

1 **3D structure and *in situ* arrangements of CatSper channel in the sperm flagellum**

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26 Running title: 3D structure and *in situ* arrangements of mammalian CatSper channel

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31

32 **Abstract**

33

34 The sperm calcium channel CatSper plays a central role in successful fertilization as a primary
35 Ca^{2+} gateway into the sperm flagellum. However, CatSper's complex subunit composition has
36 impeded its reconstitution *in vitro* and structural elucidation. Here, we applied cryo-electron
37 tomography to visualize the macromolecular organization of the native CatSper channel complex
38 in intact mammalian sperm, as well as identified three additional CatSper-associated proteins.
39 The repeating CatSper units form long zigzag-rows in four nanodomains along the flagella. In
40 both mouse and human sperm, each CatSper repeat consists of a tetrameric pore complex.
41 Murine CatSper contains an additional outwardly directed wing-structure connected to the
42 tetrameric channel. The majority of the extracellular domains form a canopy above each pore-
43 forming channel that interconnects to a zigzag-shaped roof. The intracellular domains link two
44 neighboring channel complexes to a diagonal array. The loss of this intracellular link in *Efcab9^{-/-}*
45 sperm distorts the longitudinally aligned zigzag pattern and compromises flagellar movement.
46 This work offers unique insights into the mechanisms underlying the assembly and transport of
47 the CatSper complex to generate the nanodomains and provides a long-sought structural basis for
48 understanding CatSper function in the regulation of sperm motility.

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50

51 Freshly ejaculated mammalian sperm must undergo a physiological process called capacitation
52 to be capable of fertilizing the egg^{1,2}. The crucial change that occurs during capacitation
53 represents a motility change, i.e. the sperm flagellum beats vigorously and asymmetrically,
54 producing a whip-like motion. This motility pattern – known as hyperactivated motility – enables
55 the sperm to reach the egg by overcoming the viscous microenvironment of the female
56 reproductive tract. Additionally, hyperactivation allows sperm to push through a sticky egg coat,
57 and eventually fertilize the egg³. Hyperactivation is triggered by the elevation of the
58 intraflagellar calcium that requires the sperm-specific and Ca²⁺-selective CatSper channel^{4,5}.
59 CatSper loss-of-function abrogates hyperactivation of the sperm flagellum and renders males
60 infertile in both mice and humans⁶.

61
62 Previous studies have found that CatSper is the most complex ion channel known, with at least
63 ten proteins: four subunits that form a heterotetrameric channel (CATSPER1-4)^{5,7}, as well as six
64 additional, non-pore forming subunits, including four transmembrane (TM) proteins with large
65 extracellular domains (ECD) (CATSPER β , γ , δ , and ϵ)⁸⁻¹¹ and two smaller cytoplasmic,
66 calmodulin (CaM)-IQ domain proteins that form the EFCAB9-CATSPER ζ complex^{8,12,13}
67 (Extended data Table 1). Deletions or mutations of any of the pore-forming or other TM-subunits
68 results in the loss of the entire CatSper channel complex⁶. Super resolution light microscopy
69 showed that the CatSper channel complex is restricted to four linear compartments within the
70 flagellar membrane^{14,15}, generating a unique longitudinal signaling nanodomain in each flagellar
71 quadrant. Genetic evidence suggested that this high-order arrangement is essential for Ca²⁺
72 signaling and sperm hyperactivation, highlighting physiological relevance of the spatial
73 organization. Disrupting the integrity of the linear nanodomains alters the flagellar waveform
74 and prevents sperm from efficiently migrating *in vivo*^{8,14,16}. Specifically, the absence of the
75 cytoplasmic EFCAB9-CATSPER ζ complex in *Efcab9*^{-/-} and/or *Catsperz*^{-/-} mutant sperm alters
76 the continuity of each CatSper nanodomain^{8,12}, suggesting a regularly repeating, quaternary
77 structure of the CatSper complex within the nanodomains.

78
79 Despite many important discoveries mentioned above, the fundamental structure of the native
80 channel complex and its molecular architectural arrangement were still not known. Here, we
81 address these questions by visualizing in-cell organization and domain structures of the CatSper
82 channel complex in intact mouse and human sperm flagella using cryo-electron tomography
83 (cryo-ET).

84
85 **Macromolecular composition of the CatSper complex.** Ten components have been validated
86 to comprise the CatSper channel complex in the linear nanodomains⁶. However, we previously
87 showed by comparative mass-spectrometry that in mouse *Catsper1*^{-/-} sperm four additional
88 proteins were reduced: C2CD6 (C2 Calcium-dependent Domain-containing protein 6, also
89 known as ALS2CR11), E3 ubiquitin-protein ligase TRIM69, SLCO6C1 (solute carrier organic

90 anion transporter family, member 6c1), and the DNA-binding ATPase FANCM (Fanconi
91 anemia, complementation group M)¹² (see also Extended Data Fig. 1a).

92

93 To test whether these four candidates are truly associated with the CatSper channel, we used
94 western blot analyses of *Catsper1*^{-/-} and *Catsperd*^{-/-} sperm that lack the entire CatSper channel
95 complex^{9,12,14}, and found that the protein levels of C2CD6, TRIM69, and SLCO6C1, but not
96 FANCM, were indeed reduced (Fig. 1a and Extended Data Fig. 1b, c). Moreover, using
97 fluorescence and 3D structured illumination microscopy (3D SIM) we showed that the three
98 CATSPER1/δ-dependent proteins, *i.e.* C2CD6, TRIM69, and SLCO6C1, localize in the principal
99 piece - the longest part of the sperm flagellum harboring the CatSper channel - and display the
100 same quadrilinear distribution with the nanodomains (Fig. 1b, c, and Extended Data Fig. 1c-f). In
101 the absence of EFCAB9 and/or ζ, the continuous distribution is disrupted (Fig. 1c and Extended
102 Data Fig. 1f, *lower*), resembling previously reported results for the known CatSper subunits^{8,12}.
103 This typical dependence of protein levels and localizations on other CatSper components suggest
104 that they are likely three new *bona fide* CatSper-associated proteins of murine sperm. In
105 particular, it is intriguing to find an organic anion transporter, SLCO6C1 that is highly expressed
106 in rodent testis^{12,17,18}, in complex with an ion channel, the murine CatSper. Whereas the loss-of-
107 function effect of C2CD6 remains to be determined, *Trim69*¹⁹ and *Slco6c1*²⁰ are not essential for
108 fertility, indicating their function on the CatSper channel is likely to be modulatory and/or
109 indirect.

110

111 **In-cell organization of CatSper complexes.** Based on the discovery of the additional CatSper
112 components here, and the recently reported 1:1 stoichiometry of seven TM CatSper subunits in
113 sea urchin sperm (*i.e.* CATSPER1-4, β, γ, and ε)²¹, we hypothesize that a single mouse or human
114 CatSper complex might form a nearly half-megadalton extracellular domain (ECD) (Extended
115 Data Table 1), a size that could be visualized by cellular cryo-ET. Therefore, we performed cryo-
116 ET on intact murine and human sperm flagella to characterize the native CatSper complex *in*
117 *situ*, which avoids potential purification artefacts. Viewing the 3D reconstructed sperm and
118 flagellar membranes in cross-section, we observed protruding particles of ~25 nm in width
119 positioned to either side of the longitudinal column of the fibrous sheath in the principal piece
120 (Fig. 1d), consistent with the localization for CatSper nanodomains as seen by immuno-electron
121 microscopy (EM)¹⁴. Out of the four quadrants, only up to two could be visualized in the cryo-
122 tomograms due to the missing wedge effect that results from a limited tilt-angle range in single-
123 axis ET. Longitudinal tomographic slices of the wild type sperm flagella revealed long
124 continuous rows of densely packed particles with an apparent periodicity of 17.6 nm (Fig. 1e).
125 The resolution of reconstructed whole murine flagella was limited due to the ~900 nm sample
126 thickness of the proximal region of the principal piece. Therefore, we also used cryo-FIB milling
127 to generate ~200 nm thick slices (called “lamella”) of murine sperm flagella (Extended Data Fig.
128 2a-g) that resulted in higher-resolution tomographic reconstructions (Extended Data Fig. 2h, i;
129 Extended Data Table 2).

130
131 Due to all-or-none assembly of CatSper TM subunits in mouse sperm, knockout of any one of
132 these TM subunits leads to mutant sperm that do not form the nanodomains as they lack the
133 entire CatSper complex⁶ (see also Extended Data Fig. 1a, b; *Catsper1*^{-/-} and *Catsperd*^{-/-}). By
134 contrast, *Catsperz*^{-/-} and/or *Efcab9*^{-/-} sperm assemble the CatSper complex missing only the two
135 interdependent non-TM EFCAB9 and CATSPERζ subunits¹² (see also Fig. 1a and Extended
136 Data Fig. 1b). Because previous observations by super resolution light microscopy and scanning
137 EM suggested the linearity of the nanodomains is discontinuous in a fragmented pattern in
138 *Catsperz*^{-/-} and *Efcab9*^{-/-} sperm^{8,12}, we next looked at *Efcab9*^{-/-} sperm for CatSper particles.
139 Indeed, we observed that the particles positioned to the corresponding locations in the flagellar
140 membrane of *Efcab9*^{-/-} sperm formed discontinuous rows, short clusters or individual repeat units
141 (Fig. 1f, g). Together with the position of these particles along flagella, this genetic evidence, *i.e.*
142 disruption of the particle-rows in *Efcab9*^{-/-} sperm, strongly supports that these particles are
143 macromolecular CatSper channel complexes that form the quadrilinear nanodomains.

144
145 **Zigzag arrangement of CatSper complexes.** Slicing the rows of CatSper channel complexes in
146 longitudinal orientation parallel to the flagellar membrane (top-down view) revealed continuous
147 rows with repeating units in a zigzag arrangement of ~25 nm in width (Fig. 2a-h), demonstrating
148 the CatSper complexes are repeated within the rows. We found that the number of zigzag rows
149 per nanodomain varies from a single row (Fig. 2a, b), two rows that can be up to 100 nm apart
150 (Fig. 2c, d and Extended Data Video 1) or merge into one row (Fig. 2e, f), or up to as many as
151 five parallel rows (Fig. 2g, h). In tomograms of *Efcab9*^{-/-} sperm flagella, we observed mostly
152 short clusters containing only 1-7 units (Fig. 2k-n) and very few continuous rows with a
153 maximum of ~70 repeats (Fig. 2i, j and Extended Data Fig. 3a, b). Interestingly, the short mutant
154 clusters are no longer well-aligned with the flagellar axis and adopt various angles - up to almost
155 perpendicular - relative to the longitudinal axis of the flagellum (Fig. 2l, n). In *Efcab9*^{-/-} sperm,
156 C2CD6, TRIM69, and SLCO6C1 proteins were reduced but detectable (Fig. 1a, b and Extended
157 Data Fig. 1b) as seen for all the previously known 8 TM subunits¹². These results suggest that the
158 absence of the EFCAB9-CATSPERζ complex from the intracellular side of the channel disrupts
159 the high-order arrangement of the CatSper channel complex and the linear alignment in the
160 nanodomains. Cryo-tomograms of human sperm flagella revealed similar linear rows that are
161 ~24 nm wide and consist of repeating units that are also arranged in a zigzag pattern (Fig. 2o-r).

162
163 **Extracellular structures of CatSper form canopy tents that connect pore-forming channels**
164 **as beads on a zigzag string.** After determining the periodicity of the CatSper complexes within
165 the zigzag rows, we performed subtomogram averaging of the repeating units to increase the
166 signal-to-noise ratio and thus the resolution. We averaged ~2500 CatSper complex repeat units
167 (which includes the application of two-fold symmetry) from continuous rows from 11 acquired
168 cryo-electron tomograms of both whole cells and cryo-FIB milled mouse wild type flagella
169 (Extended Data Table 2). The averages depict unprecedented details of CatSper complexes *in*

170 *situ* (Fig.3a-h) with up to 22 Å resolution (0.5 FSC criterion; Extended Data Fig.3h, Extended
171 Data Table 2).

172

173 As shown in Figure 3 and Extended Data Video 2, the averaged 3D structure of the zigzag row
174 reveals that the CatSper complexes are evenly spaced in two anti-parallel lines, *i.e.* the
175 complexes are 180° rotated between the two lines. The appearance of an ~25-nm-wide zigzag-
176 pattern results from the staggering of the rows of channels and the ECDs connecting across the
177 lines (Fig. 3c, d, g). Several structural features of the whole channel unit are visualized from
178 extra- to intracellular domains across the inner and outer leaflet of the membrane bilayer (Fig.
179 3a-h). In the side view (Fig. 3a), the most prominent structural feature of each CatSper complex
180 is the uniquely shaped ECDs that form a 11.2 nm high canopy tent in which the majority of the
181 ECD mass forms the canopy roof (Fig. 3a, f, g). The roof is connected between neighboring
182 complexes to a continuous zigzag ribbon around 6.6 nm away from the flagellar membrane (Fig.
183 3c, g).

184

185 Tangential slices (*i.e.*, top views) through this extracellular part, show closest to the membrane
186 clearly the asymmetric unit: the tetrameric arrangement of the CATSPER1-4 subunits with an
187 additional density that we named “wing” at the outside corners (Fig. 3d, white arrowheads). The
188 position of the wing clearly reveals the 180° rotation between connected neighboring CatSper
189 complexes. At an inside corner of the tetramer – opposite to the wing-connected subunit – at
190 least one of the pore-forming channel subunits forms a fine but clearly visible connection to the
191 identical subunit of the “forward slash”-neighboring, 180° rotated CatSper channel (Fig. 3d, blue
192 arrowheads). The diameter of the tetrameric channel is 10.6 nm, which is in a similar range with
193 the size observed for other tetrameric channels such as 10 nm wide Cav1.1²². The center-to-
194 center spacing between channels along the zigzag string is 15 nm.

195

196 The ECD canopy roof is positioned right above the tetrameric channel (*i.e.*, the four tent poles)
197 (Fig. 3a-d). Interestingly, the roof ridge (Fig. 3b; Fig. 3f-g, dark blue) is off-center and tilted in
198 the same “forward slash” direction as the two connected inner pore-forming subunits (Fig. 3b, d).
199 Based on the subtomogram average, the mass estimation of the ECDs of one CatSper channel
200 complex is ~430 kDa, close to the sum of the ECDs predicted for the eight known TM subunits
201 (CATSPER1-4, β, γ, δ, and ε) with 1:1 stoichiometry (Extended Data Table 1). We speculate that
202 each TM auxiliary subunit specifically pairs with a particular pore-forming subunit.

203

204 **Intracellular structures of CatSper connect two channel units as diagonal arrays.** Markedly,
205 the intracellular domains observed underneath the channel form a continuous diagonal array
206 between two staggered channel complexes of the zigzag string (Fig. 3e, h). The diagonal stripes
207 are spaced by 17.6 nm and are oriented in the same forward slash direction as the two connected
208 inner pore-forming subunits (Fig. 3d). The side view of the complex shows that the intracellular

209 protrusion of an individual channel is not coaxial with the center of the tetrameric channel (Fig.
210 3a).

211
212 The mass estimation of the intracellular domains corresponding to one wild type CatSper
213 channel complex is ~200 kDa (Fig. 3e, h), which is ~40 kDa smaller than the combined
214 molecular weights of the cytoplasmic domains from the 10 reported CatSper subunits (Extended
215 Data Table 1). As all the eight known TM subunits are required to make one channel unit^{6,21},
216 these results suggest that two forward slash neighboring channels may form an intercomplex of
217 2:1 stoichiometry such that an EFCAB9-CATSPER ζ pair links two channel units like an
218 intracellular bridge, using two channels as a building block of the zigzag assembly. At this point,
219 it remains unclear whether C2CD6 and TRIM69 would stabilize the CatSper complex or interact
220 rather transiently. Their stoichiometry to other subunits also needs to be determined.

221
222 **EFCAB9-CATSPER ζ complex has profound impact on the long- and short-range**
223 **architecture of CatSper channels.** Interestingly, we observed that not only the length and
224 alignment of the nanodomain rows was changed in *Efcab9*^{-/-} sperm as compared to wild type
225 sperm (Fig. 2), but also the arrangement between neighboring CatSper complexes were different
226 (Fig. 3i-l and Extended Data Fig. 3c-g). The mutant averages showed that complexes are still
227 arranged in two staggered and anti-parallel lines, as is evident from the preserved location of the
228 wing density (Fig. 3k). However, in the mutant the usual zigzag pattern is disrupted, and instead
229 neighboring complexes forms arrays of diagonal stripes that are oriented either in a backslash
230 (Fig. 3i-k) or a forward slash direction (Extended Data Fig. 3d-f).

231
232 In *Efcab9*^{-/-} sperm, the intracellular domain of the CatSper complex is visible but appears
233 reduced (Fig. 3l; Extended Data Fig. 3c, g) and is possibly mis-oriented in the predominant
234 backslash arrangement (Fig. 3l). In the mutant with backslash phenotype, at the roof level the
235 usual forward slash connection is greatly reduced (Fig. 2j, l, 3j) and a different subunit of the
236 tetrameric channel forms the inner connection between neighboring complexes (Fig. 3k). The
237 forward slash arrangement resembles the wild type organization at the tetrameric channel level,
238 but at the roof level the usual backslash connection is missing (Fig. 2n; Extended Data Fig. 3b,
239 e). Despite this re-arrangement, in both configurations, the rows or clusters have a width and
240 ECD mass that is comparable with the wild type zigzag ribbon.

241
242 **Similarities and differences between mouse and human sperm CatSper structures.** Cryo-
243 tomograms and subtomogram average of human sperm flagella also revealed a ~24 nm wide
244 zigzag string of staggered and anti-parallel arranged complexes on the extracellular side of the
245 flagellar membrane (Fig. 3m-o). Although the resolution of the subtomogram average of human
246 sperm flagella was limited by a low number of averaged repeats (Extended Data Fig. 3j and
247 Extended Data Table 2), a 11.3 nm wide tetrameric channel with forward slash connection (Fig.
248 3o), a center-to-center spacing between channels along the zigzag string of 15.2 nm, and the

249 canopy roofs in a zigzag-pattern were clearly visible (Fig. 3n), suggesting that these unique rows
250 (Fig. 2o-r) are likely arrays of human CatSper complexes. We observed only two differences
251 between the mouse and human sperm CatSper complex arrays: first, the human roof ridges were
252 oriented opposite to that of the mouse CatSper, *i.e.* in the backslash direction (Fig. 3m), possibly
253 reflecting an $\sim 45^\circ$ counterclockwise rotation of each channel unit within the zigzag row; second,
254 human CatSper appears to be missing the wing structure observed for the mouse CatSper
255 tetrameric channel (compare Fig. 3o vs. 3d, Extended Data Fig. 5a). The molecular identity of
256 the wing structure remains unclear. However, we propose that this wing consists of SLCO6C1 as
257 it is a rodent-specific, multi-pass TM protein with small ECD, and its quadrilinear localization to
258 the flagellar membrane is dependent on other TM CatSper subunits in mouse sperm (Fig. 1c and
259 Extended Data Fig. 1c).

260

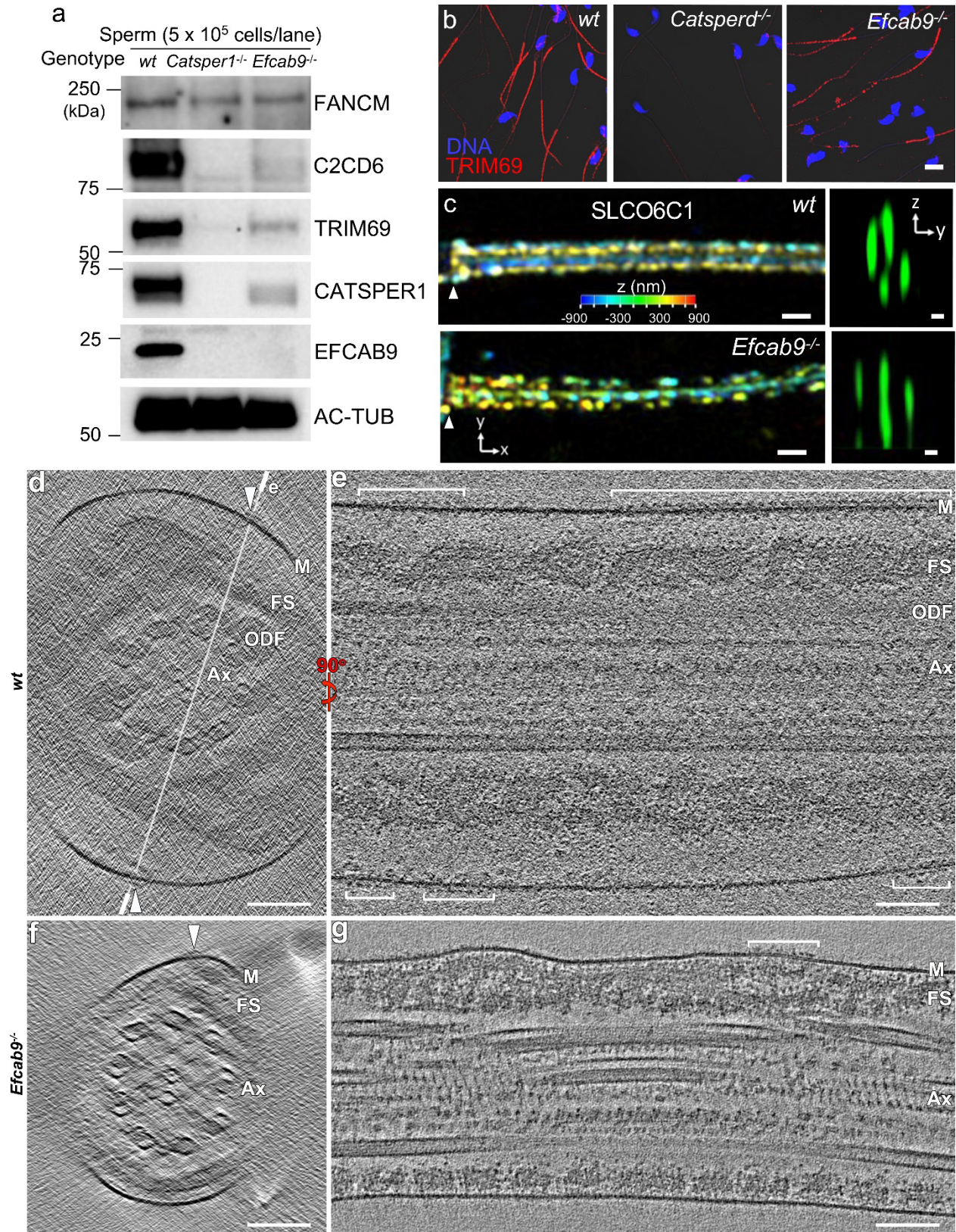
261 Physiological substrates are not yet identified for SLCO6C1¹⁷. However, the International Mouse
262 Phenotyping Consortium reports that *Slco6c1*^{-/-} mice show decreased circulating phosphorus
263 level²⁰, suggesting SLCO6C1 could be a potential phosphate transporter. As CatSper is required
264 to sustain motility for extended period of time^{7,12} which in turns is dependent on flagellar energy
265 metabolism^{23,24}, the association of SLCO6C1 with murine CatSper channel complex might be a
266 species-specific molecular mechanism linking Ca²⁺ homeostasis to ATP production via
267 glycolysis. Compared with mouse sperm, which use glycolysis as a dominant source of ATP
268 production²⁵, human sperm might split ATP production differently between oxidative
269 phosphorylation and glycolysis.

270

271 **Structural defects of mutant CatSper correlates with proximally stiff flagellum and**
272 **compromised motility.** The CatSper-mediated increase in intracellular Ca²⁺ initiates in the
273 principal region of the tail and propagates towards the sperm head²⁶. Our previous flagellar
274 waveform analyses of head-tethered sperm showed that *Efcab9*^{-/-} sperm display stiff flagella in
275 the proximal region¹². To better understand how the structural alterations of the mutant CatSper
276 channel complexes are translated into altered flagellar curvature and motility in *Efcab9*^{-/-} sperm,
277 we characterized the flagellar waveform and swim paths of free-swimming sperm in detail over
278 time using 3D high-speed Digital Holographic Microscopy²⁷ (Fig. 4, Extended Data Fig. 4).
279 Capacitation dramatically increased *xy*-excursion with respect to the laboratory-fixed frame of
280 reference, *i.e.* the out-of-plane beating of wild type sperm, which is abolished in *Efcab9*^{-/-} sperm
281 (Fig. 4a, c, d and Extended Data Fig. 4a). By contrast, capacitation did not significantly affect the
282 flagellar *z*-excursion, *i.e.* the waveform amplitude (Fig. 4a, e), suggesting that Ca²⁺ influx by
283 CatSper activation mainly regulates asymmetric out-of-plane beating in the *xy*-direction, but not
284 flagellar movement in the *z*-direction. Interestingly, the *z*-amplitude of non-capacitated *Efcab9*^{-/-}
285 sperm is smaller than that of wild type sperm (Fig. 4a, e), likely due to the proximally stiff
286 flagellum of *Efcab9*^{-/-} sperm. The proximally stiff mutant flagella might result from a lower basal
287 level of intracellular calcium, balanced by basal CatSper activity and Ca²⁺ extrusion pump¹².

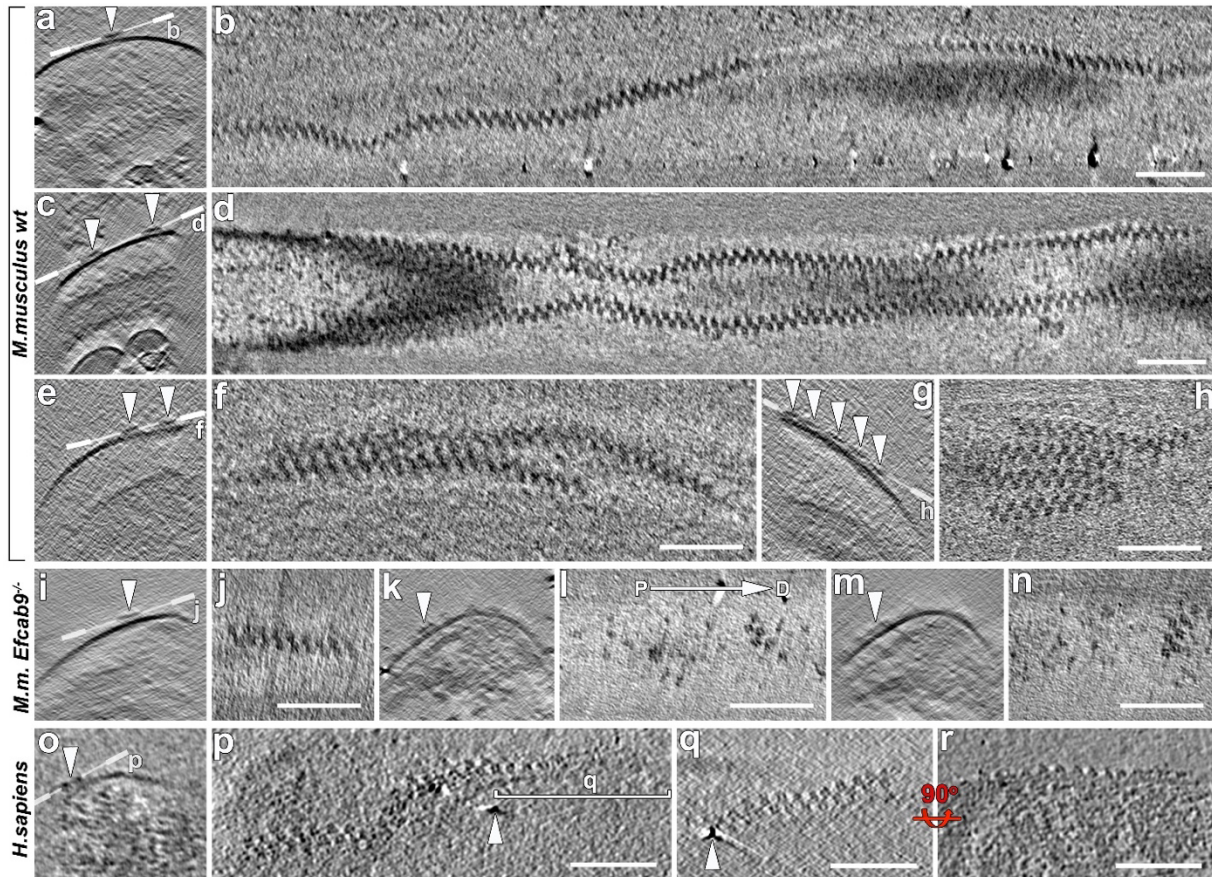
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289 To further unravel the effect of the altered beat patterns on sperm swim paths, we determined the
290 3D trajectories of free-swimming sperm by tracing the head positions using 3D high-speed
291 Digital Holographic Microscopy. Consistent with the increase of curvilinear velocity (Extended
292 Data Fig. 4b), the swimming trajectory of capacitated wild type sperm increase the range of
293 excursion in all dimensions (Fig. 4b, left; compare Extended Data Video. 3 vs 4). In contrast,
294 *Efcab9*^{-/-} sperm fail to expand the excursion range during capacitation (Fig. 4b, right), suggesting
295 the importance of CatSper channel and higher-order integrity on the effective sperm navigation
296 during capacitation. Capacitation likely requires Ca²⁺ signaling through the coordinate activity of
297 many CatSper channels. Based on our structural findings and the motility defects in *Efcab9*^{-/-}
298 sperm, we propose that in wild type sperm the extracellular connected zigzag arrangement within
299 a longitudinal nanodomain could coordinate the opening of the entire array of CatSper channels
300 along the flagellar axis, ensuring a large and synchronous Ca²⁺ influx to generate strong bending
301 force (Extended Data Fig. 5b). In contrast, disruption of the CatSper zigzag-rows and
302 misalignment from the longitudinal axis would dysregulate this domino-effect, thus preventing
303 efficient intracellular Ca²⁺ propagation towards the sperm head and resulting in a proximally stiff
304 flagellum and altered sperm motility (Extended Data Fig. 5b). Taken together, this work provides
305 an unprecedented structural basis for understanding the CatSper channel function in motility
306 regulation of mammalian sperm.



307

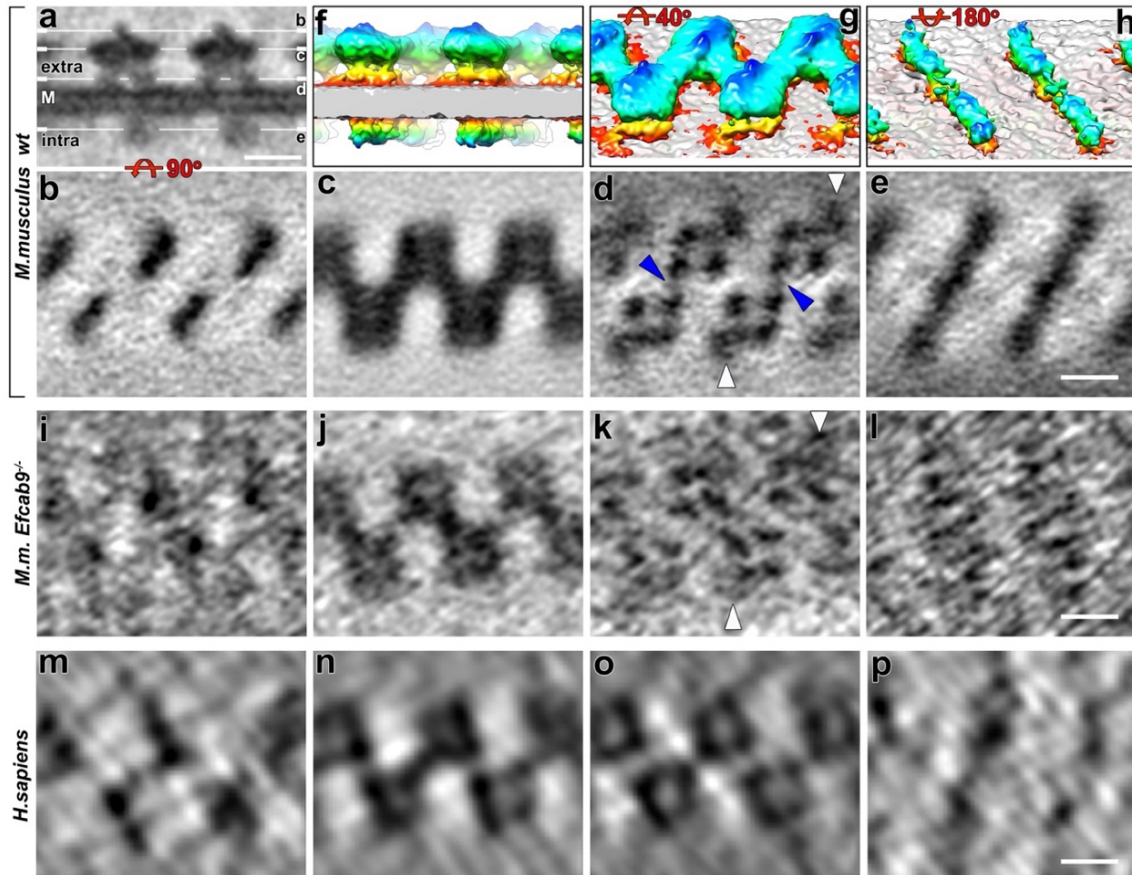
308 **Fig. 1 New CatSper components and cryo-ET of mouse sperm flagella visualizing particles**
309 **of CatSper complexes. a.** Western blot analyses of four candidate CatSper-associated proteins
310 in the whole cell proteome of mouse wild type, *Catsper1*^{-/-} and *Efcab9*^{-/-} sperm. **b-c.**
311 Immunolocalization of TRIM69 (**b**, confocal light microscopy; sperm head stained blue using
312 Hoechst) and SLCO6C1 (**c**, 3D SIM) in sperm from wild type, *Catsper1*^{-/-}, and/or *Efcab9*^{-/-} mice.
313 In (**c**) colors in *xy* projection encode the relative distance from the focal plane along the *z* axis.
314 Arrowheads in each panel indicate the annulus, the junction between the midpiece and principal
315 piece of the sperm tail. *y-z* cross sections are shown on the right. Scale bar, 10 μm in **b**; 500 nm
316 in **c** (left); 200 nm in **c** (right). **d-g.** Tomographic slices of representative principal piece regions
317 of mouse sperm flagella show CatSper complexes (arrowheads) viewed in cross section (left)
318 and longitudinal section (right): intact wild type (**d, e**) and *Efcab9*^{-/-} (**f, g**) sperm in non-
319 capacitated state. Other labels: M, membrane; ODF, outer dense fiber; FS, fibrous sheath; Ax,
320 axoneme; CP, central pair. Scale bar, 100 nm in **d-g**.



321

322 **Fig. 2 In-cell structure of the native CatSper complexes in intact sperm flagella. a-h.**

323 Representative tomographic slices of the repeating CatSper channel complexes arranged as
324 zigzag-rows along the longitudinal axis of wild type flagella (cross view from proximal to distal:
325 **a, c, e, g**; top-down view with proximal side of the flagellum on the left: **b, d, f, h**). The number
326 of zigzag-rows (arrowheads) varied from a single row (**a, b**), two rows (**c, d**), merging rows (**e,**
327 **f**), to up to five rows (**g, h**). **i-n**. Representative tomographic slices of *Efcab9*^{-/-} sperm show
328 fragmented, short CatSper complex clusters with altered orientation relative to the flagellar axis
329 (cross section view: **i, k, m**; top-down view: **j, l, n**, the direction from proximal (P) to distal (D)
330 as indicated). **o-r**. Zigzag-arrangement of CatSper in human sperm flagellum (cross section view:
331 **o**; top-down view: **p, q**; side view, **r**). Scale bar, 100 nm.

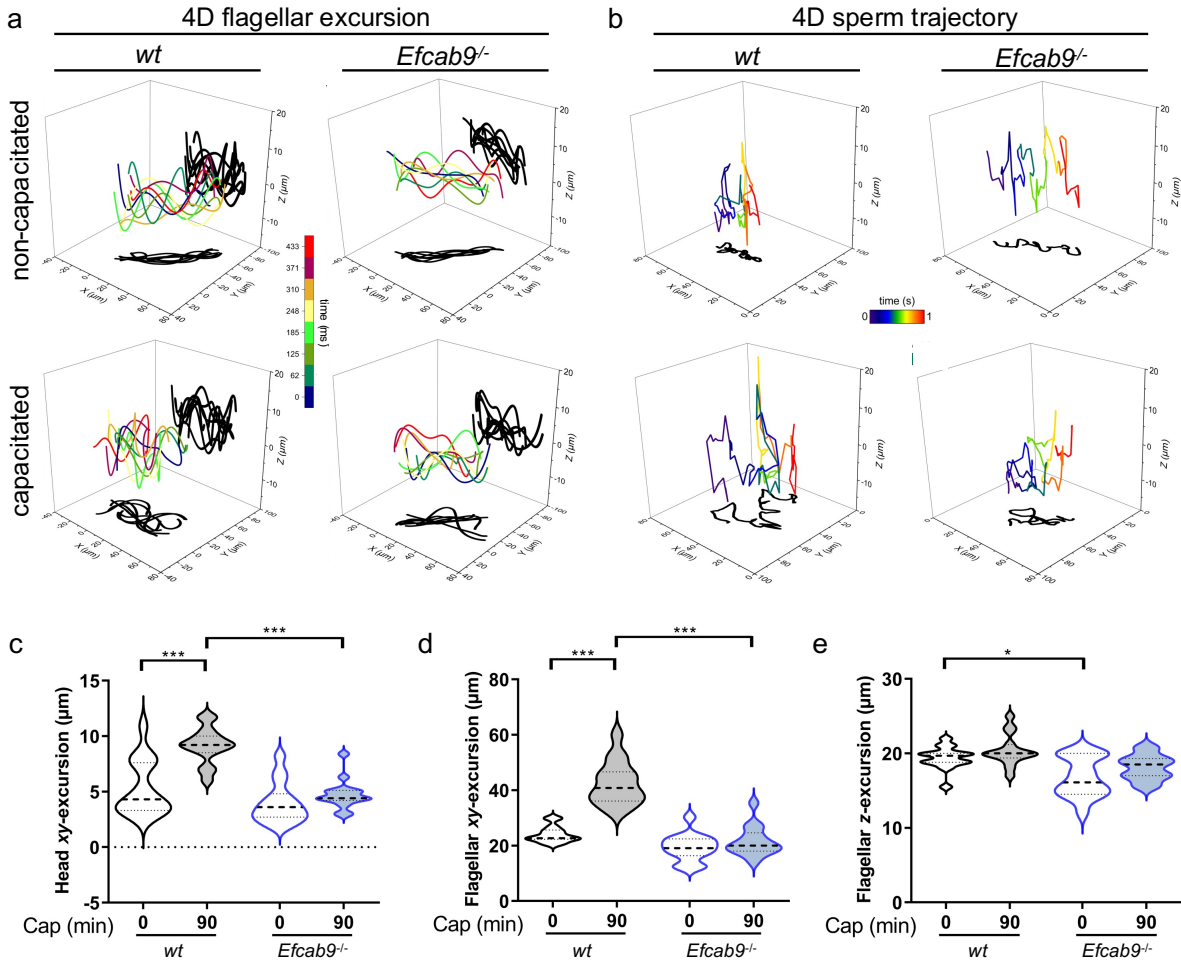


332

333 **Fig. 3 Structural features of CatSper complex in three dimensions.** a-e. Tomographic slices
334 show the averaged CatSper complex structure from wild type mouse sperm in side (a) and top
335 views (b-e). f-h. 3D isosurface renderings of the averaged CatSper complexes in wild type
336 mouse sperm: f, side view; g, extracellular domain; h, intracellular domain. i-l. Tomographic
337 slices show the averaged CatSper complex structure in *Efcab9*^{-/-} sperm in a backslash direction.
338 m-p. Tomographic slices show the averaged CatSper complex structure in human sperm
339 flagellum. Lines in (a) indicate the slice positions showing the following structural features: b, i,
340 m, roof ridge; c, j, n, canopy roof; d, k, o, tetrameric channel; e, l, p, channel intracellular
341 domain. Other labels: white arrowheads, wing structure; blue arrowheads, inner connection
342 between channel subunits, M, membrane. Scale bar, 10 nm.

343

344



346 **Fig. 4 Flagellar beating waveform of free-swimming sperm in four dimensions.** **a.** 4D
 347 flagellar beating waveform analyses of *wt* and *Efcab9*^{-/-} sperm by high-speed digital holographic
 348 microscopy (DHM). The time-lapse trace of a flagellum at 3D position (laboratory-fixed frame
 349 of reference *xyz*) is visualized in color and its projections onto *xy*- and *xz*- planes are shadowed in
 350 black. **b.** 4D sperm trajectory analyses of *wt* and *Efcab9*^{-/-} sperm by DHM. The swimming
 351 trajectory of sperm is visualized by tracing the head position. **c-e.** Statistical analyses of head (**c**)
 352 and flagellar (**d**) *xy*-excursion, and flagellar *z*-excursion (**e**) from (**a**). $n=15$ each group, $*p <$
 353 0.05 , $***p < 0.001$; the medians (thick dash lines) and interquartile ranges (thin dash lines).

354

355 **Materials and Methods**

356

357 **Human subjects**

358 A total of 3 healthy volunteers aged 25-39 were recruited for this study. Freshly ejaculated
359 semen samples were obtained by masturbation and spermatozoa purified by the swim-up
360 technique at 37°C as described in detail in²⁸. All processed samples were normozoospermic with
361 a cell count of at least 30×10^6 sperm cells per mL. The experimental procedures utilizing
362 human-derived samples were approved by the Committee on Human Research at the University
363 of California, Berkeley, IRB protocol number 2013-06-5395.

364

365 **Animals**

366 *Catsper1*^{-/-}, *Catsperd*^{-/-}, *Catsperz*^{-/-} and *Efcab9*^{-/-} mice generated in the previous studies^{5,8,9,12} are
367 maintained on a C57/BL6 background. Mice were treated in accordance with guidelines
368 approved by the Yale Animal Care and Use Committees (IACUC).

369

370 **Antibodies**

371 Rabbit polyclonal antibodies specific to mouse CATSPER1⁵, δ ⁹, and EFCAB9¹² were described
372 previously. Briefly, to produce antibodies, peptides corresponding to mouse C2CD6
373 (ALS2CR11) (359-377, EKLREKPRERLERMKEEYK) (Open Biosystems) and SLCO6C1 (1-
374 14, MAHVRNKKSDDKKA) (GenScript) were synthesized and conjugated to KLH carrier
375 protein. Antisera from the immunized rabbits were affinity-purified using the peptide
376 immobilized on Amino Link Plus resin (Pierce). Other antibodies used in this study are
377 commercially available as follows (TRIM69, Origene; Fancm, Affinity Biosciences; acetylated
378 tubulin, Sigma). All the chemicals were from Sigma Aldrich unless otherwise indicated.

379

380 **Western blot analysis**

381 Whole mouse sperm protein content was extracted as previously described^{8,9,14}. In short, mouse
382 epididymal spermatozoa washed in PBS were directly lysed in a 2×SDS sample buffer. The
383 whole sperm lysate was centrifuged at 15,000 g, 4°C for 10 min. After adjusting DTT to 50 mM,
384 the supernatant was denatured at 95°C for 10 min before loading to gel. Antibodies used for
385 Western blotting were antibodies against CATSPER1 (1 µg/mL), δ (1 µg/mL), EFCAB9 (1
386 µg/mL) and C2CD6 (1 µg/mL), TRIM69 (0.5 µg/mL), SLCO6C1 (2 µg/mL), FANCM (1
387 µg/mL) and acetylated tubulin (1:10000 µg/mL). Secondary antibodies were anti-rabbit IgG-
388 HRP (1:10,000), anti-goat IgG-HRP (1:10,000) and anti-mouse IgG-HRP (1:10,000) from
389 Jackson ImmunoResearch (West Grove).

390

391 **Sperm immunocytochemistry**

392 Sperm were washed in PBS twice, attached on glass coverslips, and fixed with 4%
393 paraformaldehyde (PFA) in PBS at room temperature (RT) for 10 minutes (mouse) or at 4°C for
394 1 hr (human). Fixed samples were permeabilized using 0.1% Triton X-100 in PBS at RT for 10

395 minutes, washed in PBS, and blocked with 10% goat serum in PBS at RT for 1 hr. Cells were
396 stained with anti-C2CD6 (10 $\mu\text{g}/\text{mL}$), TRIM69 (5 $\mu\text{g}/\text{mL}$), SLCO6C1 (10 $\mu\text{g}/\text{mL}$), FANCM (10
397 $\mu\text{g}/\text{mL}$) in PBS supplemented with 10% donkey serum at 4°C overnight. After washing in PBS,
398 the samples were incubated with donkey anti-goat Alexa 568 (Invitrogen, 1:1,000) in 10%
399 donkey serum in PBS at RT for 1 hr. Hoechst was used to counterstain nuclei for sperm head
400 visualization. Immunostained samples were mounted with Prolong gold (Invitrogen) and cured
401 for 24 hr.

402

403 **Confocal and 3D structured illumination microscopy (SIM) imaging**

404 Confocal imaging was performed on the Cured samples by a Zeiss LSM710 using a Plan-
405 Apochromat 63X/1.40 and an alpha Plan-APO 100X/1.46 oil objective lens (Carl Zeiss). 3D
406 SIM imaging was performed with a Zeiss LSM710 Elyra P1 using an alpha Plan-APO
407 100X/1.46 oil objective lens. A laser at 561 nm (200 mW) was used for Alexa 568 (Invitrogen).
408 A z-stack was acquired from 42 optical sections with a 200 nm interval. Each section was
409 imaged using 5 rotations with a 51 nm grating period. 3D SIM Images were rendered using Zen
410 2012 SP2 software.

411

412 **Sperm sample preparation for cryo-electron microscopy**

413 Epididymal spermatozoa from adult male mice (wild type and *Efcab9*^{-/-} in the C57BL/6
414 background) were collected by swim-out from caudal epididymis as described²⁹. Briefly, male
415 mice were euthanized, and the cauda isolated from the mouse carcass and placed into a 1.5 mL
416 tube with standard HEPES saline HS medium (in mM: 135 NaCl, 5 KCl, 1 MgSO₄, 2 CaCl₂, 20
417 HEPES, 5 D-glucose, 10 Lactic acid, 1 Na pyruvate, pH 7.4 adjusted with NaOH, osmolarity 320
418 mOsm/L) at room temperature. To retrieve the mature spermatozoa, the caudal epididymis was
419 cut into several pieces with a scalpel and placed into a 37°C incubator for 10-30 min to let the
420 sperm swim out into the HS buffer. Then, the sample was placed at room temperature for 30 min
421 to let the debris sediment by passive sedimentation, before separating the supernatant with
422 swimming sperm cells from the debris. The supernatant with the sperm was washed one time in
423 PBS, which involved centrifugation at 700g for 5 min at room temperature.

424

425 Human sperm were allowed to settle at the base of a conical tube and the excess buffer was
426 removed. The sperm sample was then passed three times through a Balch ball bearing
427 homogenizer (Isobiotech, 15 μm clearance).

428

429 **Cryo-sample preparation for cryo-ET**

430 Small aliquots of freshly prepared mouse sperm at a concentration of 1-5 x 10⁶ cells/mL were
431 gently mixed with 10-fold concentrated, BSA-coated 10-nm colloidal gold solution (Sigma
432 Aldrich) at 3:1 ratio, before applying 4 μL of the solution to a glow-discharged (30s at 35 mA)
433 copper R 2/2 200-mesh holey carbon grid (Quantifoil Micro Tools). The grids were blotted
434 manually from the back side with Whatman filter paper #1 for 2-4 s, before plunge-freezing the

435 grid in liquid ethane using a homemade plunge-freezer. Grids were stored under liquid nitrogen
436 until either further preparation by cryo-focused ion beam (FIB) milling or imaging by cryo-ET.
437 For mechanical support, grids were mounted into Autogrids (ThermoFisher).

438

439 3 μ l of the human sperm sample were applied to glow discharged copper R2/2 200-mesh holey
440 carbon grid (Quantifoil Micro Tools) and plunge frozen in liquid ethane using an automatic
441 plunge freezer (Vitrobot, FEI, blot force 8, blot time 8s, Whatman filter paper #1).

442

443 **Cryo-electron tomography**

444 Tilt series of whole or cryo-FIB milled mouse sperm flagella were acquired using a Titan Krios
445 (Thermo Fisher Scientific) operated at 300 keV with post-column energy filter (Gatan) in zero-
446 loss mode with 20 eV slit width. Images were recorded using a K3 Summit direct electron
447 detector (Gatan) in counting mode with dose-fractionation (12 frames, 0.05s exposure time per
448 frame, dose rate of 28 electrons/pixel/s for each tilt image). Tilt series were collected using
449 SerialEM³⁰ with the Volta Phase Plate and a target defocus of -0.5 μ m. Images were recorded at
450 26k x magnification resulting in a pixel size of 3.15 \AA . Dose-symmetric tilt series³¹ were
451 recorded under low-dose conditions, ranging from $\pm 60^\circ$ with 2° angular intervals with the total
452 electron dose limited to $\sim 100 \text{ e}^-/\text{\AA}^2$.

453

454 Frozen grids of human sperm were loaded into a Jeol3100 TEM operating at 300kV equipped
455 with an in-column energy filter and a direct electron detector (K2, Gatan). Dose-fractionated, bi-
456 directional tilt series were acquired using SerialEM³⁰ with the following parameters: angular
457 increment 1.5° , angular range about $\pm 60^\circ$ starting at -20° , energy filter slit width 30 eV,
458 nominal magnification 10k x resulting in a detector pixel size of 3.98 \AA (which was binned by x2
459 resulting in a pixel size of 7.96 \AA in the reconstruction), defocus -2.5 μ m, exposure time 1s x
460 $1/\cos(\text{tilt angle})$, fraction interval 0.2 s, dose rate $1 \text{ e}^-/\text{\AA}^2/\text{s}$, total dose $\sim 80 \text{ e}^-/\text{\AA}^2$.

461

462 **Cryo-FIB milling of mouse sperm**

463 For lamella (section) prepared by cryo-FIB-milling, clipped grids (modified Autogrids with FIB
464 notch) with plunge-frozen mouse sperm were transferred to an Aquilos dual-beam instrument
465 with cryo-sample stage (Thermo Fisher Scientific). Two layers of platinum were added to the
466 sample surface to enhance sample protection and conductivity (sputter-coater: 1 keV and 30 mA
467 for 20s; gas injection system (GIS): when needed, heated up to 28°C , and then deposited onto the
468 grid for 15 seconds)³². Scanning electron beam imaging was performed at 2 kV and 25 pA, and
469 Gallium ion beam imaging for targeting was performed at 30 kV and 1.5 pA. The target region,
470 *i.e.* a sperm flagellum, was oriented for milling by tilting the cryo-stage to a shallow angle of 14
471 $- 16^\circ$ between the ion beam and the grid. Cryo-FIB milling was performed using a 30 keV
472 gallium ion beam with a current of 30 pA for bulk milling, 30 pA for thinning, and 10 pA for
473 final polishing, resulting in 100-200 nm thick self-supporting lamella, that could then be imaged
474 by cryo-ET.

475

476 **Image processing of cryo-ET data**

477 For tilt series of both mouse and human sperm flagella, movie frames were aligned using
478 Motioncor2 1.2.3³³. The IMOD software³⁴ was used to align the tilt serial images using the 10-
479 nm gold particles as fiducial markers and to reconstruct the tomograms by weighted back-
480 projection. For subtomogram averaging, the repeating units were picked manually from raw
481 tomograms. The repeat orientation was determined based on the polarity of the axoneme at the
482 core of the sperm flagella. The alignment and missing-wedge-compensated averaging were
483 performed using the PEET software³⁵. After initial averaging a two-fold symmetry was applied.
484 Visualization of the 3D structures of the averaged repeat units was done using the UCSF
485 Chimera software package³⁶. Mass estimations from a repeat unit in the subtomogram averages
486 were calculated using the average density of 1.43 g/cm³ for proteins³⁷ and normalization of the
487 isosurface-rendering threshold in Chimera. The number of tomograms of whole cell and lamella,
488 number of averaged repeats and estimated resolutions of the averages (using the FSC 0.5
489 criterion), are summarized in Extended Data Table 2.

490

491 **Analysis of mouse sperm motility and flagellar beating in 4D**

492 Epididymal spermatozoa from adult male mice were collected by swim-out from caudal
493 epididymis in standard HEPES saline HS medium.

494

495 For 4D analysis, mouse sperm were washed twice in HS medium and resuspended to a final
496 concentration of 1-2 x 10⁶ cells/mL either under non-capacitating (HS medium) or under
497 capacitating (HS medium, 15 mM NaHCO₃⁻, 5 mg/mL BSA) conditions. To induce capacitation
498 *in vitro*, sperm were incubated for 90 min at 37°C and 5% CO₂. 4D motility analysis was done at
499 37°C and 5% CO₂ using an off-axis transmission digital holographic microscope DHMTM T-1000
500 (Lyncée Tec SA, Geneva, Switzerland) equipped with a 666 nm laser diode source, a 20x/0.4
501 NA objective and a Basler aca1920-155um CCD camera (Basler AG, Ahrensburg, Germany).
502 Holographic imaging was performed as previously described²⁷. In short, mouse sperm were
503 placed in a 100 µm deep chamber slide (Leja) and were recorded at 100 Fps. Offline processing
504 was done using proprietary Koala (Vers. 6; Lyncée Tec SA) and open-source Spyder (Python
505 3.6.9) software. Using Koala software, *xy*-plane (parallel to the objective slide) projection images
506 of sperm were numerically calculated at different focal planes (*z*-height)^{38,39}, followed by sperm
507 head tracking using Spyder to receive *x*, *y* and *z*-coordinates for the entire trajectory. Using these
508 coordinates, motility parameters including 3D curvilinear velocity (VCL, in µm/s) and 2D
509 amplitude of lateral head displacement (ALH, in µm) were analyzed. For each condition, 15 free-
510 swimming single sperm were analyzed using three males from each genotype (wild type, *Efcab9*
511 ^{-/-}, *Catsper1*^{-/-}).

512

513 For 4D flagellar beating analysis, a macro written in Igor ProTM Vers. 6.36 (Wavemetrics) was
514 used to perform frame-by-frame tracking of flagellar images in stacks of reconstructed *xy*-

515 projections (8-bit TIFF format, 100 Fps, 10 Frame Time) with a resolution of 800 x 800 pixels as
516 well as automatic brightness and contrast adjustments applied by ImageJ V1.50i (National
517 Institutes of Health). A P/U value (3.7466), which is defined as the quotient from the objective
518 magnification (20x) and the pixel size (5.34 μm) of the camera (Basler aca1920-155um), was
519 used to convert pixel to micrometer. Calculation of z -coordinates was performed utilizing the
520 received x , y -coordinates from flagellar traces and Koala. A specific script in Spyder was used to
521 load flagellar x , y -coordinates into Koala. Smoothing of z -plane data was conducted with Igor
522 ProTM by fitting to 7th order polynomials. The determination of the distance along the flagellum
523 in the xy projections (Dx , y) was carried out geometrically from adjacent pairs of x , y -
524 coordinates, also using a macro in Igor ProTM.

525
526 4D visualization of sperm flagellum and sperm swimming trajectories with respect to the
527 laboratory fixed frame of reference (x , y , z) was done using OriginPro 2020 software (OriginLab
528 Corporation). Therefore x , y , and z -coordinates of head and flagellar tracking were imported to
529 the software. Analysis was performed for one whole beat-cycle, but for better illustration only,
530 every 6th flagellar excursion between maxima of one beat cycle was illustrated (frame 0, 6, ...,
531 42, every 60 ms) in Fig. 4. The associated movies (Extended Data Videos 3 and 4) of
532 reconstructed trajectories of free-swimming single sperm were created with Cinema4D Vers. 18
533 (Maxon) using x , y , and z -values of the 4D head tracking. Adobe After Effects software Vers.
534 CS6 (Adobe Systems Software Ireland Limited) was used for video composing and time duration
535 adding. In each supporting video two different perspectives were used to show the 3D movement
536 of sperm during 1s record. The rolling ball represents the sperm head, and the color code of the
537 trajectory displays the z -excursion.

538
539 **Quantification and statistical Analysis** Statistical analyses were carried out with GraphPad
540 Prism 9 (Statcon GmbH) by using a two-way analysis of variance (ANOVA). Differences were
541 considered significant at $p < 0.05$. Numerical results are presented as medians and interquartile
542 ranges with n = number of determinations and N = number of independent experiments.

543
544 **Data and software availability**

545 The averaged 3D structure of CatSper channel from mouse wild type sperm flagella has been
546 deposited in the Electron Microscopy Data Bank (EMDB) under accession code EMD-24210.

547
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565

566 **Author Contributions**

567 J.-J.C. and P.L. conceived the study. D.N., and J.-J.C. designed and oversaw the project. H.W.,
568 J.Y.H., and X.H. performed biochemical characterization of novel CatSper components and
569 confocal and SR imaging experiments. Y.Z., H.W., and N.B.S. performed EM sample
570 preparation and screening. Y.Z., D.N. (mouse sperm) and N.B.S., K.M.D. (human sperm)
571 performed cryo-ET, Y.Z. performed subtomogram averaging and 3D visualization. E.R.
572 performed cryo-FIB milling. C.W. performed sperm motility experiments and 4D flagellar
573 beating analysis. Y.Z., H.W., and C.W. made figures. Y.Z., H.W., P.L., K.M.D., G.W., D.N., and
574 J.-J.C. interpreted data. H.W., and J.-J.C. prepared the initial draft of the manuscript. Y.Z., H.W.,
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577

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579

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