

1 Systematic studies of all PIH proteins in zebrafish
2 reveal their distinct roles in axonemal dynein assembly
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25 **Abstract**

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27 Construction of motile cilia/flagella requires cytoplasmic preassembly of axonemal dyneins before transport into
28 cilia. Axonemal dyneins have various subtypes, but the roles of each dynein subtype and their assembly processes
29 remain elusive in vertebrates. The PIH protein family, consisting of four members, has been implicated in the
30 assembly of different dynein subtypes, although evidence for this idea is sparse. Here, we established zebrafish
31 mutants of all four PIH-protein genes: *pih1d1*, *pih1d2*, *ktu*, and *twister*, and analyzed the structures of axonemal
32 dyneins in mutant spermatozoa by cryo-electron tomography. Mutations caused the loss of specific dynein subtypes,
33 which was correlated with abnormal sperm motility. We also found organ-specific compositions of dynein subtypes,
34 which could explain the severe motility defects of mutant Kupffer's vesicle cilia. Our data demonstrate that all
35 vertebrate PIH proteins are differently required for cilia/flagella motions and the assembly of axonemal dyneins,
36 assigning specific dynein subtypes to each PIH protein.

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49 **Introduction**

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51 Motile cilia/flagella are hair-like organelles that project from various types of eukaryotic cells. In humans,
52 malfunctions of motile cilia often cause primary ciliary dyskinesia (PCD), a syndrome characterized by recurrent
53 respiratory infections, male infertility, hydrocephalus, and inversion of visceral laterality (Knowles et al., 2013;
54 Brown and Witman, 2014). Motile cilia have a microtubule-based structure called an axoneme, consisting of nine
55 peripheral doublet microtubules (DMTs) with or without central-pair microtubules (so called 9+2, 9+0,
56 respectively). Ciliary motility is driven by axonemal dyneins, which have multiple subtypes such as outer arm
57 dyneins (OADs) and seven different types of inner arm dyneins (IADs; IAD a to g; Kagami and Kamiya, 1992).
58 Biochemical analyses of green algae *Chlamydomonas* revealed that each axonemal dynein consists of multiple
59 subunits (Hom et al., 2011; Sakato and King, 2004); OAD is composed of three heavy chains (α -, β -, and γ -HC),
60 two intermediate chains (IC1 and IC2), and ten light chains. Six types of IADs (IAD a, b, c, d, e, and g) have single
61 HCs with several light chains such as p28, centrin, and actin. IAD f has two heavy chains (f α - and f β -HC), four
62 intermediate chains, and five light chains.

63 In the process of ciliary construction, axonemal dyneins and all other large ciliary molecules are synthesized
64 in the cytoplasm and undergo gated entry into the ciliary compartment (Takao and Verhey, 2016). In the cytoplasm,
65 the components of OADs and IADs are detected as preassembled complexes, rather than individual components
66 (Fok et al., 1994; Fowkes and Mitchell, 1998; Viswanadha et al., 2014). This cytoplasmic preassembly of axonemal
67 dyneins requires various proteins collectively called dynein axonemal assembly factors (DNAAFs; Kobayashi and
68 Takeda, 2012; Mitchison et al., 2012). As cilia/flagella require multiple types of axonemal dyneins for their motions
69 (Kamiya, 1995), proper assembly of each dynein complex is essential for ciliary motility. However, the assembly
70 processes of each dynein subtype and their roles in cilia/flagella motions remain elusive in vertebrates.

71 The PIH protein family has been implicated in the preassembly of different subsets of axonemal dyneins. The
72 PIH protein, which contains a PIH1-domain, was first identified in budding yeast (*Saccharomyces cerevisiae*) as an

73 interactor of HSP90 and named as Pih1 (Protein Interacting with HSP90; also known as Nop17; Zhao et al., 2005;
74 Gonzales et al., 2005). Yeast Pih1 is required for the assembly of various multi-subunit protein complexes, but is
75 not involved in the assembly of axonemal dyneins, as yeast do not have a cilium. In vertebrates, there are four PIH
76 proteins: PIH1D1, PIH1D2, KTU/DNAAF2, and PIH1D3/TWISTER (Fig. 1 A), and PIH1D1 is the orthologue of
77 yeast Pih1. Similar to yeast, the human PIH1D1 is a subunit of the R2TP complex (RUVBL1, RUVBL2,
78 RPAP3/Tah1, and PIH1D1), which interacts with HSP90 to promote assembly of various protein complexes for
79 cellular activities such as box C/D snoRNP and RNA polymerase II (Kakihara and Houry, 2012).

80 KTU/DNAAF2 is the first protein that was identified as a DNAAF (Omran et al., 2008). Genetic and
81 biochemical analyses of KTU/DNAAF2 in medaka (Japanese killifish), human, and *Chlamydomonas* revealed that
82 KTU/DNAAF2 is required for the assembly of OAD and a subset of IADs (Omran et al., 2008). Subsequently, the
83 function of *Chlamydomonas* MOT48, a possible orthologue of vertebrate PIH1D1, was reported; MOT48 is one of
84 three PIH proteins in *Chlamydomonas* (MOT48, PF13/KTU, and TWI1) and is involved in the assembly of another
85 subset of IDAs (Yamamoto et al., 2010). These pioneering studies proposed that preassembly of different subsets of
86 axonemal dyneins is mediated by distinct PIH proteins. However, evidence for this hypothesis is sparse, due to a
87 lack of systematic studies of all PIH proteins. Although PIH1D3/TWISTER recently turned out to be one of the
88 DNAAFs (Dong et al., 2014; Paff et al., 2017; Olcese et al., 2017), the function of vertebrate PIH1D1 and PIH1D2
89 has not been addressed in terms of ciliogenesis and ciliary motility. Furthermore, two *Pih1d3* paralogues in mice,
90 *Pih1d3* and *Twister2*, are differently expressed in ciliary/flagellar organs (*Pih1d3* for testis, while *Twister2* for both
91 testis and the others; Dong et al., 2014), suggesting a divergence of their functions.

92 In this study, we performed systematic and functional analyses of all four PIH genes (genes encoding PIH
93 proteins; Fig. 1 B) by generating zebrafish (*Danio rerio*) mutants of each PIH gene. We compared the functions of
94 all PIH proteins in one platform, zebrafish, because functional divergence of PIH proteins among organisms is
95 highly probable. Although zebrafish is often used to analyze the functions of PCD related genes, the detailed
96 structure of their cilia/flagella has not been studied so far. We applied cryo-electron tomography (cryo-ET) for the

97 first time to zebrafish sperm, which enabled us to observe the detailed structure of wild-type and mutant axonemal
98 dyneins. Mutations of each PIH gene caused defects of different subtypes of axonemal dyneins, which was
99 correlated with abnormal sperm motility. Interestingly, some mutants showed different phenotypes of ciliary
100 motility between sperm flagella and Kupffer's vesicle cilia. Together with different expression patterns of various
101 DNAH (dynein axonemal heavy chain) genes, we also discuss the organ-specific compositions of axonemal
102 dyneins assembled by PIH proteins. This is the first report that shows all vertebrate PIH proteins as DNAAFs,
103 assigning their functions to specific types of axonemal dyneins and to cilia/flagella motions. Our data provide
104 evidence for the above-mentioned hypothesis that the cytoplasmic assembly of different dynein subtypes is
105 mediated by distinct PIH proteins.

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121 **Results**

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123 **Generation of zebrafish mutants of the *pih1d1*, *pih1d2*, *ktu* and *twister* genes**

124 To find all PIH proteins encoded in the zebrafish genome, we performed BLASTp search with the consensus
125 sequence of PIH proteins as a query. Although teleost fish are known to have undergone an additional genome
126 duplication (Kasahara et al., 2007), only four hits were obtained, similar to the human genome and consistent with a
127 previous report (Yamamoto et al., 2010). We thus conclude that zebrafish has four PIH proteins: Pih1d1, Pih1d2,
128 Ktu, and Twister. Their domain structures are well conserved among vertebrates (Fig. 1 A; Fig. S1 A and B).

129 Transcripts of these four PIH genes were all detected in ciliated organs such as Kupffer's vesicle, floor plate,
130 otic vesicle and pronephric duct (Fig. 1, D-G), suggesting their involvement in ciliary functions. The transcript of
131 *pih1d1* was also detected in the whole body of 12 hpf (hours post-fertilization) embryos (Fig. 1 D; black asterisk),
132 which is consistent with the reported cellular functions of human PIH1D1 (Kakihara and Houry, 2012). *ktu* was
133 also expressed in brain rudiments at 32 hpf (Fig. 1 D; black arrowhead), but KTU function in brain was reported
134 only for ciliated ependymal cells in mouse (Matsuo et al., 2012). These results suggest that *pih1d2*, *ktu*, and *twister*
135 have cilia specific functions, while *pih1d1* has ubiquitous cellular functions in addition to ciliary function.

136 Since DNAAFs including PIH proteins are known to be localized to the cytoplasm (Kobayashi and Takeda,
137 2012), we examined the subcellular localizations of zebrafish PIH proteins by immunoblot analysis, using specific
138 polyclonal antibodies made in this study. All PIH proteins were detected in both testis lysate and sperm lysate.
139 However, when spermatozoa were fractionated into sperm heads and flagella, PIH proteins were detected only in
140 the sperm head fraction, indicating that like other DNAAFs, zebrafish PIH proteins are indeed specifically present
141 in the cytoplasm, but not in the flagellar compartment (Fig. 1 C; asterisk).

142 To analyze the functions of the four PIH proteins in zebrafish, we generated mutant alleles by
143 genome-editing with TALEN (*pih1d1* and *pih1d2*) or CRISPR/Cas9 (*ktu* and *twister*) (Fig. 1 B; Fig. S1, C and D).
144 Immunoblot analysis confirmed that all mutations resulted in a null of each PIH protein (Fig. S1 E). Since

145 homozygous mutants of each PIH gene were viable, we established homozygous mutant lines. These were used in
146 the following experiments.

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148 ***pih1d1*^{-/-}, *twister*^{-/-}, and double mutant of *pih1d2*^{-/-};*ktu*^{-/-} showed abnormal sperm motility**

149 Ciliary functions of PIH genes were first examined by observing the motility of mutant spermatozoa using a
150 high-speed camera. Spermatozoa whose heads were attached to a coverslip were selected and subjected to the
151 analyses of beating frequencies and waveforms. In *pih1d1*^{-/-}, spermatozoa showed a slight reduction of beating
152 frequency (Fig. 2 G), and propagation of flagellar bending was disturbed, as slopes of shear angle curves changed
153 between traces (Fig. 2 B'; asterisk). In *twister*^{-/-}, almost all spermatozoa were immotile, but a few were found to be
154 motile with decreased beating frequencies and severely disturbed waveforms (Fig. 2 E). In *pih1d2*^{-/-} and *ktu*^{-/-}, a
155 significant difference was not observed in either beating frequencies or waveforms (Fig. 2, C and D). We suspected
156 functional compensation of these two genes, and thus generated double mutants of *pih1d2*^{-/-};*ktu*^{-/-}. Double mutant
157 spermatozoa exhibited abnormal waveforms; motile in the proximal half, while immotile in the distal half (Fig. 2 F).
158 In the proximal region, beating frequency was about twice as high as that of wild type (Fig. 2 G). This could be
159 caused by the reduction of sliding distance of DMTs, rather than the change of the sliding velocity of DMTs,
160 because the slopes of shear angle curves was decreased (Fig. 2 F'; dotted line), indicating that the bending of the
161 proximal flagella was smaller than that of wild type. We also analyzed the length of sperm flagella, but did not find
162 any significant differences between wild type and PIH gene mutants (Fig. S2 C).

163 We then examined the motility of free swimming spermatozoa by CASA (computer-assisted sperm
164 analysis) modified for zebrafish (Wilson-Leedy and Ingermann, 2007). In this analysis, traced paths of swimming
165 sperm heads were used to calculate sperm motility (Fig. S2 B). In all mutants, the ratios of motile (locomotive)
166 spermatozoa were significantly decreased, and no sperm in *twister*^{-/-} showed significant locomotion (Fig 2 H). For
167 motile spermatozoa, swimming velocity did not change in *pih1d1*^{-/-}, *pih1d2*^{-/-}, and *ktu*^{-/-}, but significantly decreased
168 in *pih1d2*^{-/-};*ktu*^{-/-} (Fig 2 I). The beating frequencies of sperm heads were decreased in *pih1d1*^{-/-}, but increased in

169 *pih1d2*^{-/-};*ktu*^{-/-} (Fig 2 J), which is consistent with Fig. 2 G.

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171 **Cryo-ET revealed native ultrastructure of zebrafish sperm axoneme**

172 To observe the ultrastructure of zebrafish axoneme, we applied cryo-ET to zebrafish spermatozoa. The axoneme of
173 zebrafish sperm had the characteristic 9+2 arrangement of DMTs surrounding central-pair microtubules (Fig. S3 A).

174 To analyze the structure of DMTs in more detail, subtomographic averaging was applied using a 96-nm repeat of
175 DMT as one particle assuming nine-fold rotational symmetry of the axoneme. The averaged structure of zebrafish
176 DMT exhibited overall similarity to that of other organisms (Fig. 3, C and D). Thus, based on the well-studied
177 structure of the *Chlamydomonas* axoneme (Bui et al., 2012), we assigned the structures of OADs, seven types of
178 IADs, radial spokes (RSs), and nexin-dynein regulatory complex (N-DRC) in the zebrafish axoneme (Fig. 3, A and
179 B).

180 To address the evolutionary conservation and diversity of cilia/flagella, we compared the ultrastructure of
181 zebrafish axoneme to that of *Chlamydomonas* and human axonemes in more detail. Compared with
182 *Chlamydomonas*, zebrafish axoneme does not have OAD α -HCs, but has longer RS3 (Fig. 3, A and D). A linker
183 between N-DRC and OAD is not observed in the zebrafish axoneme unlike *Chlamydomonas* (Fig. 3 D; red
184 arrowhead). These features of the zebrafish axoneme are also found in human respiratory cilia (Fig. 3 C; Lin et al.,
185 2014). The same features were also reported in mouse respiratory cilia (Ueno et al., 2012) and found in sea urchin
186 spermatozoa (Lin et al., 2012), indicating that these features are common among metazoans.

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188 **Mutations of each PIH gene caused structural defects of different subtypes of axonemal dyneins**

189 To gain structural insights into abnormal motility of mutant spermatozoa, we observed the structure of mutant
190 axonemes by cryo-ET and subtomographic averaging. Compared to wild type, mutant axonemes exhibited
191 structural defects of various types of axonemal dyneins (later summarized in Fig. 7 A). While no significant
192 difference was observed in *pih1d2*^{-/-} (Fig. 3 G), IAD c was missing in *pih1d1*^{-/-} (Fig. 3 F). In *ktu*^{-/-}, smaller IAD c

193 density was observed, suggesting that IAD c is partially missing in *ktu*^{-/-} spermatozoa (Fig. 3 H). In *twister*^{-/-},
194 reflecting severe motility defects, OADs and IAD c were missing and smaller IAD g and d were observed (Fig. 3 I).
195 Intriguingly, in *pih1d2*^{-/-};*ktu*^{-/-}, averaging of all DMT particles did not converge into one structure, thus tomograms
196 were classified as follows. We noticed that out of nine tomograms of axonemes, four axonemes had OADs but five
197 lacked OADs. Using this difference, we divided axonemes into two classes (+OAD and -OAD) and averaged,
198 respectively. The +OAD class possessed a full set of axonemal dyneins, except for a smaller IAD c, like the *ktu*^{-/-}
199 axoneme (Fig. 3 J). By contrast, the -OAD class lost not only OADs, but also IAD b, c, and e (Fig. 3 K). Although
200 we found structural defects of axonemal dyneins, no significant defect was observed in other DMT structures, such
201 as RSs, in all mutants we examined.

202 To correlate the structural defects of mutants with biochemical data, we performed immunoblot analysis
203 of axonemal dynein components (Fig. 4 A). We made specific antibodies against zebrafish Dnah8 (OAD γ -HC) and
204 Dnah2 (IAD f β -HC). Dnai1 is a component of OADs and is also known as IC1. Dnali1 is the orthologue of
205 *Chlamydomonas* p28, which is the subunit of three types of IADs: IAD a, c, and d (Piperno *et al.*, 1990; Hom *et al.*,
206 2011). Consistent with the above structural analysis, Dnah8 and Dnai1 were missing from the axoneme of *twister*^{-/-}
207 (Fig. 4 A; asterisks). In *pih1d2*^{-/-};*ktu*^{-/-}, the amount of Dnah8 and Dnai1 was decreased, possibly reflecting the
208 presence of the two types of DMT structures (+OAD and -OAD). Dnah2 was not affected in any mutants, and so
209 was the case of IAD f in the structural analysis. Dnali1 was slightly decreased in *pih1d1*^{-/-}, *ktu*^{-/-}, and *pih1d2*^{-/-};*ktu*^{-/-}
210 (Fig. 4 A; filled circles), confirming the loss of IAD c (one of three IADs containing p28 in *Chlamydomonas*) in
211 these mutants. In *twister*^{-/-}, the structural analysis revealed the loss of IAD c and d (two of three IADs containing
212 p28), and the amount of Dnali1 was strongly reduced. Interestingly, shifted bands of Dnai1 were observed in
213 *pih1d1*^{-/-} and *pih1d2*^{-/-} (Fig. 4 A; open circles), indicating abnormal construction of OADs in these mutants.
214 However, the structure of OADs in these mutants appeared normal as far as our structural analysis showed at the
215 current resolution. Taken together, all biochemical results are largely consistent with our structural data.

216 From these results, we conclude that all PIH proteins are responsible for the assembly of specific

217 subtypes of axonemal dyneins. Together with their specific cytoplasmic localizations, we identified all vertebrate
218 PIH proteins (not only Ktu and Twister, but also Pih1d1 and Pih1d2) as DNAAFs. Abnormal sperm motility
219 observed in *pih1d1*^{-/-}, *twister*^{-/-} and *pih1d2*^{-/-};*ktu*^{-/-} can be explained by the loss of specific subtypes of axonemal
220 dyneins. On the other hand, spermatozoa of *pih1d2*^{-/-} and *ktu*^{-/-} appeared to have normal motility, although the
221 structural or biochemical analyses revealed abnormal axonemal dyneins in these mutants. A likely explanation for
222 this discrepancy is that the defects of axonemal dyneins in *pih1d2*^{-/-} or *ktu*^{-/-} spermatozoa are so subtle that other
223 normal axonemal dyneins can compensate their loss of function. However, it is worth noting that the affected
224 axonemal dyneins were different between *pih1d2*^{-/-} (OAD Dnai1) and *ktu*^{-/-} (IAD c), which indicates distinct
225 functions of Pih1d2 and Ktu, although functional compensation of these two genes was also revealed by
226 *pih1d2*^{-/-};*ktu*^{-/-}.

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228 ***pih1d2*^{-/-};*ktu*^{-/-} spermatozoa have different axonemal structures between proximal and distal regions**

229 The two types of DMT structures in *pih1d2*^{-/-};*ktu*^{-/-}, (Fig. 3, J and K; +OAD and -OAD classes) led us to examine
230 their distribution in the mutant axoneme. For this, we stained mutant spermatozoa with the anti-Dnah8 (OAD
231 γ -HC) antibody (Fig. 4 B). In wild type, Dnah8 was localized along the entire length of the flagellum. However, in
232 *pih1d2*^{-/-};*ktu*^{-/-}, Dnah8 was consistently absent in the distal region, while it remained in the proximal (Fig. 4 B;
233 white arrowhead). Thus, the +OAD class structure was localized in the proximal region, while the -OAD class was
234 in the distal. We also analyzed the localization of Dnah8 in other mutants. Consistent with our structural analysis, in
235 *pih1d1*^{-/-}, *pih1d2*^{-/-}, and *ktu*^{-/-} spermatozoa, Dnah8 was normally distributed along the entire length of their flagella,
236 while *twister*^{-/-} spermatozoa completely lost Dnah8.

237 Different structural defects of IADs were also observed between the +OAD and -OAD classes. To assess
238 the distribution of IADs in *pih1d2*^{-/-};*ktu*^{-/-}, we analyzed the structure of proximal and distal axoneme directly.
239 Among many cryo-prepared *pih1d2*^{-/-};*ktu*^{-/-} axonemes, we found one axoneme suitable for observing both proximal
240 and distal regions by cryo-ET (Fig. S4 A). Although the obtained subtomograms are noisy due to a smaller number

241 of averaged particles, the DMT structure of a proximal subtomogram possessed OADs and the densities of all IADs,
242 consistent with the structure of +OAD class (Fig. S4, B and D). On the other hand, the DMT structure of a distal
243 subtomogram lost OADs, IAD b, c, and e, which corresponds to -OAD class (Fig. S4, C and E). Therefore, the
244 distribution of not only OADs but also IADs is different between the proximal and distal regions in *pih1d2^{-/-};ktu^{-/-}*
245 spermatozoa.

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247 **Kupffer's vesicle cilia showed different ciliary phenotypes from sperm flagella**

248 In humans, organ-specific compositions of OAD HCs were reported between sperm flagella and respiratory cilia
249 (Fig. 7 E; Fliegauf et al., 2005; Dougherty et al., 2016), and mouse *Pih1d3* was reported as a testis-specific gene
250 (Dong et al., 2014). To assess organ-specific functions of zebrafish PIH proteins, we focused on a second ciliated
251 organ, Kupffer's vesicle, which is orthologous to the mammalian embryonic node. In Kupffer's vesicle, epithelial
252 cells project mono-cilia that have rotational motility to produce leftward fluid flow in the organ (Fig 5, A and B).
253 Like in the mouse node, this leftward flow is required for the determination of visceral asymmetry, and thus defects
254 of Kupffer's vesicle cilia cause abnormal left-right patterning of the fish (Essner et al., 2005).

255 Mutations of each PIH gene caused abnormal motility of Kupffer's vesicle cilia. To describe ciliary
256 motility, we categorized motion patterns into three classes: rotating, irregular, and immotile (Fig. 5, C and D).
257 Rotational frequencies were measured from rotating class cilia (Fig. 5 E). The resulting left-right patterning of
258 embryos was assessed by observing the direction of heart looping (normally rightward; Fig. 5, F and G). In
259 *pih1d1^{-/-}*, rotational frequencies of cilia were significantly reduced, but almost all cilia were motile and the ratio of
260 heart-looping reversal was not largely affected. By contrast, in *twister^{-/-}* and *pih1d2^{-/-};ktu^{-/-}*, all cilia were immotile,
261 leading to complete randomization of their left-right patterning. In *pih1d2^{-/-}* and *ktu^{-/-}*, the proportions of rotating
262 class cilia were decreased to ~40% and ~15%, respectively, with reduced rotational frequencies, resulting in
263 significant levels of heart-looping defects (Fig. 5 D). Regarding the structure and localization of axonemal dyneins,
264 due to technical difficulties, we were unable to apply cryo-ET and immunohistochemistry with anti-dynein

265 antibodies to the axonemes of Kupffer's vesicle cilia.

266 Together with sperm analyses, we conclude that all PIH genes of zebrafish are essential for normal
267 motility of both sperm flagella and Kupffer's vesicle cilia. Intriguingly, however, in *pih1d2*^{-/-} and *ktu*^{-/-}, only
268 Kupffer's vesicle cilia showed motility defects, while sperm flagella beat normally, indicating that Pih1d2 and Ktu
269 have organ-specific functions.

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271 **Testis and Kupffer's vesicle showed different expression patterns of DNAH genes**

272 The organ-specific phenotypes of PIH mutants could reflect organ-specific compositions of axonemal dyneins. To
273 address this, we performed whole-mount *in situ* hybridization of various DNAH genes. Zebrafish have three OAD
274 β -HC genes: *dnah9*, *dnah9l*, and *dnah11*, and two OAD γ -HC genes: *dnah5* and *dnah8*. As for IAD, *dnah2* is an
275 IAD β -HC gene, and *dnah3* and *dnah7l* are other IAD HC genes.

276 Zebrafish embryos and testes showed distinct expression patterns of DNAH genes (Fig. 6 A). When
277 comparing Kupffer's vesicle and testis, *dnah11* expression was specifically detected in Kupffer's vesicle, while
278 *dnah8* and *dnah3* were specifically detected in testis (Fig. 6 B). At the embryonic stages, *dnah9l* and *dnah8* were
279 detected only in the otic vesicle and the pronephric duct, respectively, which also suggested specific combinations
280 of DNAH genes in these organs. These results indicate that components of axonemal dyneins are indeed
281 organ-specific. Intriguingly, however, Kupffer's vesicle and floor plate, whose cilia exhibit similar rotational
282 motility (Kramer-Zucker et al., 2005), showed the same expression patterns of DNAH genes. It is likely that the
283 same transcriptional regulation is required in Kupffer's vesicle and floor plate to construct similar types of cilia.

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289 **Discussion**

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291 In the process of ciliary construction, the PIH protein family has been implicated in the preassembly of different
292 subsets of axonemal dyneins, but thus far there has been insufficient evidence to support this idea. We performed
293 the systematic analysis of all PIH proteins using zebrafish, and demonstrated that all of vertebrate PIH proteins
294 (including two novel ciliary factors: Pih1d1 and Pih1d2) are required for cilia/flagella motions and the assembly of
295 axonemal dyneins. Each PIH protein was responsible for the construction of different subsets of axonemal dyneins,
296 suggesting the cytoplasmic assembly pathways for different axonemal dyneins through distinct PIH proteins.

297

298 **PIH proteins are required for the constructions of specific subsets of axonemal dyneins**

299 Our cryo-ET and biochemical analyses revealed that the PIH proteins are required for the assemblies of specific
300 subsets of axonemal dyneins (Fig. 7 C): Pih1d1 for OAD (Dnai1 construction) and IAD c; Pih1d2 and Ktu for OAD,
301 IAD b, c, and e; and Twister for OAD, IAD c, g, and d. In *Chlamydomonas*, the mutation of KTU/PF13 affected the
302 assembly of OAD and IAD c, while the mutation of MOT48 affected the assembly of OAD, IAD b, c, d, and e
303 (Yamamoto et al., 2010). Although the affected subtypes of axonemal dyneins are not the same, the mutations of
304 PIH genes resulted in the loss of specific subsets of axonemal dyneins in both organisms. Remarkably, OAD and
305 IAD c are most sensitive to the mutations of various PIH genes in both zebrafish and *Chlamydomonas*. Consistent
306 with this, Dong et al. (2014) suggested that the assembly of OAD proceeds in a stepwise manner mediated by
307 different PIH proteins. This might also be the case for IAD c, though no experimental evidence has been obtained.

308 In our research, IAD a or f were not affected in any PIH gene mutants (Fig. 7 C), which was essentially
309 the same as in *Chlamydomonas* mutants of KTU/PF13 and MOT48. This suggests that these axonemal dyneins are
310 assembled independently of PIH proteins. Alternatively, multiple PIH proteins redundantly participate in their
311 assembly. IAD a or f may not be constructed automatically, because a defect of DYX1C1 (a known DNAAF other
312 than PIH family proteins; Tarkar et al., 2013) affects the normal assembly of all types of IADs in *Chlamydomonas*

313 (Yamamoto et al., 2017). Double, triple or quadruple mutants of PIH genes will be needed to answer this question.

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315 **Comparison of zebrafish PIH protein functions with other reports**

316 The phenotypes of zebrafish PIH gene mutants are summarized in Fig. 7 A and B. Although PIH1D1 has been
317 known to serve as a component of R2TP complex, which has various important cellular functions (Kakihara and
318 Houry, 2012), the role of PIH1D1 in vertebrate development remains poorly understood. Zebrafish *pih1d1*^{-/-}
319 mutants were viable and exhibited only ciliary defects as far as we observed. Thus, Pih1d1 could be mostly
320 redundant in cellular functions. In fact, yeast *PIH1*-deletion cells were also reported to be viable (Gonzales et al.,
321 2005), like zebrafish *pih1d1*^{-/-}.

322 Accumulated knowledge about DNAAFs has suggested the involvement of R2TP-like complex in the
323 process of axonemal dynein assembly (Li et al., 2017). We identified Pih1d1 as a novel DNAAF, which strongly
324 support this idea. Since KTU and PIH1D3 are also suggested to participate in R2TP-like complexes (Tarkar et al.,
325 2013; Olcese et al., 2017), each of PIH proteins may serve as a component of R2TP-like complexes. Intriguingly,
326 however, our expression analysis of PIH genes suggested that *pih1d2*, *ktu*, and *twister* have cilia specific functions,
327 while *pih1d1* has ubiquitous cellular functions in addition to ciliary function. Further analysis of binding partners of
328 PIH proteins can provide us the mechanism of how PIH1D1 promote the assembly of various types of protein
329 complexes and how distinct PIH proteins modulate the assembly of different types of axonemal dyneins.

330 It was surprising that the spermatozoa of zebrafish *ktu*^{-/-} showed normal motility, since abnormal sperm
331 motility was reported in both human *KTU/DNAAF2*^{-/-} patients and medaka *ktu* mutants (Omran et al., 2008). This
332 suggests that the function of Ktu and other PIH proteins in zebrafish could have diverged during evolution.
333 Intriguingly, double mutants of *pih1d2*^{-/-};*ktu*^{-/-} showed phenotypes similar to those of the medaka *ktu* mutant, in
334 terms of complete loss of ciliary motility in Kupffer's vesicle (Fig. 5 D) and the expansion of pronephric ducts (Fig.
335 S5 F). Furthermore, the waveform of *pih1d2*^{-/-};*ktu*^{-/-} spermatozoa highly resembles that of medaka *ktu* mutant
336 spermatozoa (bends do not propagate to the tip of the sperm tail; Omran et al., 2008). The function of medaka Ktu

337 is thus partially shared by Ktu and Pih1d2 in zebrafish. Such functional divergence of PIH proteins was also
338 reported in human and mouse; human *PIH1D3* has functions in various ciliated organs (Paff et al., 2017; Olcese et
339 al., 2017), while mouse *Pih1d3* is a testis-specific gene (Dong et al., 2014). *Twister2* (paralogue of mouse *Pih1d3*)
340 is expressed in various ciliary organs including testis in mice, but is not able to rescue the loss of *Pih1d3* in testis.
341 Therefore, PIH proteins tend to be functionally diverse and sometimes interchangeable, even though they stay in
342 the category of DNAAFs.

343

344 **Sperm axoneme of *pih1d2*^{-/-};*ktu*^{-/-} exhibited distal-specific loss of axonemal dyneins**

345 In *pih1d2*^{-/-};*ktu*^{-/-} spermatozoa, OAD, IAD b, c, and e were missing only from the distal region of the flagella. One
346 possible explanation for this phenotype is that the lack of both PIH1d2 and Ktu causes decreased efficiency of
347 axonemal dynein assembly, leading to a shortage of dyneins to be loaded in the distal axoneme. Actually, the
348 axoneme is known to continue elongating by adding flagellar components to its distal end during ciliogenesis
349 (Johnson and Rosenbaum, 1992), and in mature spermatozoa, the transport of flagellar components is highly
350 improbable, because IFT components disappear as spermatozoa mature (San Agustin et al., 2015). Alternatively, the
351 distal and proximal region of zebrafish sperm could differ in the composition of axonemal dyneins. Indeed, human
352 respiratory cilia are known to have two types of OADs, i.e. DNAH11/DNAH5-containing OADs in the proximal
353 and DNAH9/DNAH5-containing OADs in the distal parts (Fig. 7 E; Fliegauf et al., 2005; Dougherty et al., 2016).
354 Intriguingly, a mutation in the human KTU/DNAAF2 gene strongly affects the assembly of only distal OADs in
355 respiratory cilia (Omran et al., 2008). However, at the moment, we do not have any evidence for the distal-specific
356 dynein composition in zebrafish spermatozoa. Although testis showed the expression of two OAD γ -HC genes:
357 *dnah5* and *dnah8*, *Dnah8* is present along the entire length of sperm flagella (Fig. 4 B) and the distribution of
358 *Dnah5* is not known. As for OAD β -HC gene, only *dnah9* was detectable in testis (Fig 6 B). Further analyses with
359 *pih1d2*^{-/-};*ktu*^{-/-} spermatozoa could shed light on the structural and functional difference between distal and proximal
360 regions of vertebrate spermatozoa.

361

362 **Different compositions of axonemal dyneins between testis and Kupffer's vesicle**

363 Expression analysis of DNAH genes suggested that the composition of axonemal dyneins differed between sperm
364 flagella and Kupffer's vesicle cilia. In sperm flagella, Dnah9/Dnah8-containing OADs could be majority as
365 discussed above, while in Kupffer's vesicle cilia, the axonemes seem to be constituted of Dnah9/Dnah5-containing
366 OADs and/or Dnah11/Dnah5-containing OADs (Fig. 7 D). We also found that *dnah3* (IAD HC gene) are differently
367 expressed between testis and Kupffer's vesicle (Fig. 6 B). Given that all PIH proteins are expressed in the two
368 organs, phenotypic differences between *pih1d2*^{-/-} and *ktu*^{-/-} can be accounted for by the different compositions of
369 axonemal dyneins. It is tempting to speculate that the dynein compositions are varies depending on the pattern of
370 ciliary beating, as cilia and flagella of the two organs exhibit the different mode of movement, planar oscillation for
371 sperm flagella and rotation for Kupffer's vesicle cilia.

1 **Materials and Methods**

2

3 **Zebrafish maintenance**

4 Zebrafish were maintained at 28.5°C on a 13.5/10.5 hours light/dark cycle. Embryos and larvae were raised at the
5 same temperature in 1/3 Ringer's solution (39 mM NaCl, 0.97 mM KCl, 1.8 mM CaCl₂, and 1.7 mM HEPES, pH
6 7.2). Developmental stages of embryos and larvae are described according to hpf at 28.5°C and the morphological
7 criteria by Kimmel et al. (1995). For embryos used in whole-mount *in situ* hybridization, 200 µM
8 1-phenyl-2-thiourea was added to 1/3 Ringer's solution to delay pigmentation. All experimental procedures and
9 animal care were done according to the animal-experimentation manual from the office for life science research
10 ethics of the University of Tokyo.

11

12 **Identification of zebrafish PIH proteins**

13 PIH1 domain is registered as PF08190 in the Pfam database. To find all PIH proteins in the zebrafish genome,
14 BLASTp search was performed with the consensus sequence of the PIH1 domain
15 (<https://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=pfam08190>). Four proteins were identified as a match:
16 Pih1d1 (NP_001153400.1, E value = 2.66e-27), Pih1d2 (NP_001008629.1, E value = 2.08e-9), Ktu
17 (NP_001028272.1, E value = 2.37e-31), and Twister (also known as Pih1d3; NP_001002309.1, E value = 4.16e-5).
18 Paralogues of each match were also checked, since teleost fish are known to have undergone an additional genome
19 duplication. BLASTn and tBLASTn search were performed using each PIH sequence as a query, however only the
20 proteins containing the query sequence were a hit in E value <10. Therefore zebrafish have four PIH proteins:
21 Pih1d1, Pih1d2, Ktu, and Twister.

22

23 **Zebrafish genome-editing**

24 Zebrafish genome-editing was performed according to previous reports of TALEN (Bedell et al., 2012) or
25 CRISPR/Cas9 (Gagnon et al., 2014). Target sites of our genome-editing are as follows: *pih1d1* (TALEN left),
26 GTTGAACACGAGCAGAAACAA; *pih1d1* (TALEN right), TGAAGCAGAAGTTGTTGGTA; *pih1d2* (TALEN
27 left), TACAGGAGCTTCATTCAG; *pih1d2* (TALEN right), TGAGTGAAACTCGGCTCCC; *ktu* (CRISPR gRNA),
28 GGAGATCCGGCCACAGCTGG; *twister* (CRISPR gRNA), GGATAATGATGAGGAAGAAG. Genomic DNA
29 was extracted from the developing embryos and target loci were amplified to check the mutations by
30 sanger-sequencing. After identifying founder fish, each mutant line underwent back-cross twice to remove the
31 effect of possible off-target mutations.

32

33 **Sperm treatment**

34 Zebrafish sperm was expelled by gently squeezing the sides of the fish, and collected in Hank's buffer (137 mM
35 NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.3 mM CaCl₂, 1.0 mM MgSO₄, and 4.2 mM
36 NaHCO₃). For the fractionation of sperm head and flagella, spermatozoa were passed through a 26 gauge needle 20
37 times in Hank's buffer with 2 mg/ml BSA, and the separated heads and flagella were collected by centrifugation
38 (head: 400 g, 3 min; flagella: 9000 g, 3 min). For purification of sperm axonemes, sperm heads and membranes
39 were removed by adding 2% Nonidet P-40 to Hank's buffer, and demembranated axonemes were collected by
40 centrifugation (10000 g, 3 min), then resuspended in HMDEKAc buffer (30 mM HEPES at pH 7.2, 5 mM MgSO₄,
41 1 mM dithiothreitol, 1 mM EGTA, and 50 mM CH₃COOK).

42

43 **Sperm motility analyses**

44 To observe proper motility, spermatozoa were kept on wet ice until analyzed, and used within 1 hour of sperm
45 collection. Zebrafish spermatozoa were inactive in Hank's buffer, but were activated by adding abundant amount of
46 1/5× Hank's buffer. Sperm motilities were observed under bright-field conditions using an inverted microscope
47 (DMI6000B; Leica) and a high-speed camera (HAS-L1; Detect). For waveform analysis, spermatozoa whose heads
48 were attached to the coverslip were selected and waveforms of flagella were filmed at 1000 fps. On the other hand,
49 for CASA, 2 mg/ml of BSA was added to buffers to prevent sperm from attaching to the glass and free swimming
50 spermatozoa were filmed at 200 fps. CASA modified for zebrafish was performed as previously reported
51 (Wilson-Leedy and Ingermann, 2007). Spermatozoa were prepared on glass slides with 10 μm spacers (200A10;
52 Kyodo giken chemical), and covered with coverslips to provide a consistent fluid depth. Eight independent
53 experiments with two times of 1 sec observations were performed to obtain sixteen technical replicates of CASA.

54

55 **Cryo-preparation of zebrafish sperm axoneme**

56 Purified sperm axoneme were incubated with anti-α-tubulin antibody (1:10000 dilution; T9026; Sigma) for 15 min
57 at 4°C in HMDEKAc buffer, and then with anti-mouse antibody conjugated with 15 nm colloidal gold (final 1:50
58 dilution; EM.GMHL15; BBInternational) and 15 nm colloidal gold conjugated with BSA (final 1:5 dilution;
59 215.133; Aurion) were added. Holey carbon grids were glow discharged before use to make them hydrophilic. 5 μl
60 of axoneme solution was loaded onto the grid, and then excess liquid was blotted away with filter paper to make a
61 thin film of the solution. Immediately after, the grid was plunged into liquid ethane at -180°C for a rapid freeze of
62 the solution. Blotting and freezing were automatically performed by an automated plunge-freezing device (EM GP;
63 Leica). Cryo-prepared grids were stored in liquid nitrogen until observation using the electron microscope.

64

65 **Cryo-image acquisition and image processing**

66 Cryo-prepared grids were transferred into a transmission electron microscope (JEM-3100FEF; JEOL) with a
67 high-tilt liquid nitrogen cryotransfer holder (914; Gatan), and kept at -180°C . Images of single axis tilt series were
68 collected using a $4,096 \times 4,096$ -pixel CMOS camera (TemCam-F416; TVIPS) and automated acquisition software
69 (EM-TOOLS; TVIPS). Tilt series were acquired by a stepwise rotation of the sample from -60 to 60° in 2.0°
70 increments. The total electron dose was limited to approximately $100 \text{ e}/\text{\AA}^2$ for an individual tilt series to avoid
71 radiation damage of the sample. Images were recorded at 300 keV, with $8.8\mu\text{m}$ defocus, at a magnification of
72 $30,000\times$ and a pixel size of 7.2 \AA . An in-column Ω energy filter was used to enhance image contrast in the zero-loss
73 mode with a slit width of 20 eV. The tilt series images were aligned and reconstructed into 3D tomograms using
74 IMOD software (Kremer et al., 1996). Alignment and averaging of subtomograms were conducted by custom
75 Ruby-Helix scripts (Metlagel et al., 2007) and PEET (Particle Estimation for Electron Tomography) software suite
76 (Nicastro et al., 2006), using a 96-nm repeat of DMT as one particle assuming a nine-fold rotational symmetry of
77 the axoneme. Effective resolutions were determined by Fourier shell correlation with a cutoff value of 0.143. For
78 the visualization of tomographic slices or 3D structures, 3dmod program (IMOD software) or isosurface rendering
79 of UCSF Chimera package (Pettersen et al., 2004) were used, respectively.

80

81 **Immunofluorescence microscopy**

82 Spermatozoa in Hank's buffer were attached to the wells of an 8-well glass slide (TF0808; Matsunami). After
83 washing out excess sperm, attached spermatozoa were briefly demembrated using 1% Nonidet P-40 for 2 min.
84 Specimens were fixed with 2% paraformaldehyde/Hank's buffer for 10 min at room temperature, followed by
85 treatment with cold acetone and methanol (-20°C). After rehydration with PBST (phosphate buffered saline
86 containing 0.1% Tween-20), specimens were treated with blocking buffer (2% normal goat serum, 1% cold fish
87 gelatin in PBST). Immunostaining was performed with anti-acetylated tubulin antibody (1:500 dilution; T6793;
88 Sigma) and anti-Dnah8 antibody (1:50 dilution; generated in this study) as primary antibodies. Alexa Fluor 488
89 Donkey anti-mouse IgG (1:250 dilution; ab150105; Abcam) and Alexa Fluor 555 Donkey anti-rabbit IgG (1:250
90 dilution; ab150074; Abcam) were used as secondary antibodies with $2.5 \mu\text{g}/\text{ml}$ DAPI (Wako) for nuclear staining.
91 Specimens were mounted with Fluoro-KEEPER Antifade Reagent (Nacalai tesque) and observed with a
92 fluorescence microscope (BX60; Olympus) and a CCD camera (ORCA-R2; Hamamatsu).

93

94 **Kupffer's vesicle cilia analysis**

95 Embryos developing Kupffer's vesicle were selected at 12 hpf and dechironated before observations. To align the
96 orientations, embryos were embedded in 0.8% of low gelling temperature agarose (Sigma) with 1/3 Ringer's

97 solution. Motility of Kupffer's vesicle cilia were observed under the bright-field conditions using an inverted
98 microscope (DMI6000B; Leica) and a high-speed camera (HAS-L1; Detect) at 1000 fps.

99

100 **RNA probe synthesis**

101 The sequences of zebrafish PIH genes and DNAH genes were subcloned into pCRII-TOPO plasmid (Invitrogen).
102 From the constructed plasmids, RNA probes were synthesized using SP6 or T7 RNA polymerase (Roche) with DIG
103 RNA Labeling Mix (Roche). RNAs were purified using RNeasy Mini Kit (Qiagen). Sequences of primers used in
104 the construction of plasmids are summarized in Table S1.

105

106 **Whole-mount *in situ* hybridization**

107 Dechorionated embryos or dissected testes were fixed with 4% paraformaldehyde (PFA) in PBST, and then stored
108 in methanol at -20°C . After rehydration with PBST, specimens were treated with proteinase K and re-fixed with
109 4% PFA/PBST solution. Hybridization was performed overnight at 63°C in hybridization buffer (750 mM NaCl, 75
110 mM trisodium citrate, 500 $\mu\text{g/ml}$ torula tRNA, 50 $\mu\text{g/ml}$ Heparin, 50% formamide, and 0.1% Tween-20) with
111 digoxigenin-labeled RNA probes. Hybridized specimens were washed with 50% formamide/ $2\times$ SSCT (saline
112 sodium citrate containing 0.1% Tween-20) followed by $2\times$ SSCT and $0.2\times$ SSCT, then treated with AP-conjugated
113 anti-digoxigenin Fab fragments (1:4000 dilution; Roche) in blocking solution (150 mM NaCl, 100 mM maleic acid
114 at pH 7.5, 5% blocking reagent (Roche), 5% normal goat serum, and 0.1% Tween-20) at 4°C overnight. After
115 washing with MABT (150 mM NaCl, 100 mM maleic acid at pH 7.5, and 0.1% Tween-20), signals were developed
116 using BM-purple (Roche). When desired intensities of staining were obtained, reactions were stopped by stopping
117 solution (PBST containing 1 mM EDTA) and 4% PFA/PBST. Before observations, specimens were transferred into
118 80% glycerol/PBS to make them transparent. For some embryos, to show stained organs clearly, posterior regions
119 were flat-mounted or yolk was removed. Images were taken by a stereoscopic microscope (MVX10; Olympus) and
120 a CCD camera (DP73; Olympus).

121

122 **Antibodies**

123 Sequences encoding full length of zebrafish PIH proteins (Pih1d1, Pih1d2, Ktu, and Twister) were subcloned into
124 the pColdI plasmid vector (Takara). Sequences encoding zebrafish Dnah8 (amino acid 895-1402) and Dnah2
125 (amino acid 802-1378) were subcloned into the pGEX-6P-2 plasmid vector (GE Healthcare). Recombinant
126 polypeptides were purified from transformed *E.coli* lysate using Ni-NTA Agarose (Qiagen) for PIH proteins or
127 Glutathione Sepharose 4B (GE Healthcare) for Dnah8 and Dnah2. Polyclonal antibodies against each purified
128 polypeptide were raised by immunization of rabbits. Antibodies were affinity purified from serum by the antigens

129 before use. Sequences of the primers used in the antigen production are summarized in Table S1. Other antibodies
130 used are as follows: anti-Dnai1 antibody (GTX109719; GeneTex), anti-Dnali1 antibody (anti-p28 antibody;
131 LeDizet and Piperno, 1995), anti- α -tubulin antibody (T9026; Sigma), and anti-acetylated tubulin antibody (T6793;
132 Sigma).

133

134 **Immunoblot analysis**

135 Proteins were separated by SDS-PAGE in 5-20% gradient polyacrylamide gels (Nacalai Tesque) and transferred
136 onto polyvinylidene difluoride (PVDF) membranes (Millipore). After blocking with 5% skim milk (Nacalai
137 Tesque) in TBST (Tris-buffered saline containing 0.1% Tween-20), membranes were incubated with primary
138 antibodies, followed by several washes and incubation with secondary antibodies (goat anti rabbit/mouse IgG
139 antibody peroxidase conjugated; Sigma). Protein signals were visualized by ECL Select Western Blotting Detection
140 Reagent (GE Healthcare) and observed using luminescent image analyzer (ImageQuant LAS4000mini; GE
141 Healthcare).

142

143 **Statistics**

144 Data with biological/technical replicates are shown with mean (bar graphs) \pm SD (error bars). Statistical
145 significances between WT and each mutant were tested by a two-tailed Dunnett's test, and P value $<$ 0.05 was
146 considered to indicate a significant difference.

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161 **Table S1. Primer sequences used in this study**

162

purpose	name	Sequence
	pih1d1.F	GAACCGGAACAGTCAGTGAA
	pih1d1.R	TAGGGCACAAAACGGAAAC
	pih1d2.F	GTGAGTCCAAGTAGGCTATT
	pih1d2.R	GGATAAACAAGAGTGAAC TATTT
	ktu.F	GACGCGCTGTTCACCGGAGC
	ktu.R	GGATCCGCCAGCTGTGGCC
	twister.F	AGAGCAGGACATTCAC TTCA
	twister.R	TTGTCTATCTTTCTCTCTGTTTCC
probes for <i>in situ</i> hybridization	dnah9.F	ATGTATGGAGTCTGTGGATGCAAAG
	dnah9.R	ACATGGCCGTACAGCAGGAA
	dnah9l.F	CGCGTGGACTTCATTAGAG
	dnah9l.R	TTTGACCGTAAGGCCTG
	dnah11.F	GTGTCAATATTTTTTAACAAGGTGT
	dnah11.R	AATATTGGACAGTTGTTCGATCA
	dnah5.F	AAATACCTCTGTCTGAATCACTG
	dnah5.R	ATCCTGTGGAGCCAATCC
	dnah8.F	ACAGAAAACGAAGAGTCTCCTC
	dnah8.R	ATCCATAAGGCTGCAGAAG
	dnah2.F	ATGGCTGACCCAGAGACC
	dnah2.R	CTCAGAATGGAACAGATTGTACAC
	dnah3.F	AGGTATTTATACTATGTCACGGAGG
	dnah3.R	AGTGACTTCACTTGTGTGTTTCA
	dnah7l.F	ATGTATTCTTTTCAGAAAGGACATC
	dnah7l.R	AGCATCTTCTTCAAGAATTTCTT
antigen generation	pih1d1_Ag.F	ATGTCTGAGGCACTCATCATG
	pih1d1_Ag.R	TCACAAAGACACGACTGGC
	pih1d2_Ag.F	ATGGCAATCCATGGCTAT
	pih1d2_Ag.R	TCATAATACATTTACCGTCACATT
	ktu_Ag.F	ATGGACGCGGACAGACTG
	ktu_Ag.R	TGCTTCCAGTTGGACTGA
	twister_Ag.F	ATGGAGGGTCTCGCGTCC
	twister_Ag.R	TCAGGTCAGATTAATGCAGTC
	dnah8_Ag.F	TATTTTCATAGGTTGTGTGTACAAG
	dnah8_Ag.R	TTCACGTGGCGCAAACC
	dnah2_Ag.F	AGCGCCTTTATCAATGACTG
	dnah2_Ag.R	GATGATCTTCTTAAACTGCTCA

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166

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168

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170 for the help of zebrafish TALEN genome-editings, and Dr H. Yanagisawa for the help of image processing of
171 cryo-ET analysis.

172

173 The authors declare no competing financial interests.

174

175

176 **Author contributions**

177

178 H. Takeda and M. Kikkawa planned and supervised this study. T. Oda and H. Yamaguchi developed cryo-ET method
179 for zebrafish sperms. All other experiments were performed by H. Yamaguchi. H. Yamaguchi, M. Kikkawa and H.
180 Takeda wrote the manuscript with input from all authors.

181

182

183 **Abbreviations**

184

CASA	computer-assisted sperm analysis
Cryo-ET	cryo-electron tomography
DMT	doublet microtubule
DNAAF	dynein axonemal assembly factor
DNAH	dynein axonemal heavy chain
fps	frames per second
HC	heavy chain
hpf	hours post-fertilization
IAD	inner arm dynein
IC	intermediate chain
N-DRC	nexin-dynein regulatory complex
OAD	outer arm dynein
PCD	primary ciliary dyskinesia
PKD	polycystic kidney disease
RS	radial spoke

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189 **References**

190

- 191 Bedell, V.M., Y. Wang, J.M. Campbell, T.L. Poshusta, C.G. Starker, R.G. Krug, W. Tan, S.G. Penheiter, A.C. Ma,
192 A.Y.H. Leung, S.C. Fahrenkrug, D.F. Carlson, D.F. Voytas, K.J. Clark, J.J. Essner, and S.C. Ekker. 2012. In
193 vivo genome editing using a high-efficiency TALEN system. *Nature*. 491:114–8. doi:10.1038/nature11537.
- 194 Brown, J.M., and G.B. Witman. 2014. Cilia and Diseases. *Bioscience*. 64:1126–1137. doi:10.1093/biosci/biu174.
- 195 Bui, K.H., T. Yagi, R. Yamamoto, R. Kamiya, and T. Ishikawa. 2012. Polarity and asymmetry in the arrangement
196 of dynein and related structures in the Chlamydomonas axoneme. *J. Cell Biol.* 198:913–25.
197 doi:10.1083/jcb.201201120.
- 198 Dong, F., K. Shinohara, Y. Botilde, R. Nabeshima, Y. Asai, A. Fukumoto, T. Hasegawa, M. Matsuo, H. Takeda, H.
199 Shiratori, T. Nakamura, and H. Hamada. 2014. Pih1d3 is required for cytoplasmic preassembly of axonemal
200 dynein in mouse sperm. *J. Cell Biol.* 204:203–13. doi:10.1083/jcb.201304076.
- 201 Dougherty, G.W., N.T. Loges, J.A. Klinkenbusch, H. Olbrich, P. Pennekamp, T. Menchen, J. Raidt, J. Wallmeier,
202 C. Werner, C. Westermann, C. Ruckert, V. Mirra, R. Hjeij, Y. Memari, R. Durbin, A. Kolb-Kokocinski, K.
203 Praveen, M.A. Kashef, S. Kashef, F. Eghtedari, K. Häffner, P. Valmari, G. Baktai, M. Aviram, L. Bentur, I.
204 Amirav, E.E. Davis, N. Katsanis, M. Brueckner, A. Shaposhnykov, G. Pigino, B. Dworniczak, and H. Omran.
205 2016. DNAH11 Localization in the Proximal Region of Respiratory Cilia Defines Distinct Outer Dynein Arm
206 Complexes. *Am. J. Respir. Cell Mol. Biol.* 55:213–24. doi:10.1165/rcmb.2015-0353OC.
- 207 Essner, J.J., J.D. Amack, M.K. Nyholm, E.B. Harris, and H.J. Yost. 2005. Kupffer’s vesicle is a ciliated organ of
208 asymmetry in the zebrafish embryo that initiates left-right development of the brain, heart and gut.
209 *Development*. 132:1247–60. doi:10.1242/dev.01663.
- 210 Fliegauf, M., H. Olbrich, J. Horvath, J.H. Wildhaber, M.A. Zariwala, M. Kennedy, M.R. Knowles, and H. Omran.
211 2005. Mislocalization of DNAH5 and DNAH9 in respiratory cells from patients with primary ciliary
212 dyskinesia. *Am. J. Respir. Crit. Care Med.* 171:1343–9. doi:10.1164/rccm.200411-1583OC.
- 213 Fok, A.K., H. Wang, A. Katayama, M.S. Aihara, and R.D. Allen. 1994. 22S axonemal dynein is preassembled and
214 functional prior to being transported to and attached on the axonemes. *Cell Motil. Cytoskeleton*. 29:215–24.
215 doi:10.1002/cm.970290304.
- 216 Fowkes, M.E., and D.R. Mitchell. 1998. The role of preassembled cytoplasmic complexes in assembly of flagellar
217 dynein subunits. *Mol. Biol. Cell*. 9:2337–47.
- 218 Gagnon, J.A., E. Valen, S.B. Thyme, P. Huang, L. Akhmetova, L. Akhmetova, A. Pauli, T.G. Montague, S.
219 Zimmerman, C. Richter, and A.F. Schier. 2014. Efficient mutagenesis by Cas9 protein-mediated
220 oligonucleotide insertion and large-scale assessment of single-guide RNAs. *PLoS One*. 9:e98186.

- 221 doi:10.1371/journal.pone.0098186.
- 222 Gonzales, F.A., N.I.T. Zanchin, J.S. Luz, and C.C. Oliveira. 2005. Characterization of *Saccharomyces cerevisiae*
223 Nop17p, a novel Nop58p-interacting protein that is involved in Pre-rRNA processing. *J. Mol. Biol.* 346:437–
224 55. doi:10.1016/j.jmb.2004.11.071.
- 225 Hom, E.F.Y., G.B. Witman, E.H. Harris, S.K. Dutcher, R. Kamiya, D.R. Mitchell, G.J. Pazour, M.E. Porter, W.S.
226 Sale, M. Wirschell, T. Yagi, and S.M. King. 2011. A unified taxonomy for ciliary dyneins. *Cytoskeleton*
227 (*Hoboken*). 68:555–65. doi:10.1002/cm.20533.
- 228 Johnson, K.A., and J.L. Rosenbaum. 1992. Polarity of flagellar assembly in *Chlamydomonas*. *J. Cell Biol.*
229 119:1605–11.
- 230 Kagami, O., and R. Kamiya. 1992. Translocation and rotation of microtubules caused by multiple species of
231 *Chlamydomonas* inner-arm dynein. *J. Cell Sci.* 103.
- 232 Kakiyama, Y., and W.A. Houry. 2012. The R2TP complex: discovery and functions. *Biochim. Biophys. Acta.*
233 1823:101–7. doi:10.1016/j.bbamcr.2011.08.016.
- 234 Kamiya, R. 1995. Exploring the function of inner and outer dynein arms with *Chlamydomonas* mutants. *Cell Motil.*
235 *Cytoskeleton.* 32:98–102. doi:10.1002/cm.970320205.
- 236 Kasahara, M., K. Naruse, S. Sasaki, Y. Nakatani, W. Qu, B. Ahsan, T. Yamada, Y. Nagayasu, K. Doi, Y. Kasai, T.
237 Jindo, D. Kobayashi, A. Shimada, A. Toyoda, Y. Kuroki, A. Fujiyama, T. Sasaki, A. Shimizu, S. Asakawa, N.
238 Shimizu, S.-I. Hashimoto, J. Yang, Y. Lee, K. Matsushima, S. Sugano, M. Sakaizumi, T. Narita, K. Ohishi, S.
239 Haga, F. Ohta, H. Nomoto, K. Nogata, T. Morishita, T. Endo, T. Shin-I, H. Takeda, S. Morishita, and Y.
240 Kohara. 2007. The medaka draft genome and insights into vertebrate genome evolution. *Nature.* 447:714–9.
241 doi:10.1038/nature05846.
- 242 Katoh, K., and D.M. Standley. 2013. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in
243 Performance and Usability. *Mol. Biol. Evol.* 30:772–780. doi:10.1093/molbev/mst010.
- 244 Kimmel, C.B., W.W. Ballard, S.R. Kimmel, B. Ullmann, and T.F. Schilling. 1995. Stages of embryonic
245 development of the zebrafish. *Dev. Dyn.* 203:253–310. doi:10.1002/aja.1002030302.
- 246 Knowles, M.R., L.A. Daniels, S.D. Davis, M.A. Zariwala, and M.W. Leigh. 2013. Primary ciliary dyskinesia.
247 Recent advances in diagnostics, genetics, and characterization of clinical disease. *Am. J. Respir. Crit. Care*
248 *Med.* 188:913–22. doi:10.1164/rccm.201301-0059CI.
- 249 Kobayashi, D., and H. Takeda. 2012. Ciliary motility: the components and cytoplasmic preassembly mechanisms of
250 the axonemal dyneins. *Differentiation.* 83:S23-9. doi:10.1016/j.diff.2011.11.009.
- 251 Kramer-Zucker, A.G., F. Olale, C.J. Haycraft, B.K. Yoder, A.F. Schier, and I.A. Drummond. 2005. Cilia-driven
252 fluid flow in the zebrafish pronephros, brain and Kupffer’s vesicle is required for normal organogenesis.

- 253 *Development*. 132:1907–21. doi:10.1242/dev.01772.
- 254 Kremer, J.R., D.N. Mastrorarde, and J.R. McIntosh. 1996. Computer Visualization of Three-Dimensional Image
255 Data Using IMOD. *J. Struct. Biol.* 116:71–76. doi:10.1006/jsbi.1996.0013.
- 256 LeDizet, M., and G. Piperno. 1995. The light chain p28 associates with a subset of inner dynein arm heavy chains
257 in *Chlamydomonas axonemes*. *Mol. Biol. Cell.* 6:697–711.
- 258 Li, Y., L. Zhao, S. Yuan, J. Zhang, and Z. Sun. 2017. Axonemal dynein assembly requires the R2TP complex
259 component Pontin. *Development*. 144:4684–4693. doi:10.1242/dev.152314.
- 260 Lin, J., T. Heuser, B.I. Carbajal-González, K. Song, and D. Nicastro. 2012. The structural heterogeneity of radial
261 spokes in cilia and flagella is conserved. *Cytoskeleton*. 69:88–100. doi:10.1002/cm.21000.
- 262 Lin, J., W. Yin, M.C. Smith, K. Song, M.W. Leigh, M.A. Zariwala, M.R. Knowles, L.E. Ostrowski, and D.
263 Nicastro. 2014. Cryo-electron tomography reveals ciliary defects underlying human RSPH1 primary ciliary
264 dyskinesia. *Nat. Commun.* 5:5727. doi:10.1038/ncomms6727.
- 265 Matsuo, M., A. Shimada, S. Koshida, Y. Saga, and H. Takeda. 2013. The establishment of rotational polarity in the
266 airway and ependymal cilia: analysis with a novel cilium motility mutant mouse. *Am. J. Physiol. Lung Cell.*
267 *Mol. Physiol.* 304:L736-45. doi:10.1152/ajplung.00425.2012.
- 268 Metlagel, Z., Y.S. Kikkawa, and M. Kikkawa. 2007. Ruby-Helix: an implementation of helical image processing
269 based on object-oriented scripting language. *J. Struct. Biol.* 157:95–105. doi:10.1016/j.jsb.2006.07.015.
- 270 Mitchison, H.M., M. Schmidts, N.T. Loges, J. Freshour, A. Dritsoula, R.A. Hirst, C. O’Callaghan, H. Blau, M. Al
271 Dabbagh, H. Olbrich, P.L. Beales, T. Yagi, H. Mussaffi, E.M.K. Chung, H. Omran, and D.R. Mitchell. 2012.
272 Mutations in axonemal dynein assembly factor DNAAF3 cause primary ciliary dyskinesia. *Nat. Genet.*
273 44:381–389. doi:10.1038/ng.1106.
- 274 Nicastro, D., C. Schwartz, J. Pierson, R. Gaudette, M.E. Porter, and J.R. McIntosh. 2006. The Molecular
275 Architecture of Axonemes Revealed by Cryoelectron Tomography. *Science (80-.)*. 313:944–948.
276 doi:10.1126/science.1128618.
- 277 Olcese, C., M.P. Patel, A. Shoemark, S. Kiviluoto, M. Legendre, H.J. Williams, C.K. Vaughan, J. Hayward, A.
278 Goldenberg, R.D. Emes, M.M. Munye, L. Dyer, T. Cahill, J. Bevillard, C. Gehrig, M. Guipponi, S. Chantot, P.
279 Duquesnoy, L. Thomas, L. Jeanson, B. Copin, A. Tamalet, C. Thauvin-Robinet, J.-F. Papon, A. Garin, I. Pin,
280 G. Vera, P. Aurora, M.R. Fassad, L. Jenkins, C. Boustred, T. Cullup, M. Dixon, A. Onoufriadis, A. Bush,
281 E.M.K. Chung, S.E. Antonarakis, M.R. Loebinger, R. Wilson, M. Armengot, E. Escudier, C. Hogg, S.
282 UK10K Rare Group, S. Amselem, Z. Sun, L. Bartoloni, J.-L. Blouin, H.M. Mitchison, S. Bhattacharya, K.
283 Carss, K. Chatterjee, S. Cirak, C. Cosgrove, D. Allan, R. Durbin, D. Fitzpatrick, J. Floyd, A.R. Foley, C.
284 Franklin, M. Futema, S.E. Humphries, M. Hurles, S. McCarthy, D. Muddyman, F. Muntoni, V. Parker, F.

- 285 Payne, V. Plagnol, L. Raymond, D.B. Savage, P.J. Scambler, M. Schmidts, R. Semple, E. Serra, J. Stalker, M.
286 van Kogelenberg, P. Vijayarangakannan, K. Walter, S. Amselem, Z. Sun, L. Bartoloni, J.-L. Blouin, and H.M.
287 Mitchison. 2017. X-linked primary ciliary dyskinesia due to mutations in the cytoplasmic axonemal dynein
288 assembly factor PIH1D3. *Nat. Commun.* 8:14279. doi:10.1038/ncomms14279.
- 289 Omran, H., D. Kobayashi, H. Olbrich, T. Tsukahara, N.T. Loges, H. Hagiwara, Q. Zhang, G. Leblond, E. O'Toole,
290 C. Hara, H. Mizuno, H. Kawano, M. Fliegauf, T. Yagi, S. Koshida, A. Miyawaki, H. Zentgraf, H. Seithe, R.
291 Reinhardt, Y. Watanabe, R. Kamiya, D.R. Mitchell, and H. Takeda. 2008. Ktu/PF13 is required for
292 cytoplasmic pre-assembly of axonemal dyneins. *Nature.* 456:611–6. doi:10.1038/nature07471.
- 293 Paff, T., N.T. Loges, I. Aprea, K. Wu, Z. Bakey, E.G. Haarman, J.M.A. Daniels, E.A. Sistermans, N. Bogunovic,
294 G.W. Dougherty, I.M. Höben, J. Große-Onnebrink, A. Matter, H. Olbrich, C. Werner, G. Pals, M. Schmidts,
295 H. Omran, and D. Micha. 2017. Mutations in PIH1D3 Cause X-Linked Primary Ciliary Dyskinesia with
296 Outer and Inner Dynein Arm Defects. *Am. J. Hum. Genet.* 100:160–168. doi:10.1016/j.ajhg.2016.11.019.
- 297 Pettersen, E.F., T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, and T.E. Ferrin. 2004. UCSF
298 Chimera--a visualization system for exploratory research and analysis. *J. Comput. Chem.* 25:1605–12.
299 doi:10.1002/jcc.20084.
- 300 Piperno, G., Z. Ramanis, E.F. Smith, and W.S. Sale. 1990. Three distinct inner dynein arms in *Chlamydomonas*
301 flagella: molecular composition and location in the axoneme. *J. Cell Biol.* 110:379–89.
- 302 Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees.
303 *Mol. Biol. Evol.* 4:406–25.
- 304 Sakato, M., and S.M. King. 2004. Design and regulation of the AAA+ microtubule motor dynein. *J. Struct. Biol.*
305 146:58–71. doi:10.1016/j.jsb.2003.09.026.
- 306 San Agustin, J.T., G.J. Pazour, and G.B. Witman. 2015. Intraflagellar transport is essential for mammalian
307 spermiogenesis but is absent in mature sperm. *Mol. Biol. Cell.* 26:4358–72. doi:10.1091/mbc.E15-08-0578.
- 308 Sun, Z., A. Amsterdam, G.J. Pazour, D.G. Cole, M.S. Miller, and N. Hopkins. 2004. A genetic screen in zebrafish
309 identifies cilia genes as a principal cause of cystic kidney. *Development.* 131:4085–93.
310 doi:10.1242/dev.01240.
- 311 Takao, D., and K.J. Verhey. 2016. Gated entry into the ciliary compartment. *Cell. Mol. Life Sci.* 73:119–127.
312 doi:10.1007/s00018-015-2058-0.
- 313 Tarkar, A., N.T. Loges, C.E. Slagle, R. Francis, G.W. Dougherty, J. V Tamayo, B. Shook, M. Cantino, D. Schwartz,
314 C. Jahnke, H. Olbrich, C. Werner, J. Raidt, P. Pennekamp, M. Abouhamed, R. Hjeij, G. Köhler, M. Griese, Y.
315 Li, K. Lemke, N. Klena, X. Liu, G. Gabriel, K. Tobita, M. Jaspers, L.C. Morgan, A.J. Shapiro, S.J.F.
316 Letteboer, D.A. Mans, J.L. Carson, M.W. Leigh, W.E. Wolf, S. Chen, J.S. Lucas, A. Onoufriadis, V. Plagnol,

- 317 M. Schmidts, K. Boldt, R. Roepman, M.A. Zariwala, C.W. Lo, H.M. Mitchison, M.R. Knowles, R.D. Burdine,
318 J.J. LoTurco, H. Omran, and H. Omran. 2013. DYX1C1 is required for axonemal dynein assembly and ciliary
319 motility. *Nat. Genet.* 45:995–1003. doi:10.1038/ng.2707.
- 320 Ueno, H., T. Ishikawa, K.H. Bui, K. Gonda, T. Ishikawa, and T. Yamaguchi. 2012. Mouse respiratory cilia with the
321 asymmetric axonemal structure on sparsely distributed ciliary cells can generate overall directional flow.
322 *Nanomedicine.* 8:1081–7. doi:10.1016/j.nano.2012.01.004.
- 323 Viswanadha, R., E.L. Hunter, R. Yamamoto, M. Wirschell, L.M. Alford, S.K. Dutcher, and W.S. Sale. 2014. The
324 ciliary inner dynein arm, I1 dynein, is assembled in the cytoplasm and transported by IFT before axonemal
325 docking. *Cytoskeleton (Hoboken).* 71:573–86. doi:10.1002/cm.21192.
- 326 Wilson-Leedy, J.G., and R.L. Ingermann. 2007. Development of a novel CASA system based on open source
327 software for characterization of zebrafish sperm motility parameters. *Theriogenology.* 67:661–72.
328 doi:10.1016/j.theriogenology.2006.10.003.
- 329 Yamamoto, R., M. Hirono, and R. Kamiya. 2010. Discrete PIH proteins function in the cytoplasmic preassembly of
330 different subsets of axonemal dyneins. *J. Cell Biol.* 190:65–71. doi:10.1083/jcb.201002081.
- 331 Yamamoto, R., J.M. Obbineni, L.M. Alford, T. Ide, M. Owa, J. Hwang, T. Kon, K. Inaba, N. James, S.M. King, T.
332 Ishikawa, W.S. Sale, and S.K. Dutcher. 2017. Chlamydomonas DYX1C1/PF23 is essential for axonemal
333 assembly and proper morphology of inner dynein arms. *PLOS Genet.* 13:e1006996.
334 doi:10.1371/journal.pgen.1006996.
- 335 Zhao, R., M. Davey, Y.-C. Hsu, P. Kaplanek, A. Tong, A.B. Parsons, N. Krogan, G. Cagney, D. Mai, J. Greenblatt,
336 C. Boone, A. Emili, and W.A. Houry. 2005. Navigating the Chaperone Network: An Integrative Map of
337 Physical and Genetic Interactions Mediated by the Hsp90 Chaperone. *Cell.* 120:715–727.
338 doi:10.1016/j.cell.2004.12.024.
- 339
340

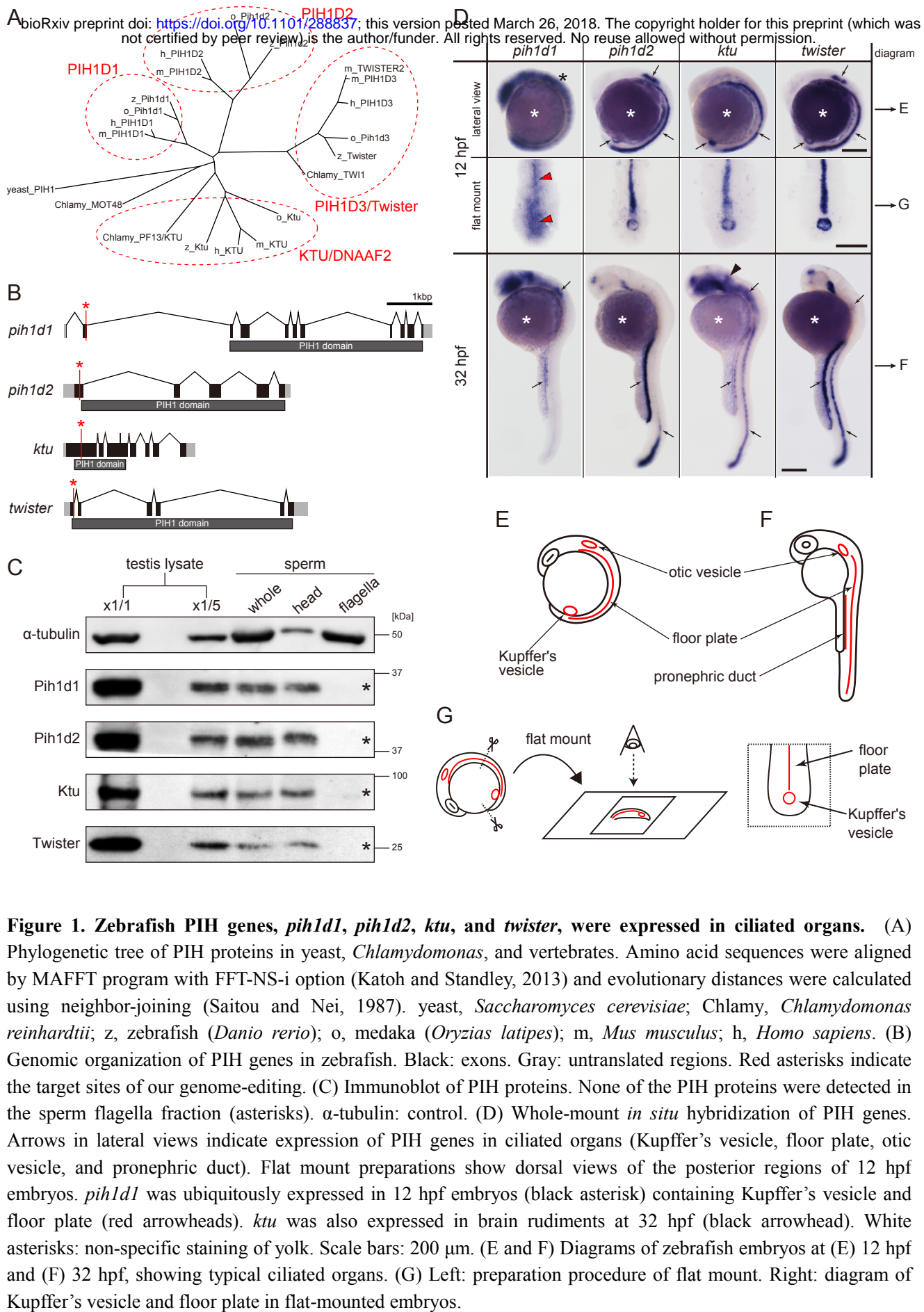


Figure 1. Zebrafish PIH genes, *pih1d1*, *pih1d2*, *ktu*, and *twister*, were expressed in ciliated organs. (A) Phylogenetic tree of PIH proteins in yeast, *Chlamydomonas*, and vertebrates. Amino acid sequences were aligned by MAFFT program with FFT-NS-i option (Kato and Standley, 2013) and evolutionary distances were calculated using neighbor-joining (Saitou and Nei, 1987). yeast, *Saccharomyces cerevisiae*; Chlamy, *Chlamydomonas reinhardtii*; z, zebrafish (*Danio rerio*); o, medaka (*Oryzias latipes*); m, *Mus musculus*; h, *Homo sapiens*. (B) Genomic organization of PIH genes in zebrafish. Black: exons. Gray: untranslated regions. Red asterisks indicate the target sites of our genome-editing. (C) Immunoblot of PIH proteins. None of the PIH proteins were detected in the sperm flagella fraction (asterisks). α -tubulin: control. (D) Whole-mount *in situ* hybridization of PIH genes. Arrows in lateral views indicate expression of PIH genes in ciliated organs (Kupffer's vesicle, floor plate, otic vesicle, and pronephric duct). Flat mount preparations show dorsal views of the posterior regions of 12 hpf embryos. *pih1d1* was ubiquitously expressed in 12 hpf embryos (black asterisk) containing Kupffer's vesicle and floor plate (red arrowheads). *ktu* was also expressed in brain rudiments at 32 hpf (black arrowhead). White asterisks: non-specific staining of yolk. Scale bars: 200 μ m. (E and F) Diagrams of zebrafish embryos at (E) 12 hpf and (F) 32 hpf, showing typical ciliated organs. (G) Left: preparation procedure of flat mount. Right: diagram of Kupffer's vesicle and floor plate in flat-mounted embryos.

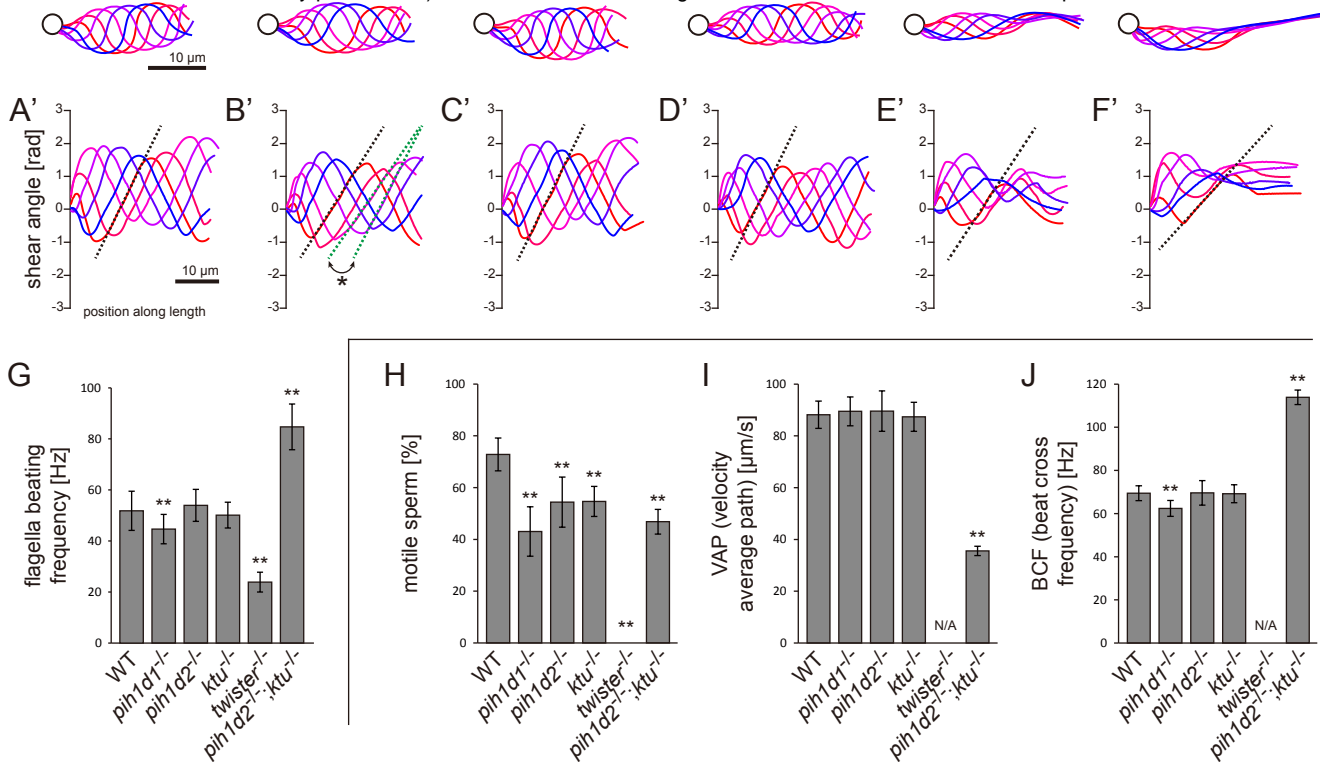


Figure 2. Mutations of PIH genes caused abnormal sperm motilities. (A-G) Waveforms and the beating frequencies of motile sperm were analyzed using a high-speed camera at 1000 fps (frames per second). (A-F) Sperm waveforms were traced six times in one beating cycle and overlaid. Intervals are as follows: WT, 3 msec; *pih1d1*^{-/-}, 4 msec; *pih1d2*^{-/-}, 3 msec; *ktu*^{-/-}, 3 msec; *twister*^{-/-}, 6 msec; and *pih1d2*^{-/-};*ktu*^{-/-}, 2 msec. Traces are painted in a color gradient of from red (first traces) to blue (sixth traces). (A'-F') Shear angles of beating flagella were calculated from each trace of A-F. Slopes of dotted lines represent the size of flagellar bending. An asterisk with green dotted lines in B' shows unstable propagation of flagellar bending in *pih1d1*^{-/-} sperm. (G) Beating frequencies of sperm flagella were measured by sperm kymographs. Kymographs of *pih1d2*^{-/-};*ktu*^{-/-} were obtained from the proximal region of sperm flagella, as distal flagella were immotile. Number of samples: WT, 23; *pih1d1*^{-/-}, 23; *pih1d2*^{-/-}, 26; *ktu*^{-/-}, 15; *twister*^{-/-}, 8; and *pih1d2*^{-/-};*ktu*^{-/-}, 14. (H-J) Motilities of free-swimming spermatozoa were observed using a high-speed camera at 200 fps and analyzed by CASA modified for zebrafish. For each zebrafish line, more than 1,200 spermatozoa were observed in total with sixteen technical replicates. Spermatozoa with less than 20 μm/s velocities were considered immotile. (H) Ratio of motile (locomotive) sperm. (I) Velocity of spermatozoa on averaged paths. Averaged paths were constructed by connecting the points of averaged sperm positions of coordinating 33 frames. (J) Frequencies at which sperm heads crossed their averaged paths. Bar graphs show mean ± SD. **: p-value < 0.01 in Dunnett's test of each mutant against WT.

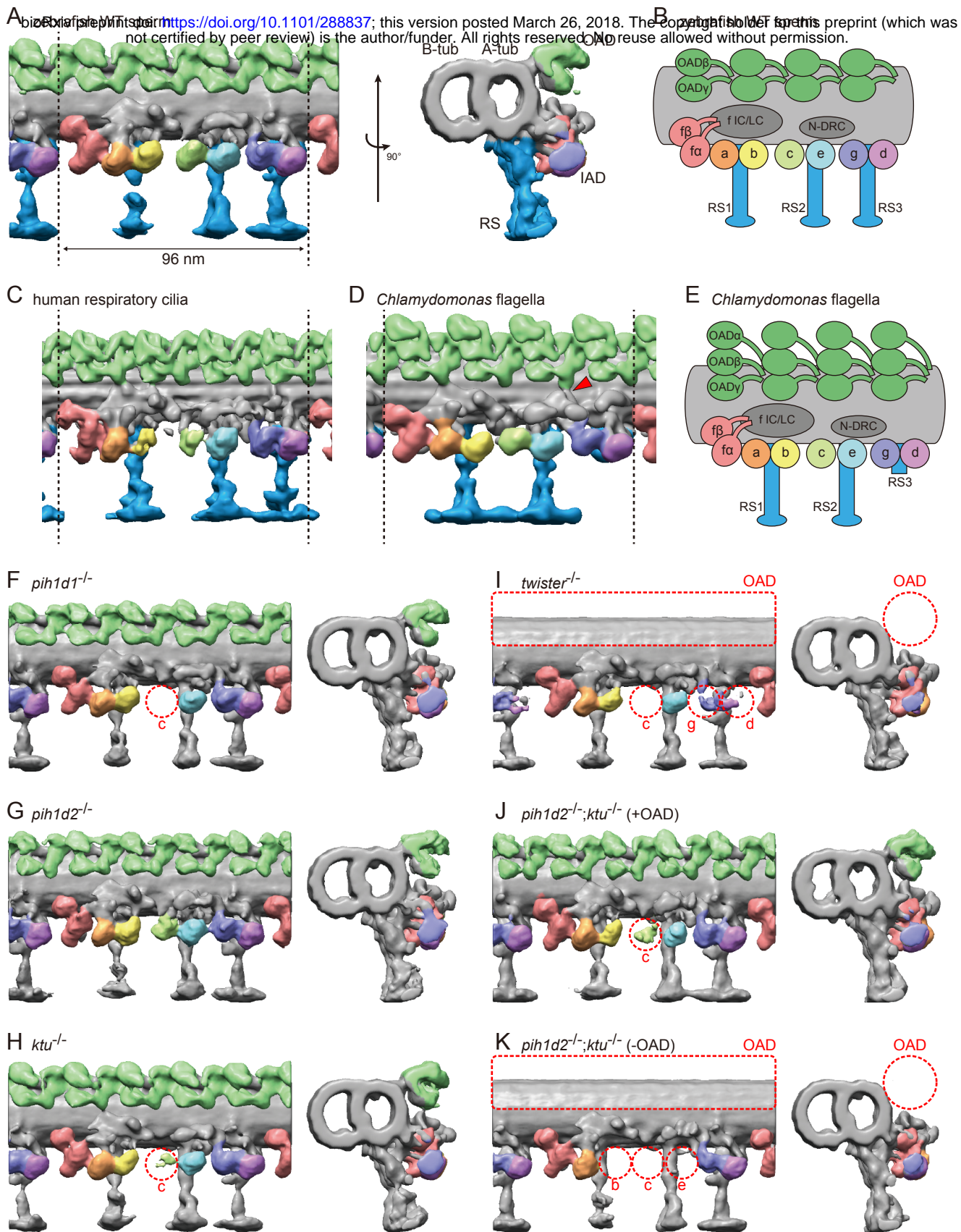


Figure 3. Cryo-ET revealed structural defects of axonemal dyneins in mutant spermatozoa. (A) DMT structure of native zebrafish sperm. Left: side view. Right: base-to-tip view. A-tub and B-tub: A- and B-tubule of DMT, respectively. (B) Diagram of the DMT structure of zebrafish sperm. f IC/LC means IAD f intermediate chain and light chain complex. (C) DMT structure of human respiratory cilia (EMD-5950; Lin et al., 2014). (D) DMT structure of *Chlamydomonas* flagella (DMT 2-8 averaged; EMD-2132; Bui et al., 2012). Red arrow indicates a linker between N-DRC and OAD. (E) Diagram of the DMT structure of *Chlamydomonas* flagella. (F-K) DMT structures of PIH gene mutant spermatozoa. For *pih1d2*^{-/-}; *ktu*^{-/-}, J (+OAD) and K (-OAD) represent subtomograms of axonemes with or without OADs, respectively. Red circles indicate the defects of axonemal dyneins. Green, OADs; red, IAD f; orange, IAD a; yellow, IAD b; light-green, IAD c; cyan, IAD e; indigo, IAD g; violet, IAD d; blue, RSs.

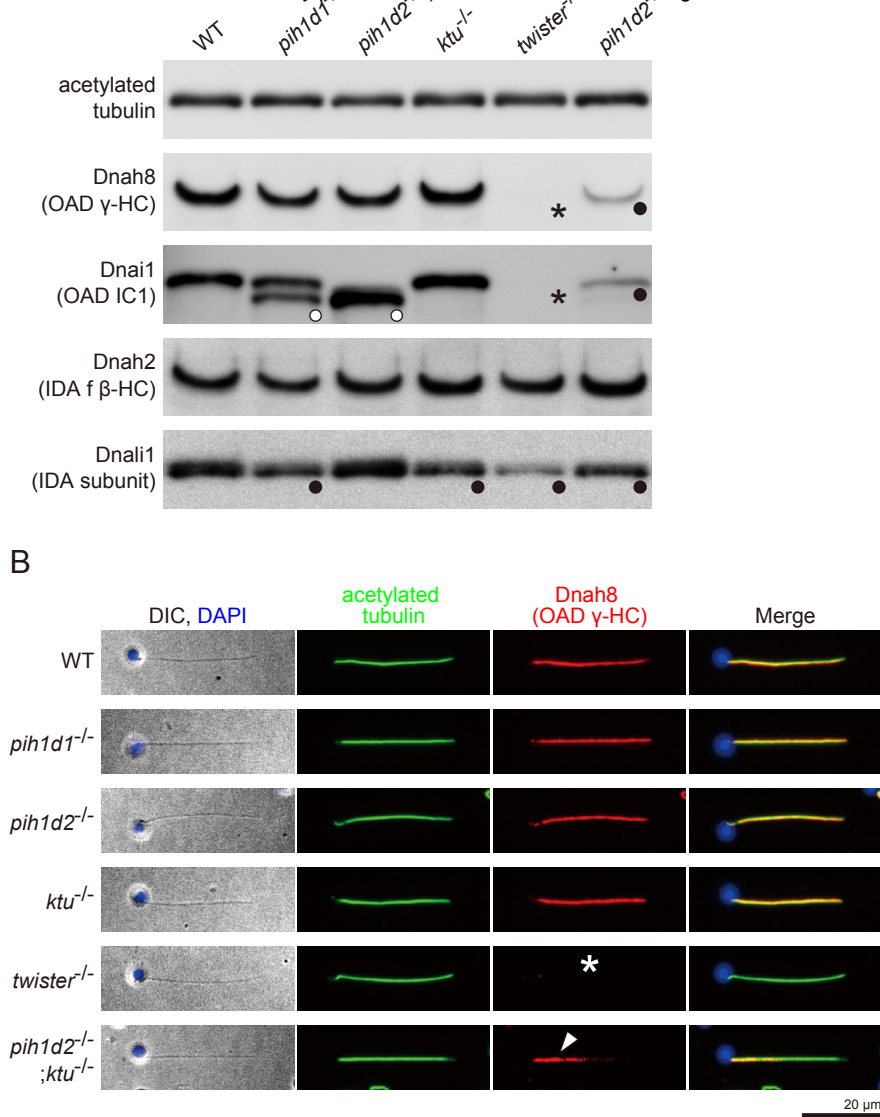


Figure 4. Immunoblot and immunofluorescence microscopy of axonemal dyneins. (A) Immunoblot of sperm axonemes. Asterisks, filled circles, and open circles indicate missing, decreased, and shifted bands, respectively. Acetylated tubulin: control. (B) Immunofluorescence microscopy of zebrafish spermatozoa. Dnah8 was localized along the entire length of sperm flagella in WT, *pih1d1^{-/-}*, *pih1d2^{-/-}*, *ktu^{-/-}*. In *twister^{-/-}*, Dnah8 was lost (white asterisk), while in *pih1d2^{-/-};ktu^{-/-}*, Dnah8 was localized only in the proximal half of the flagella (white arrowhead).

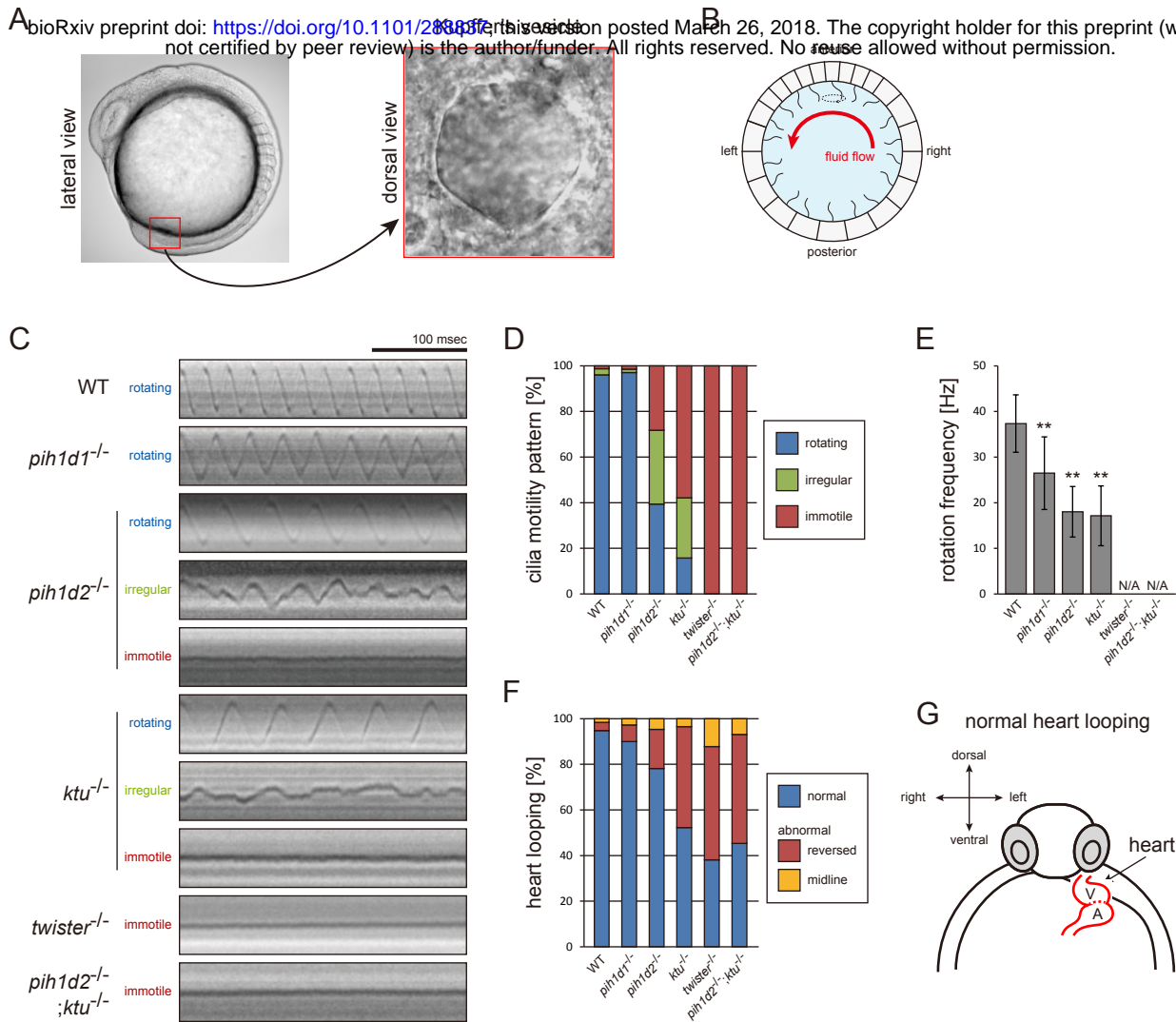
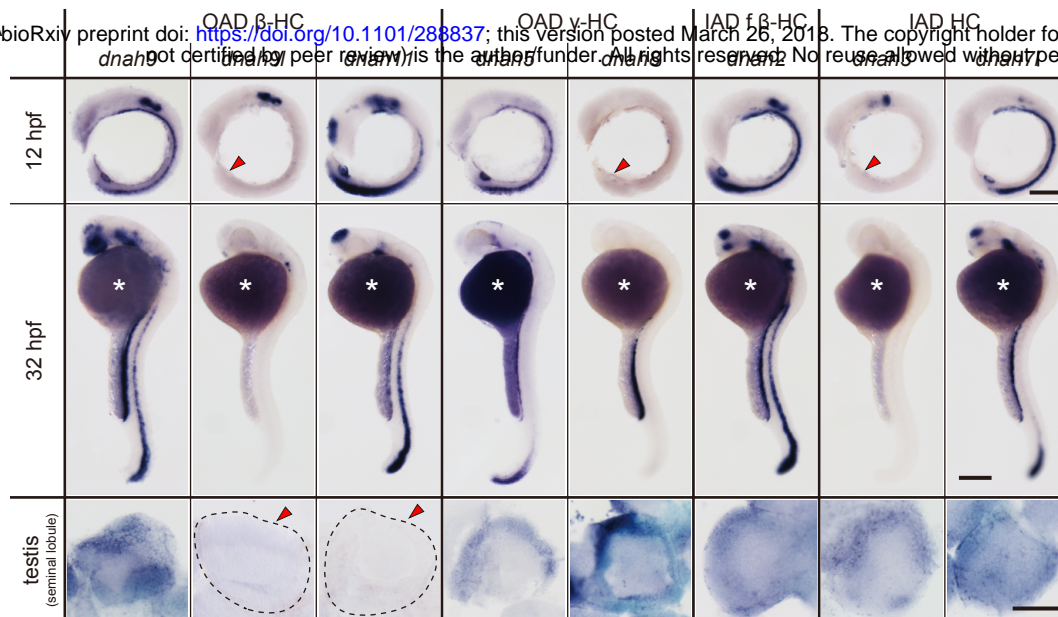


Figure 5. Mutations of PIH genes caused abnormal motilities of Kupffer's vesicle cilia. (A) Images of zebrafish embryo and Kupffer's vesicle. Red square in left-hand image indicates the position of the Kupffer's vesicle in a 12 hpf embryo. Right-hand image shows the dorsal view of the Kupffer's vesicle. (B) Diagram of zebrafish Kupffer's vesicle. Mono-cilia, which project from epithelial cells, have rotational motilities to produce left-ward fluid flow (red arrow) in the organ. (C) Typical kymographs of Kupffer's vesicle cilia in WT and PIH gene mutants. Patterns of the kymographs were categorized into three classes: rotating (blue), irregular (green), and immotile (red). (D) Ratios of each motility class. Number of observed cilia: WT, 76; *pih1d1*^{-/-}, 67; *pih1d2*^{-/-}, 198; *ktu*^{-/-}, 190; *twister*^{-/-}, 53; and *pih1d2*^{-/-};*ktu*^{-/-}, 48. (E) Rotational frequencies of Kupffer's vesicle cilia. Bar graphs show mean \pm SD. **: p-value < 0.01 in Dunnett's test of each mutant against WT. (F) Heart looping of WT and mutant embryos at 30 hpf. Number of samples: WT, 247; *pih1d1*^{-/-}, 288; *pih1d2*^{-/-}, 275; *ktu*^{-/-}, 285; *twister*^{-/-}, 139; and *pih1d2*^{-/-};*ktu*^{-/-}, 276. (G) Diagram represents normal heart looping of the 30 hpf embryo. V, ventricle; A, atrium.



B

		gene expression	
		Kupffer's vesicle	testis
	<i>dnah9</i>	+	+
OAD β -HC	<i>dnah9l</i>	-	-
	<i>dnah11</i>	+	-
OAD γ -HC	<i>dnah5</i>	+	+
	<i>dnah8</i>	-	+
IAD f β -HC	<i>dnah2</i>	+	+
IAD HC	<i>dnah3</i>	-	+
	<i>dnah71</i>	+	+

Figure 6. DNAH genes showed distinct expression patterns in zebrafish embryos and testis. (A) Whole-mount *in situ* hybridization of DNAH genes with embryos (12 and 32 hpf) and dissected testis (seminal lobule). For embryos, lateral views are shown. Yolk of 12 hpf embryos was removed before observation to show Kupffer's vesicle clearly. Red arrowheads indicate Kupffer's vesicles or testes in which DNAH gene expressions were not detected. White asterisks: non-specific staining of yolk. Scale bars: 200 μ m for embryos; 100 μ m for testes (B) Comparison of DNAH gene expression between Kupffer's vesicle and testis. +, expressed; -, expression not detected. Red indicates when DNAH gene expression was difference between the two organs.

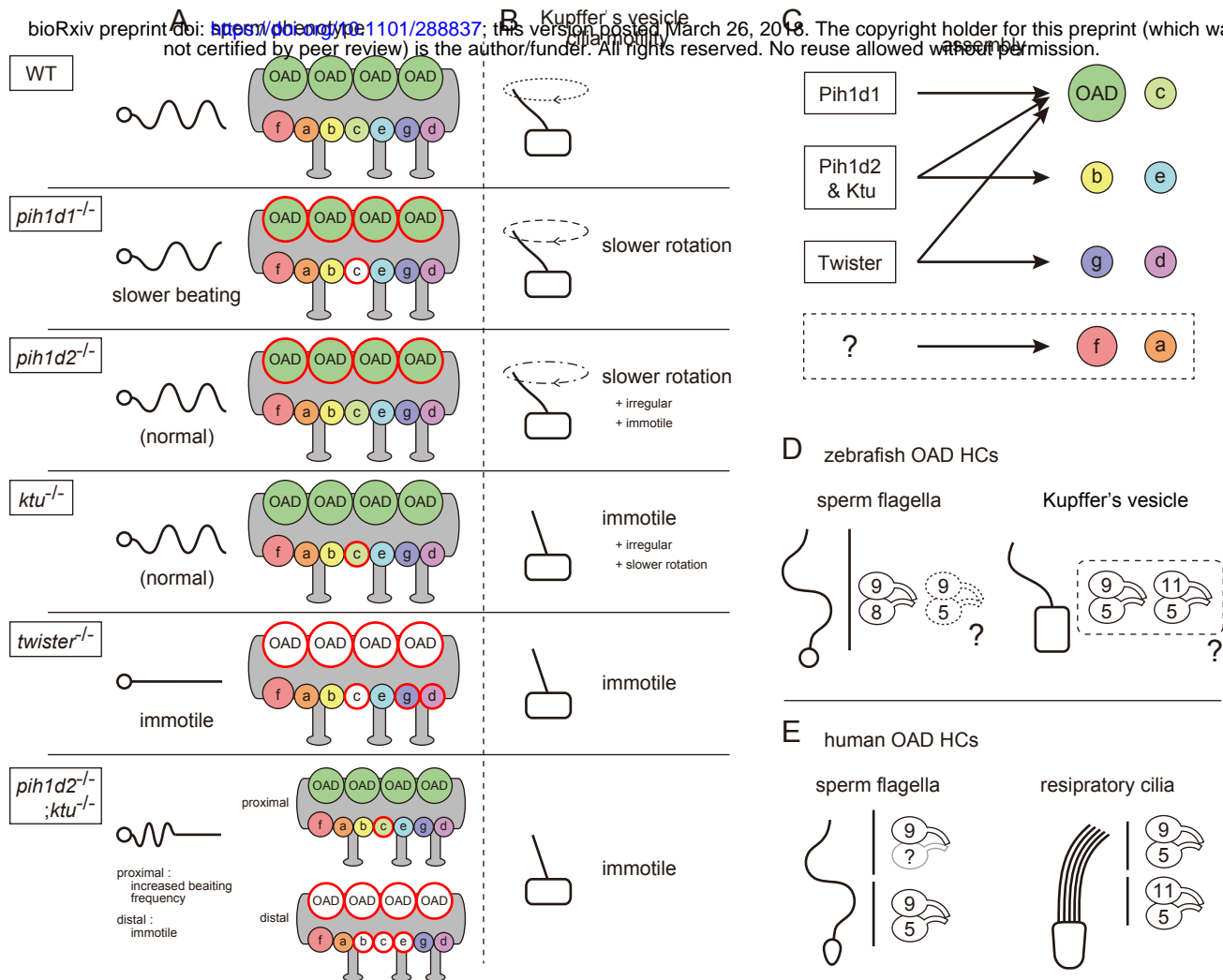


Figure 7. Mutant phenotypes revealed axonemal dyneins assembled by distinct PIH proteins in zebrafish.

(A) Summary of the sperm motilities and the axonemal structures of WT and each PIH gene mutant. Abnormal axonemal dyneins are indicated by red circles filled with white (missing) or color (diminished or Dna11 defects).

(B) Summary of the motilities of Kupffer's vesicle cilia.

(C) Proposed model of the axonemal dynein assembly by distinct PIH proteins. Pih1d1, Pih1d2 and Ktu, and Twister are required for the assembly of specific subsets of axonemal dyneins. Factors responsible for the construction of IAD a or IAD f were not revealed in our research.

(D) Possible combinations of OAD HCs in zebrafish sperm flagella and Kupffer's vesicle cilia, suggested by the expression analysis of DNAH genes. Although testis showed expression of both γ -HC genes (*dnaH5* and *dnaH8*), *Dnah8* were present along the entire length of sperm flagella, while the distribution of *Dnah5* is not known.

(E) Possible combinations of OAD HCs in human sperm flagella and respiratory cilia. Localizations of DNAH9 and DNAH5 in human spermatozoa were reported by Fliegauf et al. (2005). Localizations of DNAH9, DNAH11 and DNAH5 in human respiratory cilia were reported by Dougherty et al. (2016).

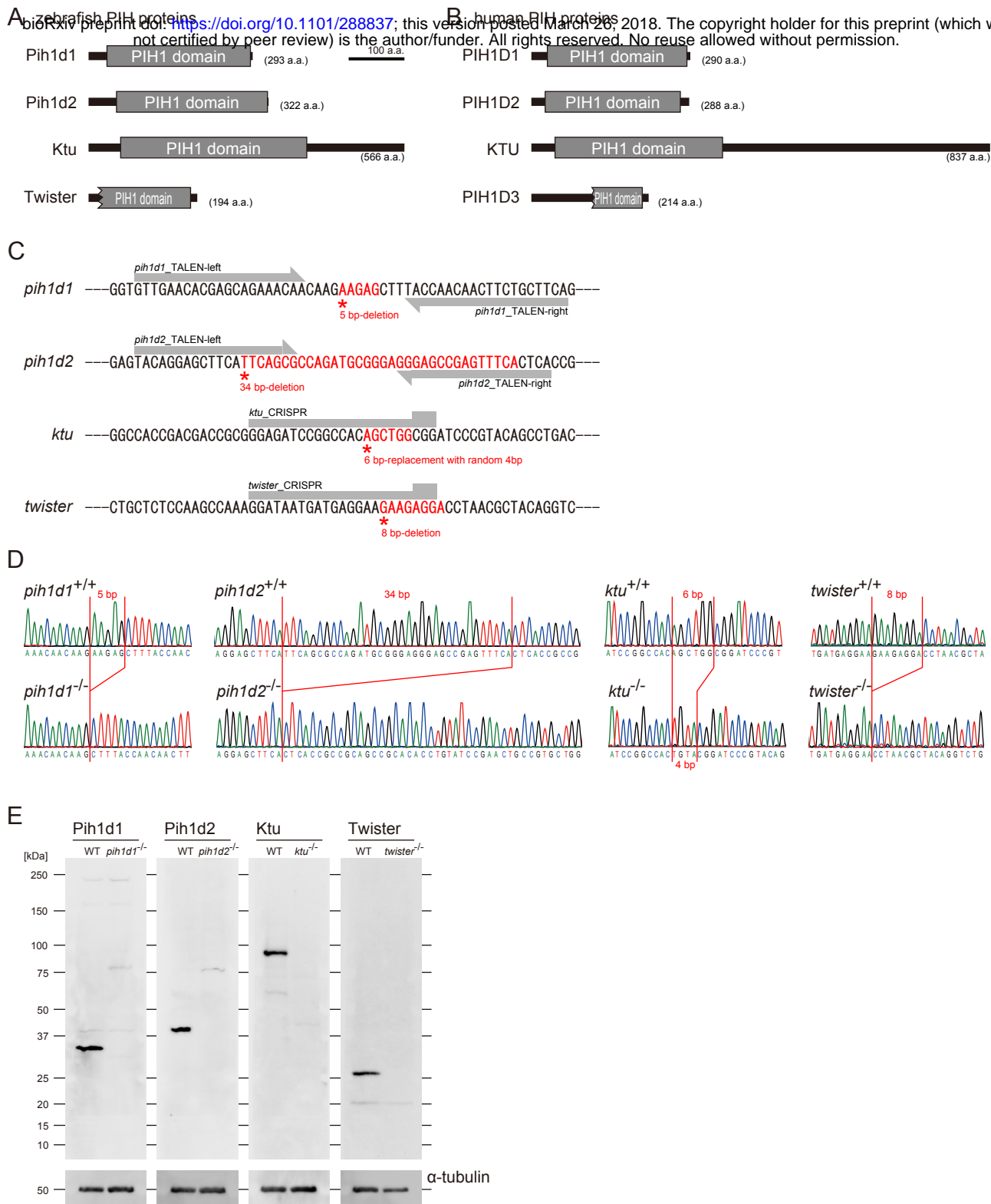


Figure S1. PIH gene mutants were established by genome-editing. (A and B) Domain structures of PIH proteins in (A) zebrafish and (B) human. Protein motifs were obtained by SMART or Pfam analyses. (C) Target sites of our genome-editing. Induced mutations changed the sequences shown in red. (D) Sanger sequencings of WT and PIH gene mutants around the target sites of our genome-editing. (E) Immunoblot of testis lysate of WT and PIH gene mutants. Induced mutations caused null of each PIH protein. α -tubulin: control.

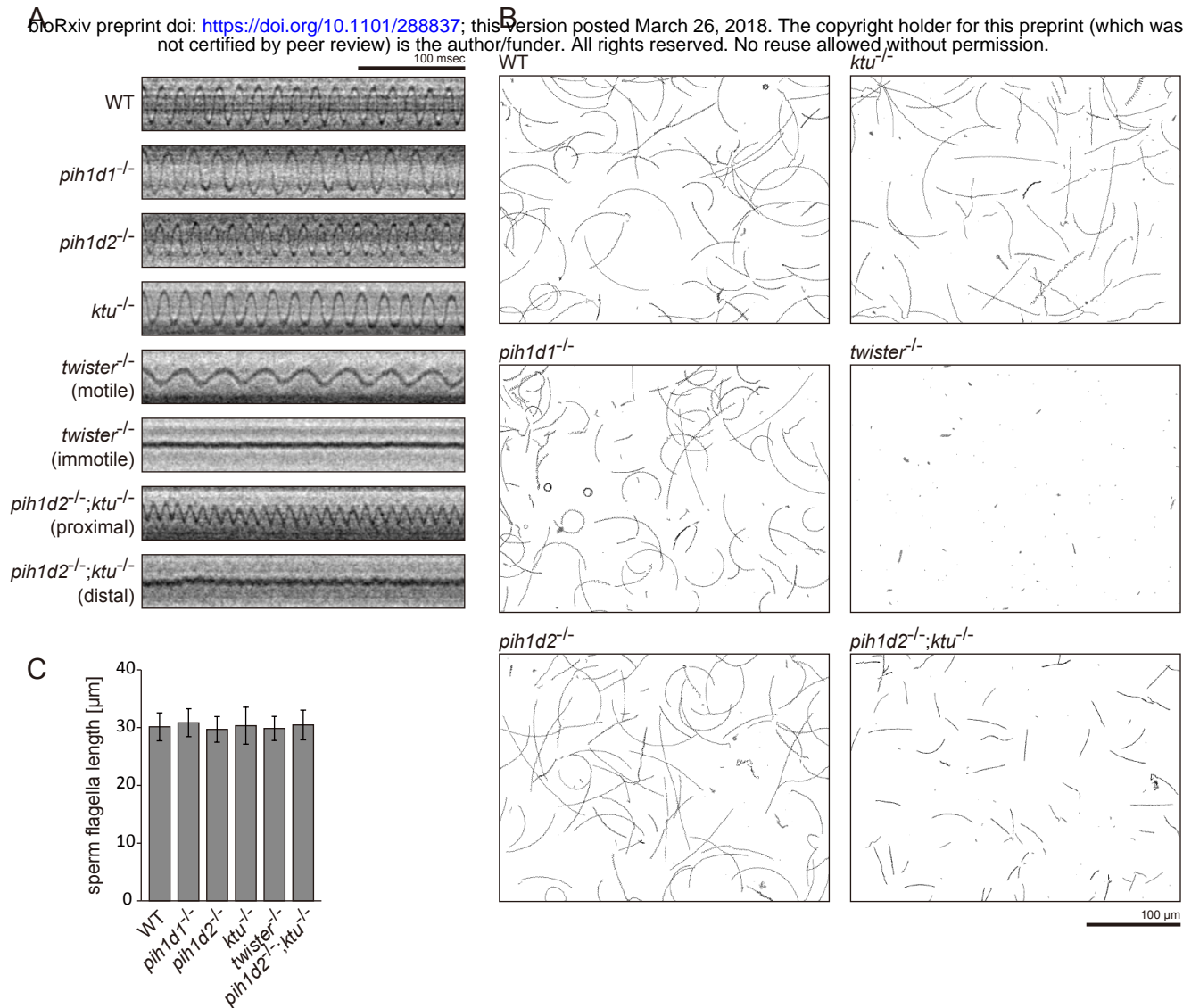


Figure S2. Sperm motilities and flagellar length. (A) Typical kymographs of sperm flagella. (B) Typical traced paths of sperm heads in CASA. Black: traced paths of motile spermatozoa. Gray: traced paths of immotile (velocity < 20 µm/s) spermatozoa. (C) Length of sperm flagella. Immunofluorescent staining of sperm with acetylated tubulin antibodies was performed to measure the length of the flagella. Number of samples: WT, 129; *pih1d1*^{-/-}, 64; *pih1d2*^{-/-}, 78; *ktu*^{-/-}, 84; *twister*^{-/-}, 81; and *pih1d2*^{-/-};*ktu*^{-/-}, 80. Bar graphs show mean ± SD. Significant differences (p-value < 0.05) were not detected in a Dunnett's test of each mutant against WT.

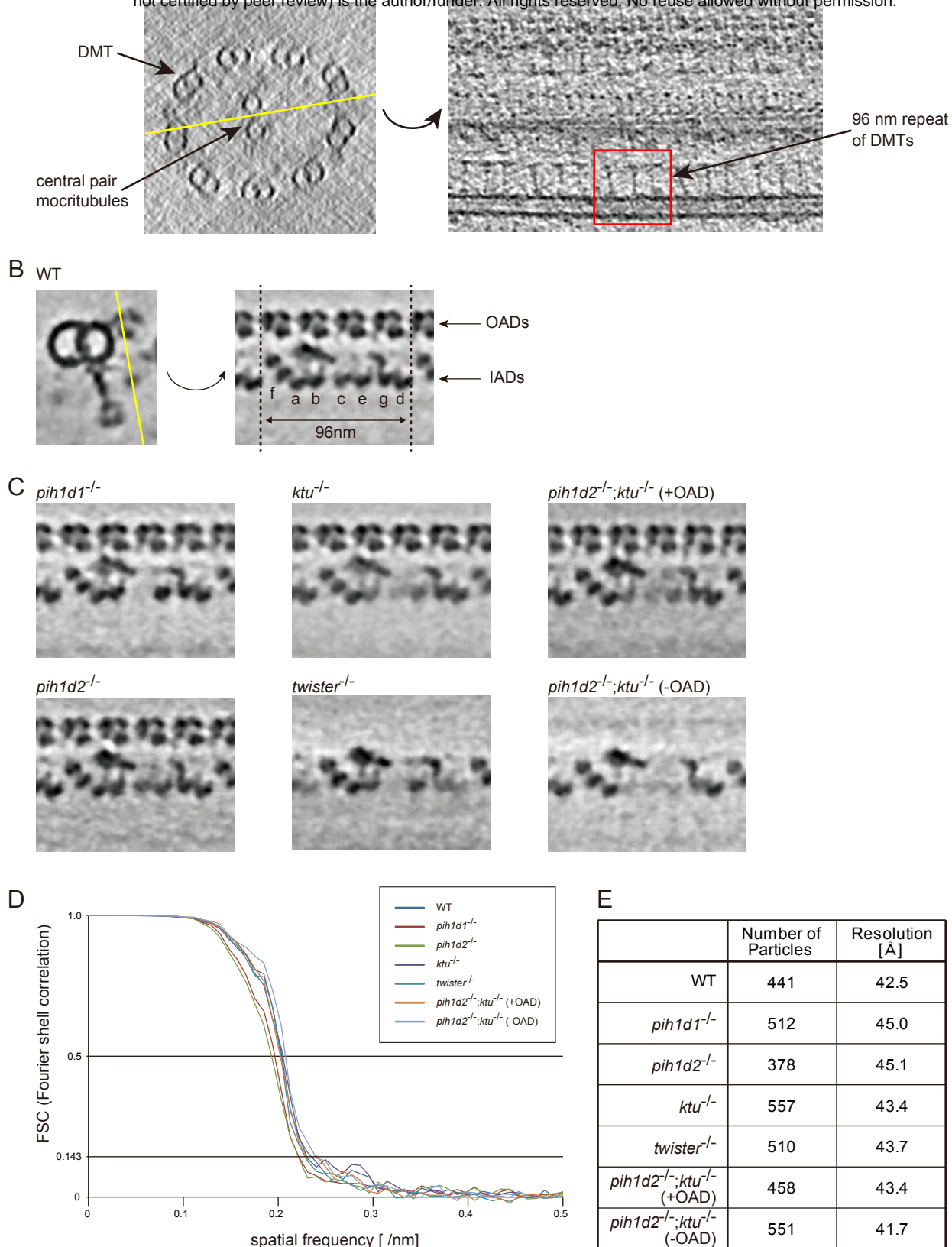


Figure S3. Tomographic slices and Fourier shell correlations of the averaged subtomograms. (A) Tomographic slices (20 nm thick) of WT sperm axoneme. Left: cross-section (base-to-tip view). Right: longitudinal section at the location indicated by the yellow line in the left-hand image. (B) 2 nm-thick slices of the WT sperm subtomogram. Cross-section is shown on the left. Yellow line indicates the position of the right-hand slice and the slices in C. (C) 2 nm-thick slices of PIH gene mutant subtomograms. (D) Fourier shell correlation curves of the averaged subtomograms in this study. Effective resolutions were determined by a cutoff value of 0.143. (E) Number of particles and effective resolutions of the averaged subtomograms.

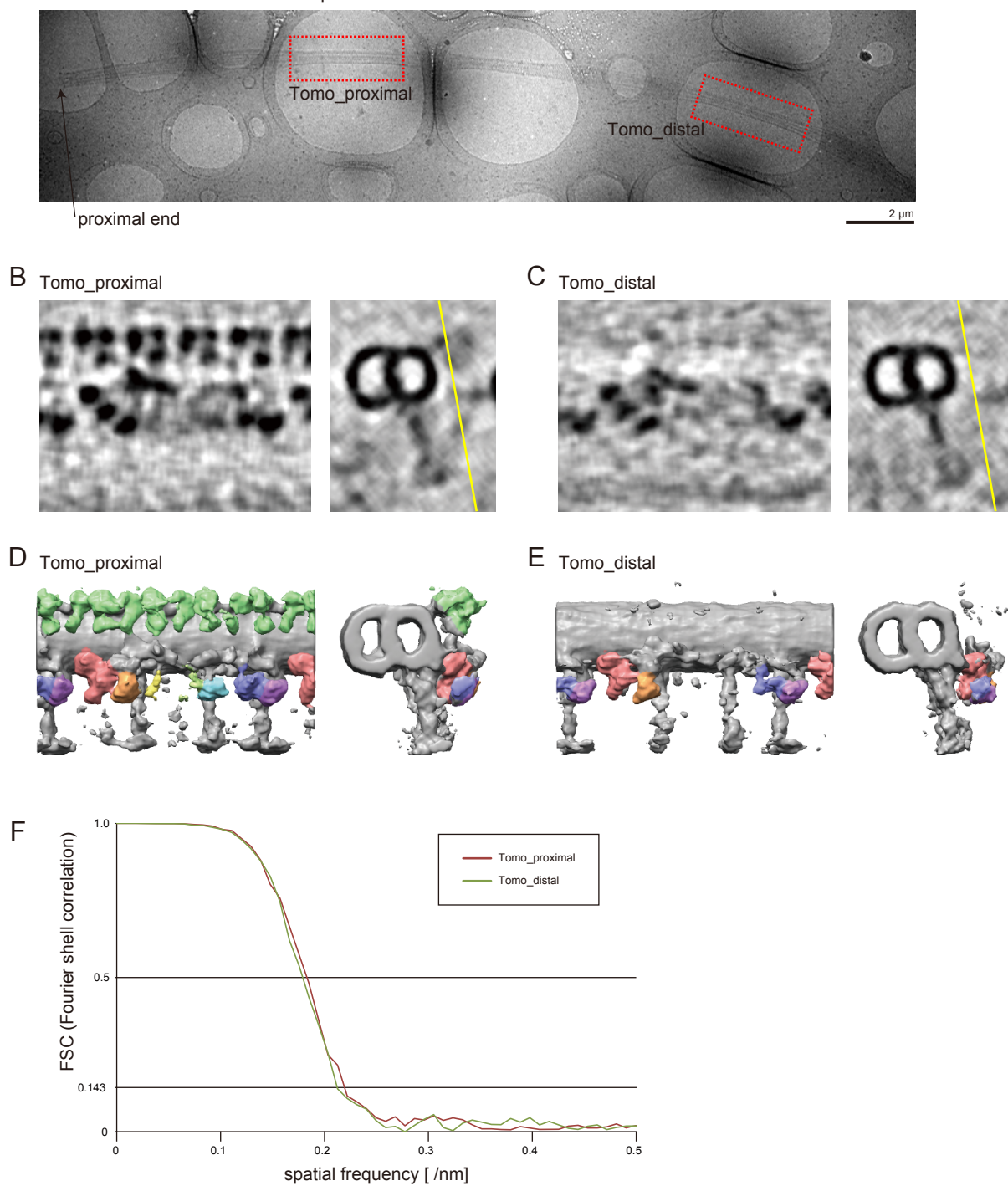


Figure S4. Axonemal structures in *pih1d2^{-/-};ktu^{-/-}* sperm. (A) Cryo-TEM image of *pih1d2^{-/-};ktu^{-/-}* axoneme, which was suitable for observing both proximal and distal regions by cryo-ET. Red squares indicate the observed areas. (B and C) 5 nm-thick slices of the subtomograms of the proximal (B; Tomo_proximal) and the distal (C; Tomo_distal) regions. Images on the left show slices of subtomograms at the point indicated by the yellow lines in the images on the right (cross-section). (D and E) 3D images of averaged subtomograms. Left: side views. Right: cross views. (F) Fourier shell correlation curves of the averaged subtomograms in this study. Number of particles: Tomo_proximal, 128; and Tomo_distal, 115. Effective resolutions were determined by a cutoff value of 0.143, and approximately 46 \AA was estimated.

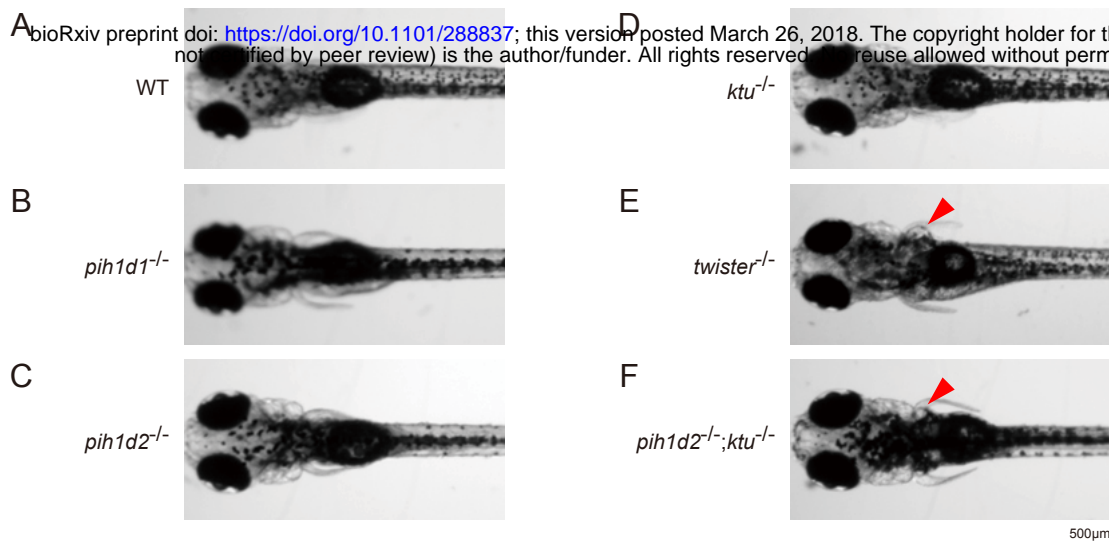


Figure S5. Expansion of pronephric duct was observed in *twister*^{-/-} and *pih1d2*^{-/-};*ktu*^{-/-}. (A-F) Dorsal views of zebrafish larvae at 4 days post-fertilization. (A) WT, (B) *pih1d1*^{-/-}, (C) *pih1d2*^{-/-}, (D) *ktu*^{-/-}, (E) *twister*^{-/-}, (F) *pih1d2*^{-/-};*ktu*^{-/-}. *twister*^{-/-} and *pih1d2*^{-/-};*ktu*^{-/-} exhibited abnormal expansions of pronephric ducts (red arrowheads), which disabled larvae from having their pectoral fins close to their bodies.