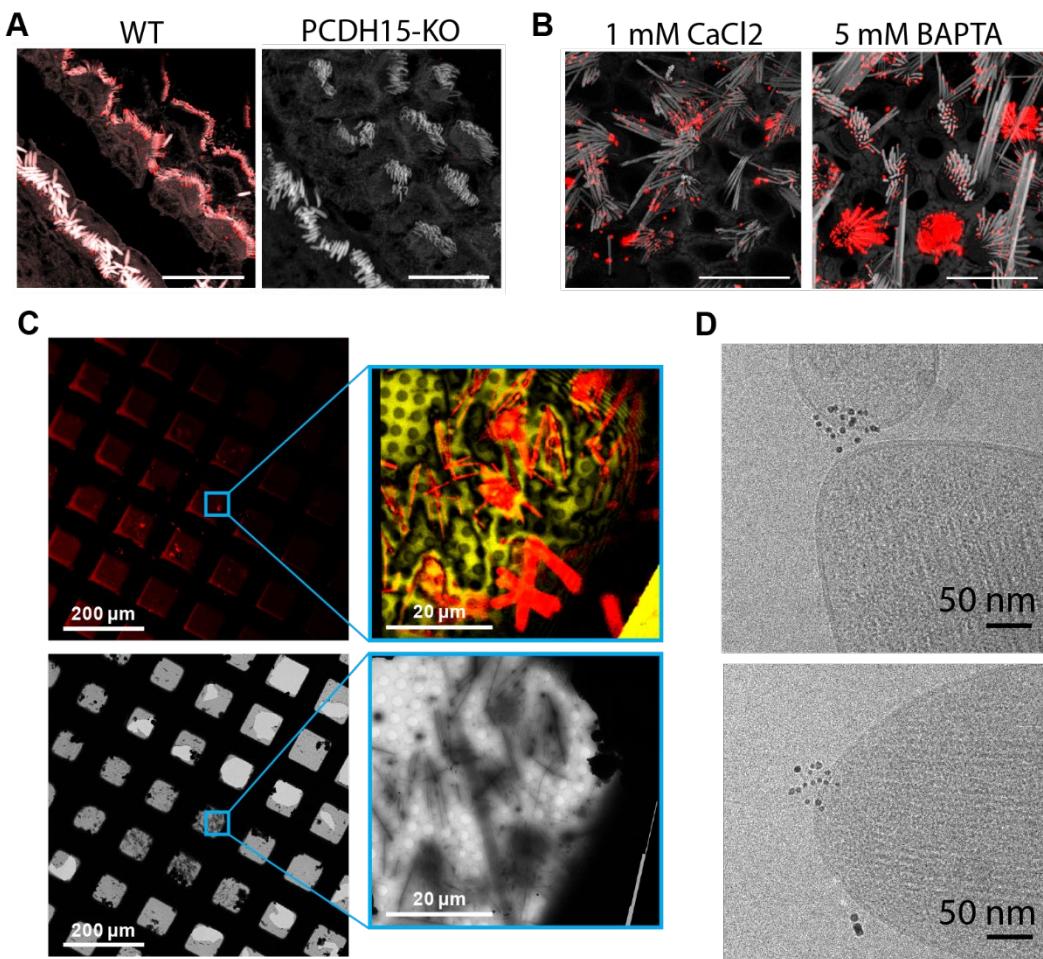
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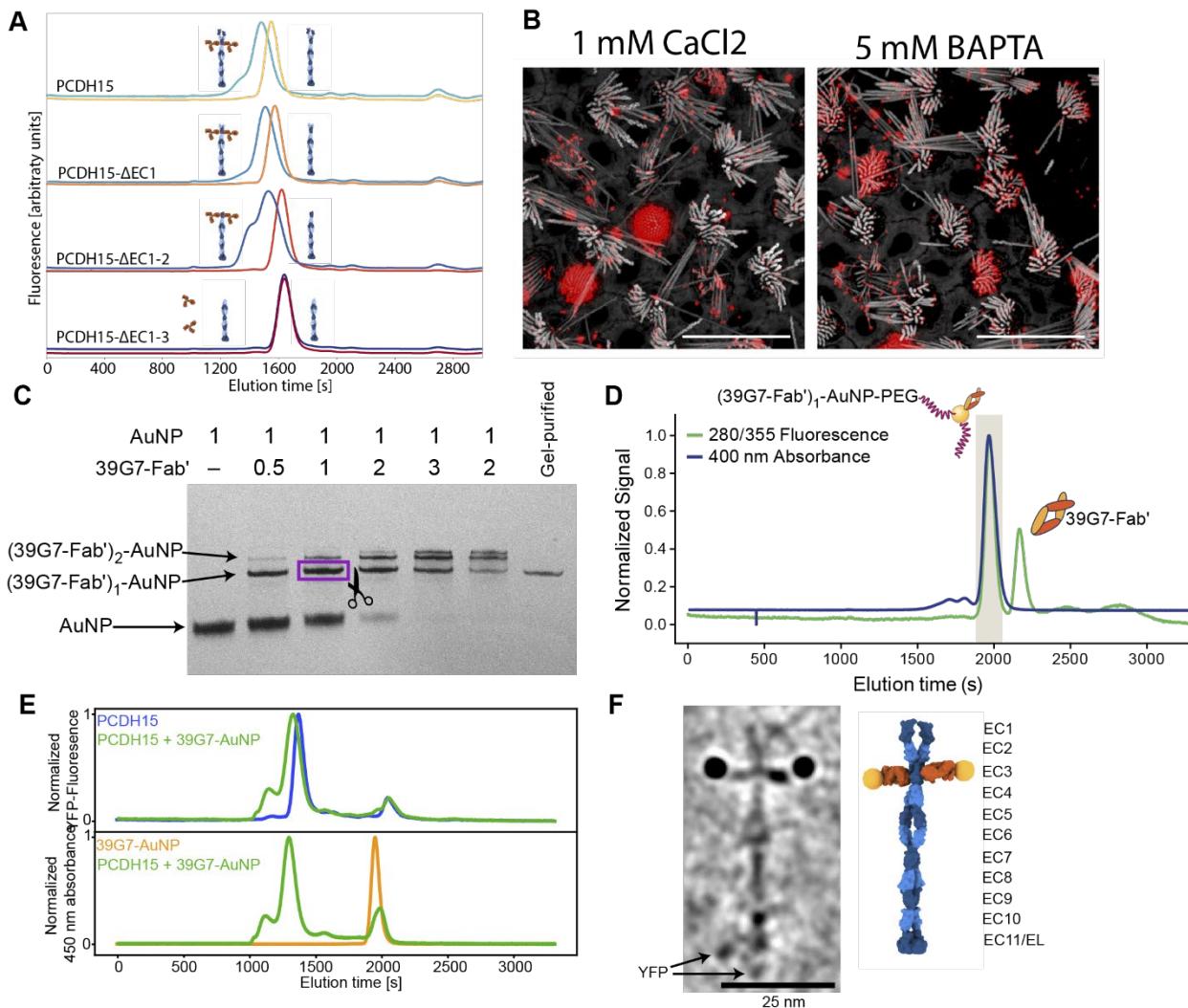
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554



556 **Figure 1. Cryo-EM data-collection of antibody stained stereocilia tips supported by cryo-**  
557 **CLEM**  
558 (A) Immunostaining of WT cochlea or PCDH15 KO with polyclonal antibody raised in rabbits  
559 against PCDH15 extracellular domain. (B) Immunostaining of WT utricle with polyclonal  
560 antibody in media containing 1mM CaCl2 or with the addition of 5mM BAPTA (C) CLEM-  
561 based screening to identify squares with thin-ice supported stereocilia. The top row shows low-  
562 and medium magnification views acquired using a cryo-light microscope. In both views  
563 SirAction fluorescence is shown in red. In the medium-magnification view laser-reflection is  
564 shown in gold. The bottom rows show the same areas as acquired in a cryo-TEM. (D) High-

565 magnification cryo-TEM micrographs of stereocilia tips stained with the anti-PCDH15 rabbit  
566 polyclonal antibody, detected with a 5nm gold coupled secondary antibody.



568 **Figure 2. Stoichiometric gold staining of PCDH15 using a monoclonal Fab AuNP conjugate**

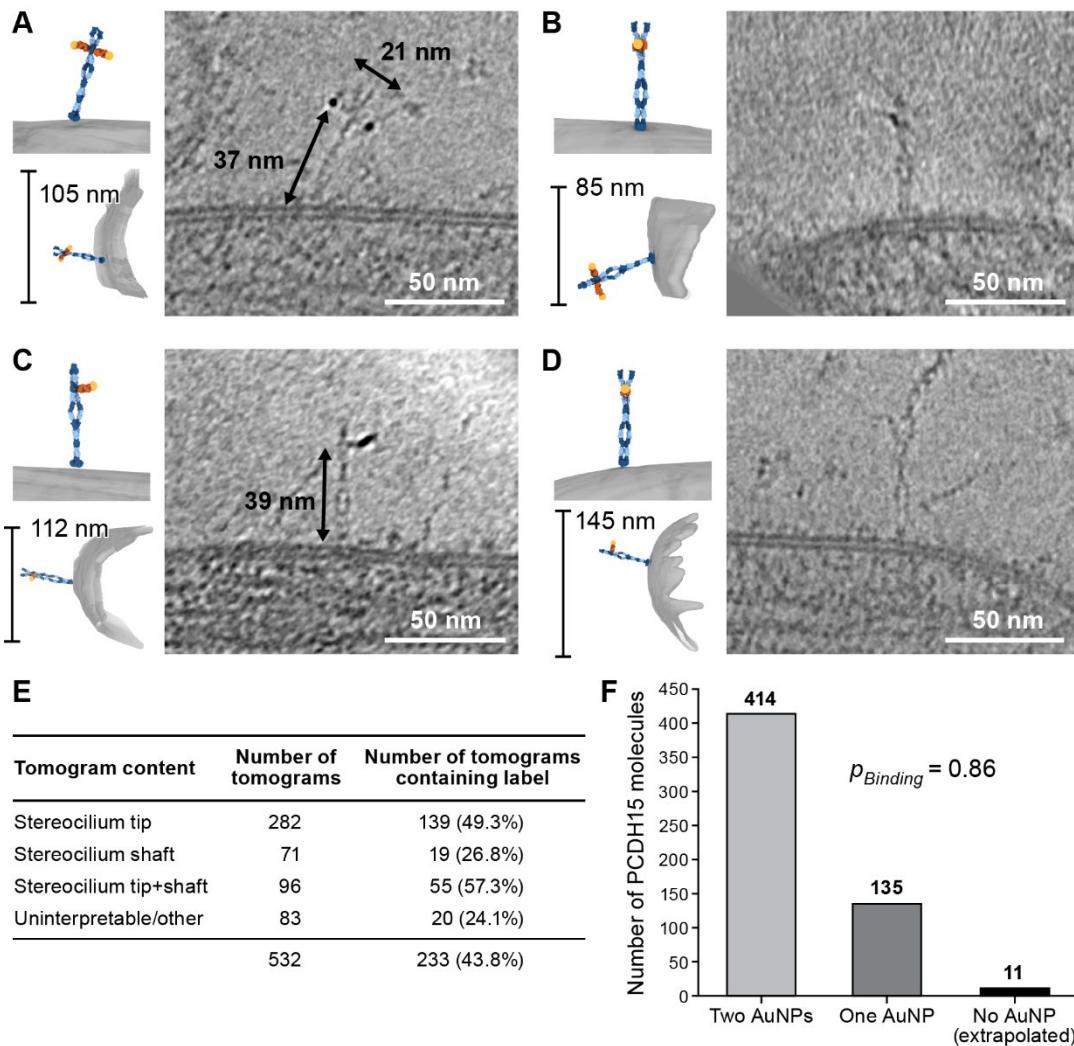
569 (A) FSEC analysis of binding of 39G7 to the PCDH15 extracellular domain. 39G7 addition leads  
 570 to earlier elution of PCDH15 in full-length constructs and after deletion of EC1 and EC2.  
 571 Deletion of EC3 abolished 39G7 binding. (B) Immunostaining of WT utricle with 39G7 in media  
 572 containing 1mM CaCl<sub>2</sub> or with the addition of 5mM BAPTA. (C) PAGE analysis and  
 573 purification of conjugation between 39G7 Fab' and a 2nm AuNP (D) SEC purification of PEG-  
 574 coated 39G7-Fab'-AuNP conjugates. (E) FSEC analysis of 39G7-Fab'-AuNP-PEG conjugate to  
 575 PCDH15 extracellular domain. (F) Cryo-TEM image of PCDH15 extracellular domain bound to

576 39G7-Fab'-AuNP-PEG conjugate together with model of the complex. In the model PCDH15 is

577 shown in blue, 39G7 Fab in orange, and AuNPs as golden spheres.

578

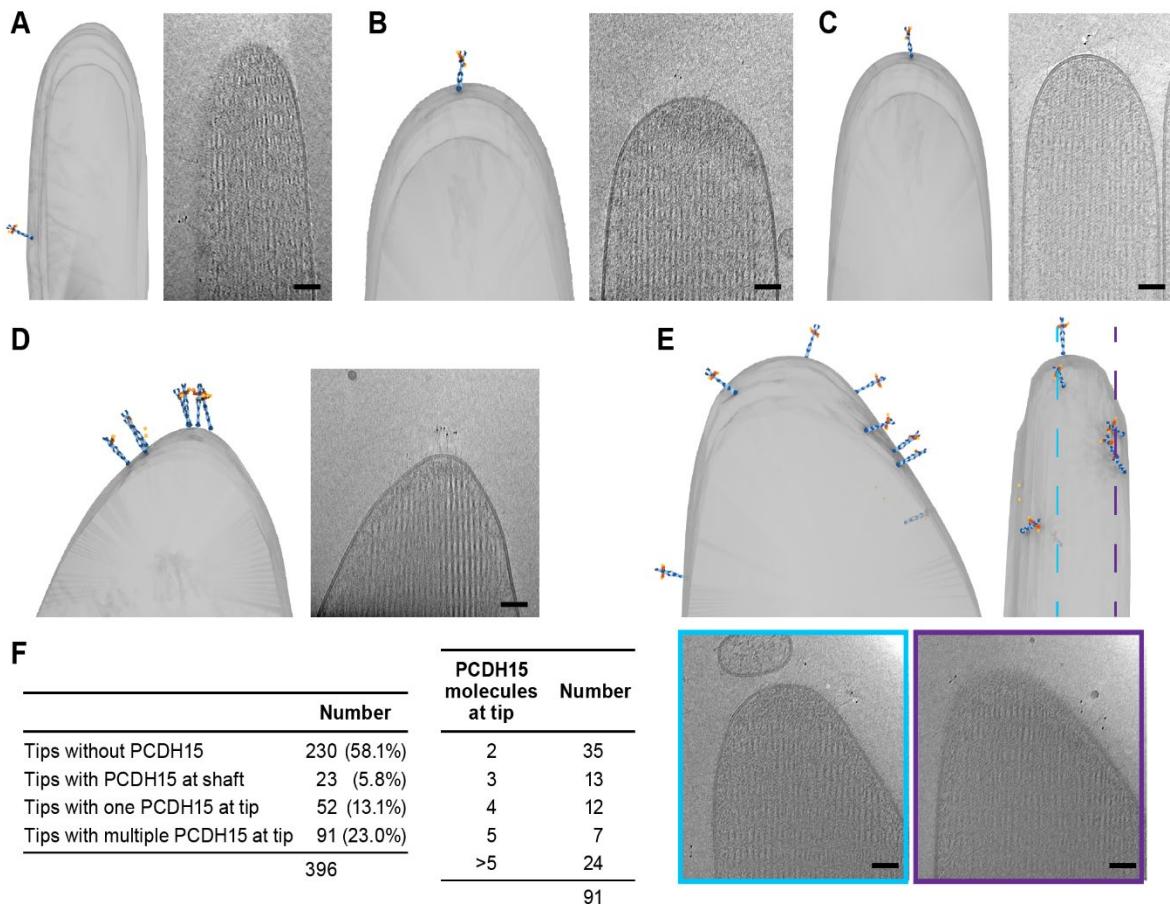
579



581 **Figure 3. 39G7-AuNP conjugate labels PCDH15 dimer in stereocilia**

582 (A-B) Representative examples of PCDH15 dimers with two bound AuNPs imaged *in situ*. (C-  
 583 D) Representative examples of PCDH15 dimers with one bound AuNPs imaged *in situ*. Dimeric  
 584 features in the PCDH15 density suggests that these molecules are PCDH15 dimers with one  
 585 epitope not bound to 39G7. (E) Table detailing numbers and content of collected tomograms. (F)  
 586 Bar chart quantifying the ratio of PCDH15 molecules labeled by one or two AuNPs. Assuming  
 587 that AuNPs bind independently to the two epitopes in the PCDH15 dimer results suggests that  
 588 86% of all epitopes were labeled and only 2% of PCDH15 molecules were unlabeled.

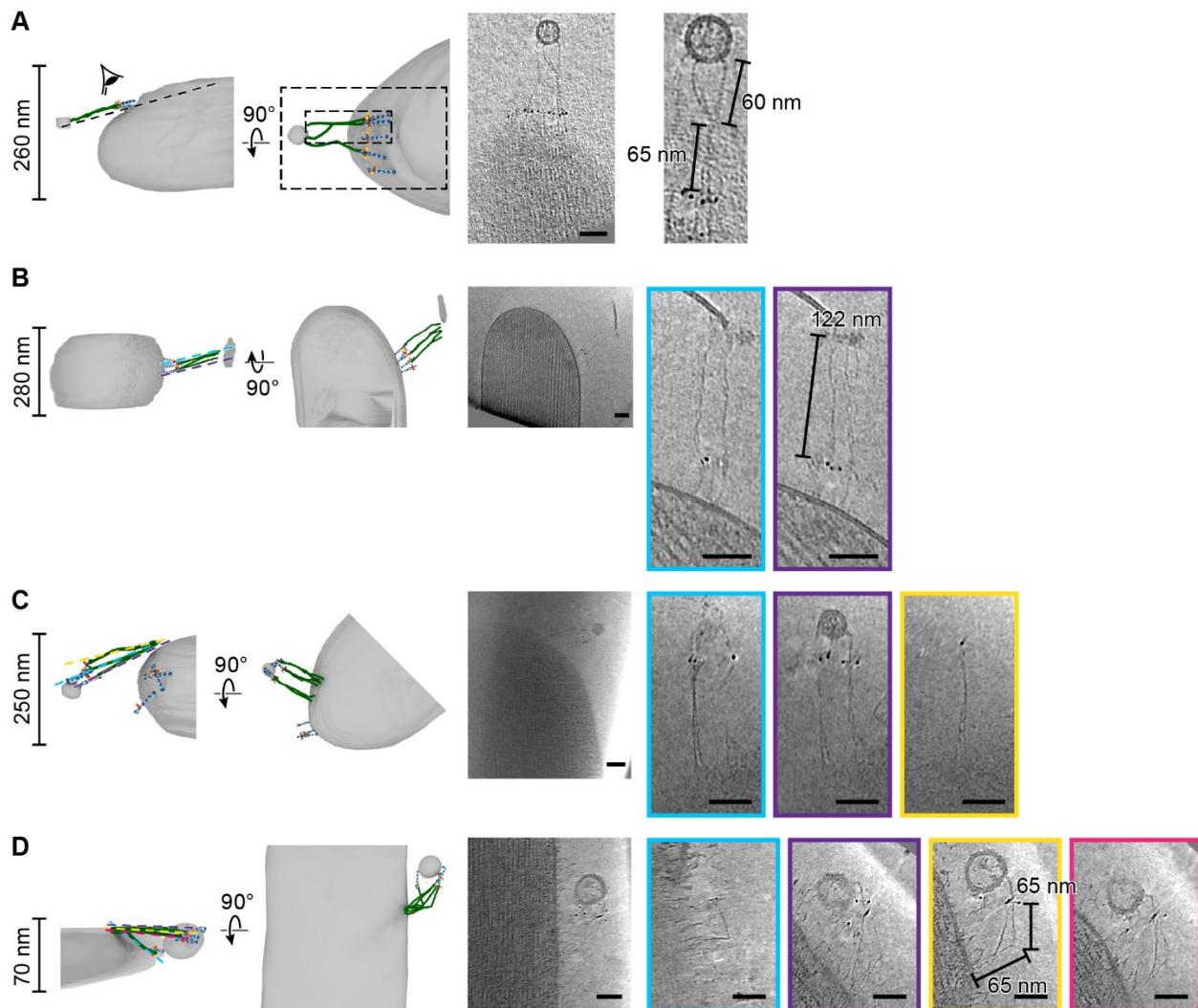
589



591 **Figure 4. Stereocilia tips frequently harbor more than one copy of PCDH15**

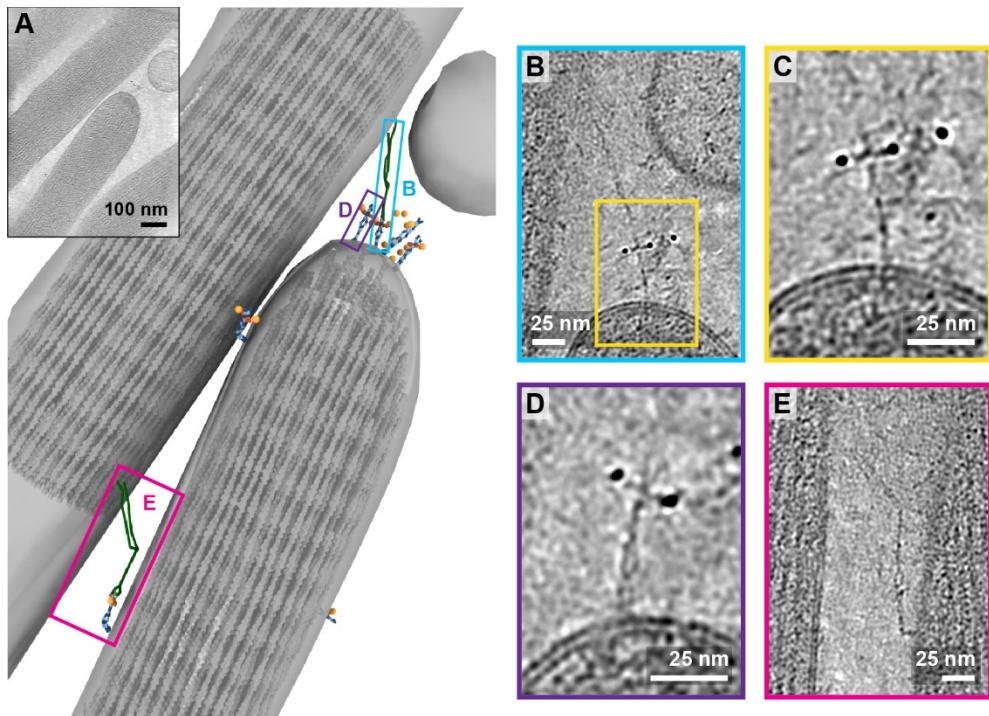
592 (A) Representative example of stereocilia tip with PCDH15 molecule only found in adjacent  
593 shaft. (B-C) Representative examples of stereocilia tips with one copy of PCDH15 at tip. (D-E)  
594 Representative examples of stereocilia tips with multiple copies of PCDH15 at tip. (F) Table  
595 detailing number of imaged stereocilia tips and distribution of PCDH15 molecules found at the  
596 tip.

597



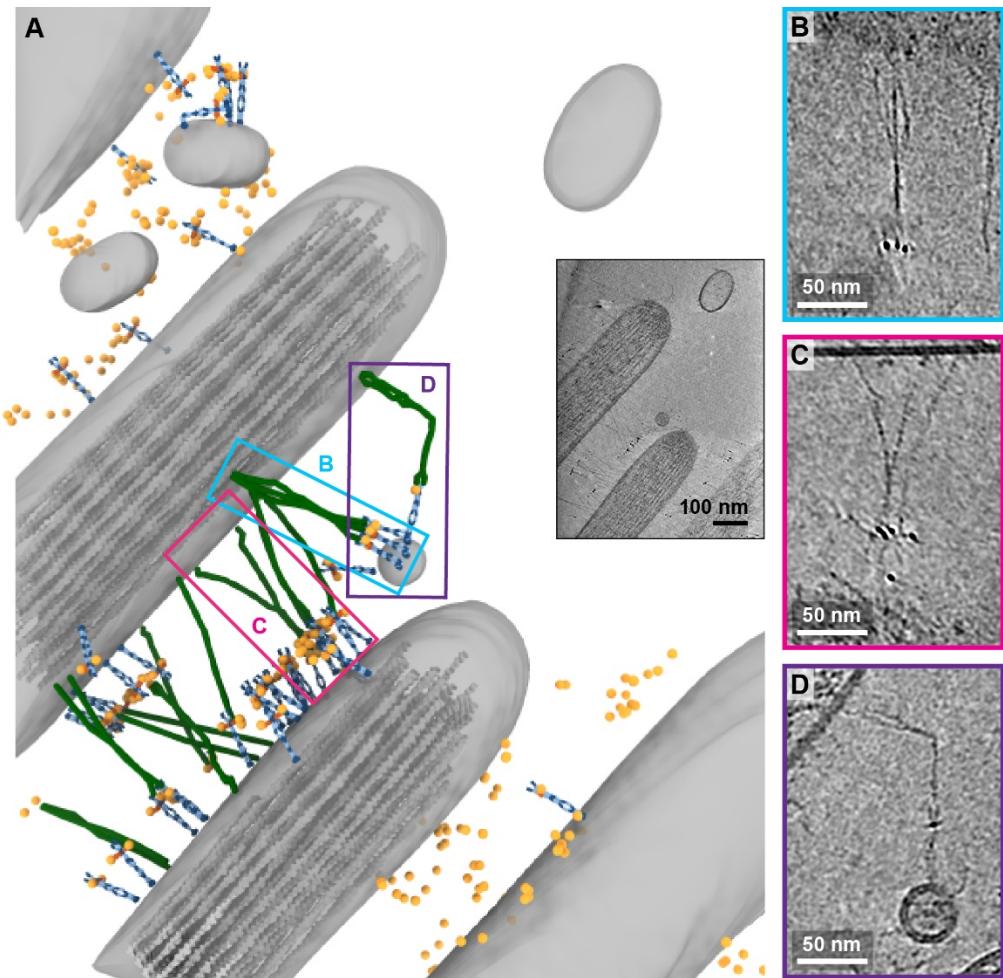
599 **Figure 5. PCDH15/CDH23 tetramers are found in clusters**

600 On the left side of each panel the tomogram annotation is shown from the side and from top. On  
601 the sideview ice thickness is indicated. To the right of the annotation is a projection of the cluster  
602 density in context of the stereocilium. Further to the right are detailed slices of the electron  
603 density. The angle and position of each slice is indicated in the sideview of the tomogram  
604 annotation. Scale bars correspond to 50nm. (A-B) Representative tomograms showing  
605 PCDH15/CDH23 heterotetramers with PCDH15 at tip of stereocilium and CDH23 in lipid  
606 fragment. (C-D) Representative tomograms showing PCDH15/CDH23 heterotetramers with  
607 CDH23 in side of stereocilium and PCDH15 in lipid fragment.



609 **Figure 6. Tomogram containing intact tip-link**

610 (A) Annotation of tomogram showing a putative intact tip-link. The inset at the top left shows a  
611 projection of the tomogram. (B) Closeup view of putative tip-link density in the tomogram. (C)  
612 Closeup of PCDH15 density in tip-link. (D) Close-up of PCDH15 molecule at tip not bound to  
613 CDH23. (E) Close-up of PCDH15/CDH23 heterotetramer in the stereocilia shaft region.  
614 PCDH15 has a 90° bend between EC9 and EC10.



616 **Figure 7. Tomogram depicting lateral links containing PCDH15 in small stereocilia**

617 (A) Annotation of tomogram showing developing stereocilia including PCDH15/CDH23 'lateral  
618 links'. The inset at the bottom right shows a projection of the tomogram. (B-C) Closeup of  
619 CDH23 molecules clustering together into single strand. (D) Closeup of CDH23 molecule with  
620 distinct bend half-way between N- and C-terminal end.

621

622 **Supplementary Materials**

623 **Movie S1.** Tomogram of recombinant PCDH15 extracellular domain in complex with 39G7-  
624 AuNP conjugate.

625 **Movie S2.** Tomogram of stereocilium displaying a PCDH15 dimer with two bound 39G7-AuNP  
626 conjugates, also depicted in Figure 3A.

627 **Movie S3.** Tomogram of stereocilium displaying a PCDH15 dimer with two bound 39G7-AuNP  
628 conjugates, also depicted in Figure 3B.

629 **Movie S4.** Tomogram of stereocilium displaying a PCDH15 dimer with one bound 39G7-AuNP  
630 conjugates also depicted in Figure 3C.

631 **Movie S5.** Tomogram of stereocilium displaying a PCDH15 dimer with one bound 39G7-AuNP  
632 conjugates, also depicted in Figure 3D.

633 **Movie S6.** Tomogram of stereocilium tip with a single PCDH15 dimer in the shaft region of the  
634 tip, also depicted in Figure 4A.

635 **Movie S7.** Tomogram of stereocilium tip with a single PCDH15 dimer at the apex of the tip, also  
636 depicted in Figure 4B.

637 **Movie S8.** Tomogram of stereocilium tip with a single PCDH15 dimer at the apex of the tip, also  
638 depicted in Figure 4C.

639 **Movie S9.** Tomogram of stereocilium tip with a cluster of multiple PCDH15 dimers at the apex  
640 of the tip, also depicted in Figure 4D.

641 **Movie S10.** Tomogram of stereocilium tip with multiple PCDH15 dimers scattered around the  
642 tip, also depicted in Figure 4E.

643 **Movie S11.** Tomogram of stereocilium tip containing multiple PCDH15 dimers connected to  
644 putative CDH23 filaments in a lipid vesicle, also depicted in Figure 5A.

645 **Movie S12.** Tomogram of stereocilium tip containing multiple PCDH15 dimers connected to  
646 putative CDH23 filaments in a lipid membrane fragment, also depicted in Figure 5B.

647 **Movie S13.** Tomogram of a stereocilium tip containing multiple putative CDH23 filaments  
648 connected to PCDH15 dimers in a lipid vesicle, also depicted in Figure 5C.

649 **Movie S14.** Tomogram of a stereocilium shaft containing multiple putative CDH23 filaments  
650 connected to PCDH15 dimers in a lipid vesicle, also depicted in Figure 5D.

651 **Movie S15.** Tomogram of a stereocilium tip and a stereocilium shaft containing a PCDH15  
652 dimer in the stereocilium tip that is connected to a putative CDH23 filament in the neighboring  
653 stereocilium shaft. The tip also contains PCDH15 dimers not bound to CDH23. An additional  
654 putative PCDH15/CDH23 complex is connecting the two stereocilia in the shaft region. Also  
655 depicted in Figure 6.

656 **Movie S16.** Tomogram of stereocilia connected by tens of PCDH15/CDH23 complexes.  
657 Noteworthy complexes, that include apparent interaction between CDH23 dimers, as well as  
658 bends in the CDH23 dimer are highlighted. Also depicted in Figure 7.