



# TTC12 loss-of-function mutations cause primary Ciliary Dyskinesia and unveil distinct dynein assembly mechanisms in motile cilia versus flagella

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**1 *TTC12* loss-of-function mutations cause primary ciliary dyskinesia and unveil distinct dynein**  
**2 assembly mechanisms in motile cilia versus flagella**

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## 52    **Abstract**

53    Cilia and flagella are evolutionarily conserved organelles whose motility relies on the outer and inner  
54    dynein arm complexes (ODAs/IDAs). Defects in ODAs/IDAs result in primary ciliary dyskinesia (PCD), a  
55    disease characterized by recurrent airway infections and male infertility. To date PCD mutations in  
56    assembly factors cause a combined ODA/IDA defect, affecting both cilia and flagella. We identified four  
57    loss-of-function mutations in *TTC12*, which encodes a cytoplasmic protein, in four independent  
58    families in which affected individuals displayed a peculiar PCD phenotype characterized by the absence  
59    of ODAs and IDAs in sperm flagella, contrasting with the sole absence of IDAs in respiratory cilia.  
60    Analyses of both primary cells from individuals carrying *TTC12* mutations and human differentiated  
61    airway cells invalidated for *TTC12* by a CRISPR-Cas9 approach revealed an IDA defect restricted to a  
62    subset of single-headed IDAs different in flagella and cilia, while *TTC12* depletion in the ciliate  
63    *Paramecium tetraurelia* recapitulated the sperm phenotype. Overall, our study, which identifies *TTC12*  
64    as a gene involved in PCD, unveils distinct dynein assembly mechanisms in human motile cilia versus  
65    flagella.

## 66 Introduction

67 Primary ciliary dyskinesia (PCD [MIM: 244400]) is a group of clinically and genetically heterogeneous  
68 disorders caused by dysfunction of motile cilia leading to impaired mucociliary clearance and early  
69 recurrent airway infections. About one person in 10,000 individuals is concerned<sup>1</sup>, and given the key  
70 role of motile cilia in the establishment of left-right asymmetry during embryogenesis<sup>2</sup>, nearly 50% of  
71 those individuals display a *situs inversus* thereby defining the Kartagener syndrome. In addition, as the  
72 microtubule-based structure of motile cilia, the axoneme, is close to that of sperm flagella, most male  
73 individuals are also infertile. The axoneme consists of nine peripheral outer microtubule doublets  
74 circularly arranged around two central microtubules surrounded by a central sheath ("9+2" pattern).  
75 Attached all along the microtubule length, the outer dynein arms (ODAs) and inner dynein arms (IDAs)  
76 are multiprotein complexes, which carry an ATPase activity and provide the sliding force for motility.  
77 In humans, ODAs are composed of two axonemal dynein heavy chains (HCs), namely the  $\beta$  and  $\gamma$   
78 chains, which are attached to a large intermediate chain/light chain complex (IC/LC). Two types of  
79 ODAs have been described in cilia: the type 1 ODAs are located at the proximal part of the cilium and  
80 contain DNAH5 ( $\gamma$ -chain) associated with DNAH11 ( $\beta$ -chain), while the type 2 ODAs are located at the  
81 distal part of the cilium and contain DNAH5 ( $\gamma$ -chain) associated with DNAH9 ( $\beta$ -chain). Noteworthy,  
82 recent studies performed in humans revealed that the ODA composition of spermatozoa differs from  
83 that found in cilia<sup>3</sup>: in spermatozoa, the  $\gamma$ - and  $\beta$ -chain consist in DNAH8 and DNAH17, respectively,  
84 both being specifically expressed in sperm cells. As for IDAs, their exact structure and composition is  
85 virtually unknown in humans. Most of the knowledge was provided by studies in the flagellated alga  
86 *Chlamydomonas reinhardtii* where seven IDA subspecies have been identified. Six distinct single-  
87 headed IDA subspecies (called IDA a, b, c, d, e, and g), each including only one HC, have been  
88 characterized, and one dimeric IDA complex (called IDA f or IDA-I1), which contains two HCs, has been  
89 identified<sup>4,5,6,7</sup>. There is growing interest in identifying the key players involved in the process of ODA  
90 and IDA assembly and transport. In this regard, one of the most frequent ciliary ultrastructural defects  
91 found in PCD corresponds to the absence of both dynein arms (DAs)<sup>8</sup> due to mutations in genes

encoding proteins involved in ODA and IDA assembly, docking and transport (i.e. *DNAAF2/KTU*<sup>9</sup> [MIM: 612517], *DNAAF1/LRRC50*<sup>10</sup> [MIM: 613190], *DNAAF3*<sup>11</sup> [MIM: 614566], *CCDC103*<sup>12</sup> [MIM: 614677], *DNAAF5/HEATR2*<sup>13</sup> [MIM: 614864], *LRRC6*<sup>14</sup> [MIM: 614930], *DNAAF4/DYX1C1*<sup>15</sup> [MIM: 608709], *ZMYND10*<sup>16</sup> [MIM: 607070], *SPAG1*<sup>17</sup> [MIM: 603395], *CFAP298/C21orf59*<sup>18</sup> [MIM: 615494], *PIH1D3*<sup>19</sup> [MIM: 300933] and *CFAP300/C11orf70*<sup>20</sup> [MIM: 618058]).

Although several of these genes have been renamed with the acronym DNAAF, which stands for Dynein Axonemal Assembly Factor, their precise role in the assembly and/or transport machinery is not known. As shown recently, multiciliated cells contain cytoplasmic liquid-like organelles that concentrate DNAAFs with dynein chains and chaperones<sup>21</sup>. To date very few investigations have been performed on spermatozoa from PCD-affected individuals; in all cases, the sperm ultrastructure defects observed by transmission electron microscopy (TEM) were always found to be identical to those reported in the cilia. In this regard, the recent observation of differences in protein composition of ODAs between human cilia and sperm flagella<sup>3</sup> raises the question as to whether the assembly and transport mechanisms of ODA and IDA components are conserved between human cilia and flagella.

In the present study, we identified independent families in which the individuals displayed a peculiar PCD phenotype characterized by the absence of IDAs in respiratory cilia, contrasting with the absence of both ODAs and IDAs in sperm. Such unusual observation prompted us to search for the molecular basis of the PCD phenotype in those individuals and to decipher the consequences of the identified molecular defects on cilia and flagella assembly, by developing an *in vivo* model in the ciliate *Paramecium tetraurelia* and a CRISPR-Cas9-mediated genome editing approach in human primary airway epithelial cells (AECs).

## **Material and Methods**

### **Affected individuals**

Written informed consent was obtained from all affected individuals and/or their parents following protocols approved by the Comité de Protection des Personnes (CPP) Ile de France III and the Institutional Review Board of the French Institute of medical research health (CPP02748 and CEEI-IRB: opinion number 15-259, respectively).

### **Genetic analyses**

Identification of *TTC12* (RefSeq NM\_017868.4) sequence variations was performed from genomic blood DNA, either by WES or by parallel sequencing with a custom targeted-capture panel. More precisely, in individual DCP791, WES was performed with the Agilent SureSelect V5 target enrichment system on a HiSeq sequencing machine (Illumina). In individuals DCP153 and DCP1606, WES was performed with the SeqCap EZ MedExome target enrichment kit on a NextSeq sequencing machine (Illumina). The DNA of individual 18GM00157 was analyzed on a MiSeq sequencer (Illumina) with a custom targeted-capture panel (SeqCap EZ Choice, Roche Diagnostics) that encompasses the 45 genes involved in PCD and the 250 candidate genes for PCD. The libraries were prepared following manufacturer's instructions. Data were analyzed through an in-house double pipeline based on Bowtie2 and BWA tools. Reads were visualized with the IGV viewer (Broad Institute). Copy number variation analysis was performed with a depth-ratio comparison between subjects sequenced in the same run, and depth ratios obtained for each of the 4 individuals were represented on a graph built with the GraphPad Prism 5 software.

Sanger sequencing was performed on genomic blood DNA with the BigDye Terminator v3.1 system (Thermo Fisher) following PCR-amplification with the Go-Taq Green Master Mix (Promega) at an annealing temperature of 60°C and purification with ExoSAP-IT(USB), following manufacturer's instructions. Fragments were analyzed on a 3730XL device (Thermo Fisher) after Sephadex G-50

138 Superfine purification (GE Healthcare). Amplification and sequencing primers are listed in **Table S1**.  
139 Sequences were analyzed with the SeqScape software (Thermo Fisher).

140

#### 141 **TEM analysis of ciliary and flagella ultrastructure**

142 AECs obtained from nasal or bronchial biopsies were fixed in 2.5% glutaraldehyde to be processed for  
143 TEM as previously described<sup>22</sup>. Ciliary ultrastructural results were quantified as a percentage of  
144 abnormal cilia among the total number of analyzed cilia. Semen was fixed in 2.0% v/v glutaraldehyde  
145 in phosphate buffer, washed for 15 min in fresh buffer containing 4% w/v sucrose and embedded in  
146 2% agar. Post-fixation lasted 1h in 1% w/v osmic acid in phosphate buffer. After dehydration in a  
147 graded series of ethanol, small pieces of agar containing sperm cells were embedded in Epon resin  
148 (Polysciences Inc., Warrington, PA, USA). Sections were cut on a Reichert OmU2 ultramicrotome  
149 (Reichert-Jung AG, Wien, Austria) using a diamond knife. Sections (70 nm) were collected on nickel  
150 grids and stained with uranyl acetate (2% in 70% ethanol, 20 min) and Reynolds lead citrate (10 min),  
151 and examined using a Zeiss transmission electron microscope 902 (Leo, Rueil-Malmaison, France).  
152 Images were acquired using a Gatan Orius SC1000 CCD camera (Gatan France, Grandchamp, France).

153

#### 154 **Immunoblotting**

155 AECs obtained by airway brushing from individuals 18GM00157, DCP1606 and control individuals, as  
156 well as spermatozoa from a control donor, were suspended in ice-cold lysis buffer (50mM Tris, 150  
157 mM NaCl, 1% Triton X-100, and Complete Protease Inhibitor Cocktail (Roche Diagnostics)). Lysates  
158 were sonicated and cleared by 10 minutes of centrifugation at 1000g, 4°C.

159 Cell lysates were loaded on 10% SDS-PAGE, transferred 2h on a PVDF membrane and analyzed by  
160 western blotting with TTC12 antibody (Santa Cruz sc-390229, 1/200), DNAJB13 antibody (HPA052465,  
161 1/300) or DNAI1 antibody (HPA021649, 1/400), all incubated overnight at 4°C in PBS - 0.1% Tween 20 -  
162 5% milk. The secondary antibody (rabbit-HRP, SIGMA, A0545) was incubated for 1h at room  
163 temperature at a final dilution of 1:5000 in PBS - 0.1% Tween 20 - 5% milk. Proteins were detected



using Amersham ECL Select Western Blotting Detection Reagent (GE healthcare) according to the manufacturer's recommendations.

#### **Immunofluorescence analyses on AECs**

All antibodies are listed in **Table S2**. AECs obtained by nasal brushing from individuals 18GM00157, DCP1606 and control individuals (i.e. individuals in whom the diagnosis of PCD was excluded on the basis of the absence of sinopulmonary symptoms and of normal values of nasal nitric oxide) were suspended in Ferticult IVF medium. Samples were cytocentrifugated onto glass slides, air-dried and directly used for immunostaining. Cells were first fixed with 4% paraformaldehyde for 15 min at room temperature, washed three times with PBS supplemented with 0.49mM MgCl<sub>2</sub> and 0.9mM CaCl<sub>2</sub>, incubated for 10 min with PBS++ NH<sub>4</sub>Cl 50mM, and permeabilized with 0.1% Triton X-100 for 10 min. Then, cells were incubated 2h at room temperature with primary antibodies before being revealed by secondary antibodies at room temperature during 1h. All antibodies were incubated in PBS++ 1% BSA. Cells were finally washed three times with PBS++, mounted in ProLong<sup>®</sup> Gold Antifade Reagent with DAPI (Cell Signaling #8961) to stain nuclei, and images were taken using an ECLIPSE 80i microscope (Nikon) with a Retiga-2000R camera (QImaging).

#### **Immunofluorescence analyses of human spermatozoa**

Semen samples were obtained by masturbation after a period of two to seven days of sexual abstinence, evaluated according to World Health Organization (WHO) guidelines<sup>23</sup> and then frozen until further analyses. 10µl of frozen semen samples was spread onto a Superfrost Plus slide (Menzel Glasbearbeitungswerk, GmbH & Co. KG, Braunschweig, Germany) and fixed by incubation with PBS/4% paraformaldehyde for 10 minutes. The slides were incubated 20 minutes at 95°C in citrate buffer (H-3300, VectorLabs, Burlingame, CA, USA), treated with 0.2% Triton in PBS for permeabilization and then blocked by incubation in 1% BSA for 1 hour. The slides were then incubated with primary antibodies overnight at 4°C and then with secondary antibodies for one hour at room temperature. The slides

were mounted in Vectashield medium (Vector Laboratories, Burlingame, USA) supplemented with 0.5mg/ml DAPI and were subsequently analyzed with a Zeiss Axiophot epifluorescence microscope. Digital images were acquired with a cooled charge-coupled device (CCD) camera (Hamamatsu Co. Japan), under identical instrument settings, with MetaMorph® software (Molecular Devices, Inc. USA).

#### ***Paramecium* experiments**

All experiments were carried out with entirely homozygous mutant strains: 7S strain for feeding experiments, and nd7 which carries a recessive monogenic mutation blocking trichocyst discharge<sup>24</sup> for *Paramecium* transformation.

##### **- *Paramecium* strain and cultivation**

Cells were grown at 27 °C in a wheat grass powder (Bio Herbe de Blé L'arbre de vie, Luçay Le Male, France) infusion medium bacterized with *Klebsiella pneumoniae* and supplemented with 0.8 µg/ml β-sitosterol according to standard procedures<sup>25</sup>.

##### **- *Paramecium* gene knockdown experiments**

RNAi gene silencing was achieved using the feeding procedure as previously described<sup>26</sup>. Plasmids used in RNAi silencing experiments were obtained by cloning PCR products, amplified from the coding region of each gene (**Table S3**), in L4440 vector<sup>27</sup> using Gibson technology (Gibson DJ 2009). These plasmids were then transformed in HT115 DE3 *Escherichia coli* strain to produce T7Pol-driven dsRNA as previously described<sup>26</sup>.

##### **- Microinjection of a transgene expressing a GFP-tagged TTC12a protein**

To become resistant to RNAi silencing, the nucleotide sequence from 471 to 745 (corresponding to dsRNA sequence) of the *TTC12a* coding region was replaced *in vitro* by a modified synthetic DNA sequence coding for the same amino-acid sequence (**Figure S1**). GFP coding sequence was fused at the 3' end of WT or *TTC12a* resistant-RNAi version and cloned (using Gibson technology (Gibson DJ 2009)) in XhoI site of pPXV-GFP vector under the control of the constitutive regulators of the *Paramecium* calmodulin gene<sup>28</sup>. Twelve nucleotides coding for four glycine amino acids were introduced between the *TTC12a* coding region and the GFP coding sequence. The plasmid was linearised by SfiI digestion

216 and microinjected with ND7-complementing plasmid into the macronucleus of nd7-1 mutant cells  
217 unable to discharge their trichocysts<sup>24</sup>. Transformants were first screened for their ability to discharge  
218 trichocysts and if so, further analyzed. Microinjection was made under an inverted Nikon phase-  
219 contrast microscope, using a Narishige micromanipulation device and an Eppendorf air pressure  
220 microinjector.

#### 221 - *Paramecium* TEM analysis

222 For ultrastructural observations, paramecia were permeabilized in PHEM Buffer (PIPES 60 mM, HEPES  
223 25 mM, EGTA 10mM, MgCL2 2 mM, adjusted to pH 6.9 with NaOH) with 1% Triton X100 for 1 min,  
224 rinsed in PHEM Buffer and fixed in 1% (v/v) tannic acid and 1% (v/v) glutaraldehyde in PHEM buffer for  
225 30 min. Cells were then rinsed in PHEM and 0.05 M Cacodylate buffers before being postfixed in 1%  
226 OsO4 (v/v) 0.05 M cacodylate buffer, pH 7.4 for 30 min. After rinsing, cells were embedded into 2%  
227 agarose. Agar blocks were then dehydrated in graded series of ethanol and propylene oxide and  
228 embedded in Epon. Ultrathin sections were contrasted with uranyl acetate and lead citrate. The  
229 sections were examined with Jeol transmission electron microscope 1400 (at 120kV). The number of  
230 ODAs and IDAs were counted in about 140 cilia cross-sections obtained from TTC12-knocked down or  
231 control cells.

#### 232 - *Paramecium* swimming speed and cell trajectory

233 To analyze paramecia swimming behavior of TTC12-depleted and control cells, 8-10 paramecia were  
234 transferred into a drop of conditioned BHB medium and tracked for 10 s every 0.3 s to assess the  
235 swimming pattern under dark field microscopy, using MetaVue software. Image analysis and the  
236 measurement of swimming velocity were then performed with the ImageJ software.

#### 237 - *Paramecium* fluorescence-microscopy analysis

238 Immunofluorescence was performed as follows: paramecia were fixed in 2% paraformaldehyde in  
239 PHEM buffer for 15 min. Cells were then permeabilized for 15 min in 1% Triton X-100 in PHEM and  
240 washed 3 times in PBS/BSA 3%. Immunostaining were performed with polyglutamylated tubulin  
241 antibody (kindly provided by Carsten Janke, 1/500) as previously described<sup>29</sup>. Cells were observed with

a confocal Leica SP8 microscope and image stacks were processed with ImageJ and photoshop softwares.

- *Paramecium* deciliation, protein extraction and western blot

Cilia were purified<sup>30</sup> and recovered by centrifugation at 28,000 × g for 30 min and protein directly extracted in SDS-Laemmli buffer. Total protein content from paramecia or paramecia cell body (deciliated paramecia) was directly boiled in 10% SDS followed by dilution in 2XSDS Laemmli buffer. Proteins were then separated on SDS PAGE, blotted on nylon membrane and probed with an anti-GFP antibody (1:1,000, Interchim, Montluçon, France).

#### **Air-liquid interface (ALI) culture and infection**

AECs obtained either from surgical material from non PCD-affected individuals suffering from nasal polyps requiring surgery or from nasal brushing of PCD-affected individuals, were cultured at ALI as previously described<sup>31</sup>. Briefly, epithelial cells were seeded in 12-well collagen IV-coated transwell inserts (Costar, corning) and cultured in PneumaCult-Exp Plus medium (#05040, Stem Cell) until confluence were reached. The medium was then removed from the apical surface and replaced in the basal chamber by the PneumaCult-ALI medium (#5001, Stem Cell) in order to expose cells to an air-liquid interface that promotes ciliogenesis during the following 21 days. Lentiviral transduction of AECs was performed as previously described<sup>32</sup>. In brief, viral particles were added in the medium together with a Rho-associated protein kinase inhibitor, during the expansion phase, before a cell selection step by puromycine (2ug/mL). Resistant cells were then cultured at ALI as described above. Cells infected with an empty vector were used as control.

## Results

### Next generation sequencing-based approaches identify homozygous loss-of-function *TTC12* mutations in four independent PCD-affected individuals with distinct ciliary and flagellar dynein arm defects

To uncover novel genetic causes responsible for PCD, we used a next generation sequencing (NGS)-based approach in four independent probands with a PCD phenotype that was not explained by mutations in genes known to be involved in PCD. As summarized in **Table 1**, individuals DCP791, 18GM00157 and DCP1606, originate from North Africa and were born from a consanguineous union, whereas the fourth individual, DCP153, originates from Europe with no known consanguinity. All four individuals displayed sinopulmonary symptoms with rhino-sinusitis and had normal *situs* position. Individuals 18GM00157 and DCP1606 also displayed bronchiectasis, and individual DCP1606 suffered from neonatal respiratory distress. Nasal nitric oxide (NO) levels were recorded for those four individuals, showing low levels for two of them. High-speed videomicroscopy (HSVM) performed on nasal brushing of three individuals, showed variable proportions of immotile cilia (70%, 50% and 10% in 18GM00157, DCP1606 and DCP153, respectively), a normal beat frequency and moderate beating defects (for the portion of motile cilia) consisting of a reduced beating angle and a lower distance swept by the cilium tip (**Table S1**). Representative movies showing immotile and beating cilia are shown in supplemental data (**Movies S1-S2, S3-S4, S5-S6** for individuals 18GM00157, DCP1606 and DCP153, respectively, and **movie S7** for a control individual). TEM analysis of respiratory cilia, which was performed for individuals DCP153, 18GM00157 and DCP1606, showed the absence of IDAs associated with some axonemal disorganization (2%, 22% and 24%, respectively) but, importantly, the presence of ODAs in all examined cilia (**Table 1**) (**Figure 1**).

Among the three male individuals, individual DCP1606 was a child (11 year-old) and individuals DCP791 and DCP153 were adults known to be infertile due to total asthenozoospermia (**Table 2**). Surprisingly, TEM analysis of sperm cells from the two infertile individuals revealed the total lack of

both IDAs and ODAs, an ultrastructural phenotype clearly different from that observed in cilia from respiratory cells where only IDAs were missing.

NGS analyses led to the identification of homozygous mutations in *TTC12* [MIM: 610732], a gene not associated with PCD so far. *TTC12* is located on chromosome 11 and consists of 22 coding exons (RefSeq NM\_017868.4) predicting a protein of 705 residues (Uniprot Q9H892). Individuals DCP1606 and 18GM00157 were shown to carry the nonsense mutations c.607del, p.Ile203\* and c.1678C>T, p.Arg560\*, respectively. Individual DCP791 carries a splice mutation (c.1614+3A>T, p.?) and individual DCP153 is homozygous for the missense mutation c.1700T>G, p.Met567Arg. Two of these mutations, c.1614+3A>T and c.607del, are not reported in gnomAD, while the c.1678C>T and c.1700T>G mutations are found at very low frequencies:  $4 \times 10^{-5}$  and  $6 \times 10^{-5}$ , respectively (**Table S4**). The presence of all these *TTC12* mutations was confirmed by Sanger sequencing, as illustrated in **Figure S2 and S3**. The expected consequences of the identified variants at the protein level are shown in **Figure 2**.

### **TTC12 is a cytoplasmic protein of ciliated cells**

*TTC12* contains three tetratricopeptide repeat (TPR) domains in its N-terminal part and three armadillo repeat domains (ARM1, ARM2 and ARM3) in its C-terminal part. A TPR domain consists of a motif of ~34-aminoacids organized in two antiparallel alpha-helices, known to mediate protein-protein interactions and the assembly of multiprotein complexes<sup>33</sup>. ARM domains, which are approximately 40-aminoacid long, have also been implicated in protein-protein interactions through their superhelix structure<sup>34</sup>. To date the biological functions of *TTC12* are unknown. To gain insight into its role, we first checked *TTC12* tissue expression by RT-PCR in AECs and in testis tissue (**Figure S4A**) with a primer set (located in exons 2 and 22) bracketing the entire coding region (**Table S1**). Sequencing of the resulting RT-PCR products allowed the identification of the same *TTC12* isoform (NM\_017868.4) in both samples. As shown by real-time PCR performed with primers located in exons 11 and 12 of *TTC12* (**Table S1**), a similar expression level of this transcript was found in AEC and testis samples (**Figure S4B**). The corresponding *TTC12* 75 KDa protein was also easily detectable by western blot in both cell

types (**Figure S4C**). We next investigated the subcellular localization of TTC12 in ciliated cells. Given the absence of available anti-TTC12 antibodies suitable for immunofluorescence (IF) studies, we performed western blot experiments on protein extracts from ciliary or cytoplasmic fractions of cultured AECs. This analysis showed that TTC12 is virtually exclusively detected in the cytosolic fraction, in contrast to DNAJB13, a component of radial spokes which, as expected, was mainly detected in the ciliary fraction (**Figure S4D**). These results are in line with data from quantitative proteomic analyses, which did not identify TTC12 in purified preparations of axonemes from human airway cells<sup>35</sup>. Overall, the cytoplasmic localization of TTC12 indicates that this protein is not a structural component of the axoneme but would rather play a role in the assembly and/or the transport of axonemal components.

#### **Functional consequences of the identified *TTC12* mutations**

The nonsense mutations carried by individuals 18GM00157 and DCP1606 might generate truncated proteins lacking the ARM superhelix structure of TTC12 (**Figure 2**). Such premature stop codons may also trigger the nonsense-mediated mRNA decay pathway and therefore might lead to the absence of protein. To test these hypotheses, AECs from individuals 18GM00157 and DCP1606, and from a control individual were cultured at ALI as previously described<sup>31</sup>. Analysis of total protein extracts by immunoblotting with an antibody raised against the N-terminal part of TTC12, led to the detection of the TTC12 protein in the control but not in samples from individuals 18GM00157 and DCP1606 (**Figure 3A**). These results clearly indicate that the two nonsense mutations identified in individuals 18GM00157 and DCP1606 are associated with TTC12 loss of function.

As for individual DCP153, he carries a missense variation replacing methionine 567, located in the second ARM domain of TTC12, by an arginine residue. Several lines of evidence support the involvement of the p.Met567Arg variation in the PCD phenotype of this individual. Firstly, as mentioned above, this variation is reported in gnomAD at a very low frequency ( $6 \times 10^{-5}$ ) and only in the heterozygous state (**Table S5**). Secondly, it predicts changes in both the polarity and the charge of this

aminoacid highly conserved in mammals. In zebrafish and xenopus, this methionine residue is replaced by a leucine, an aminoacid also apolar (**Figure S5A**). In addition, alignments of the well-conserved ARM2 and ARM3 domains of TTC12 with the ten ARM domains found in ARMC4 - also involved in PCD<sup>36</sup> - unveil (i) that the methionine found in ARM2 at position 567 also corresponds to a methionine in the ARM3 domain (Met610) (**Figure S5B**), and (ii) the high conservation among all those ARM domains of a hydrophobic residue at position corresponding to Met567 in TTC12 (**Figure S5C**). Thirdly and most importantly, when performing western blot analyses on protein extracts from AECs and from spermatozoa of individual DCP153 who carries the missense mutation in the homozygous state, the amount of TTC12 was found to be dramatically reduced (**Figure 3A, 4B**), thereby attesting the pathogenicity of the p.Met567Arg mutation.

The last individual, DCP791, carries the c.1614+3A>T transversion involving the splice donor site of *TTC12* intron 18. As predicted by the MaxEntScan webtool, this nucleotide substitution is expected to have a strong impact on the splicing of *TTC12* transcripts (score decreasing from 9.49 to 2.03). In addition, a recent study described a deep neural network that precisely models mRNA splicing from a genomic sequence and accurately predicts the effects on splicing of all possible genomic SNVs<sup>37</sup>. Based on this dataset, the probability that the *TTC12* c.1614+3A>T mutation alters the intron 17 canonical splice site is 88%. To further confirm the deleterious effect of this mutation on splicing, we generated minigene constructs containing a genomic DNA region spanning exons 17 to 19 of *TTC12*, with or without the A>T transversion. Analysis of the corresponding transcripts in HEK293 cells transfected with those plasmid constructs showed that the mutation induces a complete skipping of exon 18 (**Figure S6A, S6B**). This in-frame deletion of exon 18 spares the exons encoding the ARM2 and ARM3 domains but, as shown with NCBI CDD webtool, the prediction of those domains is lost (**Figure S6C**).

#### ***TTC12* mutations affect a subset of single-headed IDAs in respiratory cilia**

To further investigate the function of *TTC12* in respiratory cilia and in sperm flagella, we first analyzed



368 by high-resolution immunofluorescence the localization of different axonemal components in AECs  
369 from three individuals (18GM00157, DCP1606 and DCP153) compared to controls: DNAH5 and DNAI1  
370 as markers of the ODAs; DNAH2 and DNAH10 for the double-headed IDA-I1 complex; RSPH3 and  
371 RSPH4a for radial spokes (RSs); GAS8 for the nexin-dynein regulatory complex (N-DRC), and CCDC40  
372 for the ciliary ruler<sup>38</sup>. As proposed by Kollmar<sup>39</sup>, the single-headed IDA subspecies (a, b, c, d, e and g)  
373 would result from gene duplications followed by generation of highly divergent N-termini, and  
374 formation, in *Chlamydomonas* but not in mammals, of chimeric single-headed heavy dynein chains.  
375 This model, which relies on thorough phylogenetic analyses, readily explains the fact that the single-  
376 headed IDAs a, c and d bind the light chain DNALI1 (both in *Chlamydomonas* and in humans), while the  
377 heavy chains belonging to another subgroup (b, e and g) bind centrin in *Chlamydomonas*, whereas, in  
378 humans, the b and e heavy chains, which are not chimeric, would bind DNALI1. We therefore  
379 performed immunostaining with antibodies directed against DNALI1 (corresponding to IDA-subspecies  
380 a, c and d, and potentially to IDA subspecies b and e), DNAH1 (corresponding to IDA-subspecies d),  
381 DNAH6 (corresponding to IDA-subspecies g), and DNAH12 and DNAH3, both corresponding to other  
382 IDA subspecies (a, b, c or e). We found that, in the respiratory cilia of the three individuals with *TTC12*  
383 mutations, the subcellular localization patterns of the markers for ODAs, radial spokes (RSs), the nexin-  
384 dynein regulatory complex (N-DRC) and the ciliary ruler were similar to those found in controls (**Figure**  
385 **S7**). Interestingly concerning IDAs, while DNAH2 and DNAH10, corresponding to the double-headed  
386 IDA-I1, were found to be normal in those same three individuals (**Figure S7**), all the markers of the  
387 single-headed IDA subspecies (i.e. DNALI1, DNAH1, DNAH6, and DNAH12) displayed an abnormal  
388 pattern (**Figure 3B**). DNALI1, DNAH1 and DNAH6 were present at low levels in the very proximal part of  
389 the cilia from the three individuals and absent from the rest of the axoneme, while DNAH12 was not  
390 detectable all along the cilia and was found to accumulate at the subapical part of the cytoplasm of  
391 ciliated cells. Overall, in cilia, *TTC12* mutations does not affect ODAs nor IDA-I1, but compromise a  
392 subset of single-headed IDAs which comprises DNALI1, DNAH1, DNAH6, DNAH12. Of note, DNAH3 was  
393 not detected in cilia from control individuals (data not shown).

### ***TTC12* mutations affect ODAs and a distinct subset of single-headed IDAs in sperm flagella**

In line with the TEM data, IF analyses in spermatozoa from the infertile PCD-affected individual DCP153 showed that DNAI1 as well as DNAH8 and DNAH17, the two dynein HCs specific of ODAs in sperm flagella<sup>3</sup>, were undetectable (**Figure 4A**), thus clearly confirming that *TTC12* mutations induce a loss of ODAs in sperm cells but not in airway cilia. As very little information on the IDA subtypes and their HC composition is available in human sperm cells, we analyzed the pattern of all described IDA HCs in humans (except DNAH7 and DNAH14 for which no antibodies are so far suitable for immunofluorescence). Interestingly, as observed in cilia, markers of the double-headed IDA-I1 complex (i.e. DNAH2 and DNAH10) were found unchanged in individual DCP153. Regarding markers of single-headed IDAs, while the dynein HC DNAH3 (a, b, c or e) was not detected in control respiratory cilia, it was found present along the axoneme of control spermatozoa. Remarkably, this dynein HC was totally absent in sperm from individual DCP153. Additionally, DNAH12 (also corresponding to the single-headed IDA subspecies a, b, c or e) was detected along the full length of the flagellar axoneme in the control but displayed an expression pattern restricted to the proximal part of the flagella in the individual (**Figure 4A**). By contrast, the dynein HCs of the single-headed IDA subspecies d and g (i.e. DNAH1, DNAH6) displayed a normal expression pattern (**Figure S8**). As for the light chain associated with the IDA subspecies a, c and d (i.e. DNALI1), it was found normally localized along the flagellar axoneme of spermatozoa from the individual; however, the IF signal intensity appeared to be reduced (**Figure 4A**), a result confirmed by a western blot performed with an anti-DNALI1 antibody on protein extracts from the individual's spermatozoa (**Figure 4C**). Taken together, these data demonstrate that in sperm cells from individuals with *TTC12* mutations, the assembly and/or the transport of ODAs and a subset of IDAs which comprises DNALI1, DNAH3 and DNAH12, are compromised, while IDA-I1 remains intact.

## TTC12-depleted paramecia reproduce the spermatozoa phenotype observed in PCD-affected individuals

To further investigate the function of TTC12 in cilia motility, we undertook studies in the free-living ciliate *Paramecium tetraurelia* which constitutes a powerful model organism to characterize potential functions of PCD candidate genes<sup>40,41</sup> as gene functional analysis by RNA interference silencing is rapid, efficient and easy to perform. Gene homology analyses performed on *P. tetraurelia* genome<sup>28</sup> identified four genes putatively encoding TTC12 proteins: GSPATT00036969001, GSPATT00034215001, GSPATT00035675001 and GSPATT00037155001 hereafter named *TTC12a*, *TTC12b*, *TTC12-like a* and *TTC12-like b*, respectively. Interpro software analysis detected both TPR and ARM repeats, only in *TTC12a* and *TTC12b*. Therefore, we focused our analysis on these two proteins, which are the closest orthologs of human TTC12 protein (**Figure S9**). RNAseq analysis showed that *TTC12a* is highly expressed by contrast to *TTC12b*, and is the only paralog overexpressed during ciliogenesis (**Table S6**<sup>42,43</sup>). By generating a GFP-tagged *TTC12a* protein in paramecia, we showed that, as observed in human cells, *TTC12a* is a cytoplasmic protein (**Figure S10**). We used the dsRNA feeding method to silence *TTC12a*, *TTC12b* and the combination *TTC12a+TTC12b*. The knockdown (KD) of *TTC12a* alone or in association with *TTC12b* led to a dramatic defect in the swimming velocity (**Figure 5A, 5B**) followed by cell death. By contrast, cells knocked down for *TTC12b* showed no obvious abnormal swimming phenotype, indicating that only *TTC12a* is essential for cilia motility (**Figure 5A, 5B**). The swimming velocity and cell death observed in *TTC12a*-depleted cells were rescued by the expression of a resistant-RNAi version of the gene (**Figure 5C, 5D, 5E**) confirming that the observed phenotypes are specific to *TTC12* knockdown. Cilia staining immunofluorescence together with TEM indicated that in *Paramecium TTC12a+b* KD, cilia were globally intact (**Figure 6A**) but contained a reduced number of ODAs and/or IDAs per cilium (**Figure 6B**). Similar results were obtained after depletion of the *TTC12a* protein alone (data not shown). Taken together, these data indicate that, similarly to what we found in human spermatozoa, the assembly and/or transport of both ODAs and IDAs was affected by the

absence of TTC12a in the *Paramecium* model, which suggests that the function of this protein is at least partly conserved throughout evolution.

#### **CRISPR-cas9-mediated *TTC12* invalidation in AECs leads to single-headed IDA subspecies defects mimicking the phenotype of AECs from individuals with *TTC12* mutations**

In order to further assess the consequences of *TTC12* loss-of-function mutations in airway ciliated cells, we invalidated *TTC12* in the model of primary AECs cultured at ALI<sup>31</sup> through a CRISPR-cas9 approach. Control AECs were transduced with lentiviruses expressing plentiCRISPRv2 carrying Cas9 and RNA guides (gRNA) targeting *TTC12*. Two gRNA targeting exon 3 were tested and the most effective one, gRNA 1 (i.e. with the higher level of insertions or deletions, as assessed by TIDE webtool), was used for further experiments. Genomic DNA (gDNA) was then extracted from ALI cultures transduced with gRNA 1. We found that 82% of gDNA carried INDELs, including 28% of single-nucleotide insertions (**Figure S11A, S11B**). *TTC12* invalidation in AECs was confirmed by western blot (**Figure S11C**), thereby allowing us to investigate the functional effects of such invalidation on the main axonemal components. Immunofluorescence staining of *TTC12*-invalidated cultured epithelial cells revealed a phenotype similar to that found in primary cells from individuals with *TTC12* mutations: only axonemal components specific of single-headed IDAs (i.e. DNAH1, DNAH6, DNAH12 and DNALI1) were found to be affected (**Figure 7A, 7B**). More specifically, the analysis of ciliated cells cultured in ALI by high-resolution IF confirmed the invalidation of *TTC12* in clusters with a combination of ciliated cells lacking DNAH6, DNAH12 and DNALI1 along the cilia length, and ciliated cells with normal staining (**Figure 7A**). Moreover, analysis of isolated ciliated cells confirmed the data obtained in AECs from individuals with *TTC12* mutations, with the abnormal localization of DNAH1, DNAH6 and DNALI1, found at very low levels at the base of the cilia, while DNAH12 was completely absent all along the cilia (**Figure 7B**). All the other tested axonemal components displayed a normal staining (i.e. DNAH5, DNAH9, DNAH11 and DNAI1 for ODAs; DNAH2 and DNAH10 for IDA-I1; RSPH1, RSPH3, RSPH4a, RSPH9 and RSPH11 for RSs; GAS8 for the N-DRC, and CCDC39 and CCDC40 for the ciliary ruler) indicating that all other axonemal

470 complexes were normally assembled (**Figure S12**). To further study the impact of *TTC2* invalidation on  
471 ciliary axonemal components, cross-sections of cilia from *TTC12*-invalidated epithelial cells were  
472 analyzed by TEM. As shown in **Figure 7C**, those cilia displayed an abnormal ultrastructure with 84% of  
473 cilia lacking IDAs, whereas a non-infected culture harbored only 3% of abnormal cilia. This  
474 ultrastructural phenotype correlates again with the one found in individuals with *TTC12* mutations.

## Discussion

We report mutations in *TTC12*, a gene encoding a protein involved in cytoplasmic assembly and/or transport of axonemal dynein arms, in PCD-affected individuals. A thorough analysis of the phenotype of sperm flagella and respiratory cilia from *TTC12*-deficient individuals revealed that the loss of function of *TTC12* affects both ODAs and a subset of single-headed IDAs in sperm flagella, but only a different subset of single-headed IDAs in respiratory cilia. These data, which are further supported by two cellular models of the pathology (i.e. the ciliate *Paramecium tetraurelia* depleted in *TTC12* and human AECs differentiated *in vitro* after a CRISPR-cas9-mediated invalidation of *TTC12*), reveal the existence of distinct dynein assembly mechanisms in human sperm flagella and respiratory cilia.

The biological function of *TTC12* is still elusive. One study has shown an upregulation of *TTC12* transcripts during mouse ciliogenesis *in vitro* and a centrosomal localization of *TTC12* fused to GFP<sup>44</sup>. In paramecia, *TTC12a*, the ortholog of human *TTC12*, was also found to be overexpressed during ciliogenesis (*ParameciumDB*). While some TTC proteins have been involved in the so-called intra flagellar transport (IFT)<sup>45–47</sup>, as for *TTC12*, the following characteristics support its possible role as a co-chaperone: (i) *TTC12* contains three TPR domains and three ARM domains, two structural motifs with alpha helices known to be involved in protein-protein interactions; and TPR-containing proteins are part of several multiprotein complexes that include co-chaperones<sup>33</sup>; (ii) noteworthy, in humans, only two other proteins are known to contain both TPR and ARM domains: UNC45A and UNC45B that have been shown to interact with HSP90 and to act as molecular co-chaperones<sup>34,48–50</sup>. These data, together with the cytoplasmic localization of *TTC12*, are therefore consistent with a role of *TTC12* as a co-chaperone involved in dynein assembly.

The cytoplasmic assembly of DAs is a complex process involving a wide and still incompletely resolved chaperone-mediated protein network. Dynein arm components are first synthesized and pre-assembled into multi-subunit structures in the cytoplasm, before being transported by IFT into the axoneme for microtubule attachment<sup>51,52</sup>. Except HSP90, all the proteins known to be involved in the axonemal dynein pre-assembly network have been implicated in PCD (i.e. CFAP300, CFAP298,

CCDC103, DNAAF1, DNAAF2, DNAAF3, DNAAF4, DNAAF5, LRRC6, PIH1D3, SPAG1 and ZMYND10). HSP90 is known to interact with TPR domains of various co-chaperones to promote their function<sup>53</sup>. Two so-called dynein assembly factors contain TPR domains: DNAAF4 and SPAG1. In recent studies, DNAAF4 was found to interact with both HSP90 and DNAAF2 through its TPR domains to form an R2TP-like complex<sup>54,55</sup>. PIH1D3, whose corresponding gene has recently been involved in PCD, might also interact with HSP90 through a TPR-containing protein which could be DNAAF4<sup>54,55</sup>. Interestingly, DNAAF4, which seems to play a central role in this network, was found to interact with ZMYND10, a partner of LRRC6 and CFAP298<sup>16,56</sup>, while the latter one also interacts with DNAAF1<sup>57</sup>. In this assembly network, it has been reported that DNAAF2 and PIH1D3 interact with DNAI2<sup>9,55</sup>, a dynein intermediate chain known to assemble early in the human ODA assembly process<sup>58</sup>. As for DNAAF5, it has been shown to interact with DNAI2, but would not bind HSP90<sup>58</sup>. Regarding CFAP300, DNAAF3, SPAG1 and CCDC103, their functions remain unclear with so far no reported interactions with HSP90 or other chaperones. In this context, TTC12, with its three TPR domains that suggest an interaction with HSP90 and/or other chaperones, emerges as a player in this complex pre-assembly network.

In most studies, the absence of both DAs in PCD-affected individuals has been identified by TEM together with high-resolution IF using mainly DNAH5 and DNALI1 antibodies directed against the ODA  $\gamma$ -heavy chain and a/c/d IDA subspecies, respectively. However, these two approaches do not allow determining which ODA type(s) and which IDA subspecies are affected. For *DNAAF4*, *DNAAF5* and *CCDC103* mutations, which are responsible for an absence of both DAs, the proximal staining of DNAH5 and DNAI2 and/or the loss of DNAH9 staining suggest that only type 2 ODAs are affected<sup>9,12,15</sup>.

In order to accurately decipher the role played by TTC12 in dynein arm assembly, we aimed to identify which single-headed IDA subspecies are affected in respiratory cilia of individuals with *TTC12* mutations. Besides the presence of ODAs, as attested to by TEM and immunofluorescence analyses, IDA staining revealed that IDA-I1 remained present while, according to the *Chlamydomonas* model, four of the six single-headed IDA subspecies were affected: a, c, d and g. No conclusion could be raised on b and e subspecies since the heavy chain DNAH12 is so far not

assigned to a specific subspecies (a, or b, or c, or e), and no efficient antibodies are available to detect the two remaining IDA heavy chains (i.e. DNAH7 and DNAH14) (**Figure 8**). Of note, proteomic analyses performed on human airway cilia and spermatozoa showed that DNAH7 is present in both proteomes, unlike DNAH14 that is undetected in both of them<sup>35,59–61</sup>. Like in cilia, spermatozoa from individuals with *TTC12* mutations conserve the dimeric IDA-I1. Nonetheless, unlike cilia, those spermatozoa display an absence of ODAs as revealed by TEM analyses and confirmed by the absence of staining of the ODA HCs specifically expressed in flagella (DNAH8 and DNAH17)<sup>3</sup>. Interestingly, as for single-headed IDA components, our results show that the IDA heavy chain DNAH3 (present in the IDA subspecies a, b, c, or e) is detected in flagellar axonemes, whereas, it is not detectable in cilia as expected from proteomic data<sup>35,59</sup>. Additionally, spermatozoa from an individual with biallelic *TTC12* mutations displayed a loss of DNAH3 together with a mislocalization of DNAH12 (another monomeric IDA heavy chain not specifically assigned to subspecies a, b, c or e), thereby unveiling the role of *TTC12* in the proper expression of DNAH3 and DNAH12 in sperm cells.

Surprisingly, although DNAH1 (d) and DNAH6 (g) are present in both normal cilia and flagella, they were not impacted by *TTC12* mutations in spermatozoa, contrasting with what was observed in cilia. Moreover, in those spermatozoa, DNALI1 amounts were found reduced but present (**Figure 8**).

Taken together, these results show that, in spermatozoa, *TTC12* defects lead to the complete absence of ODAs and of a set of monomeric IDA subspecies, which is different from the one impacted in respiratory cilia. While the absence of ODAs in flagella could be explained by differences in ODA composition between cilia and flagella (DNAH5/DNAH9/DNAH11 and DNAH8/DNAH17, respectively), the differences affecting single-headed IDAs are not exclusively due to distinct dynein HC composition (e.g. DNAH1 and DNAH6 are both present in cilia and flagella).

In paramecia, *TTC12* knock-down led to the absence of both dynein arms and to a drastic impairment of the swimming velocity. This suggests similarities in the ODA/IDA assembly process at play in this protist and in human sperm flagella. In human cilia, except for *TTC12*, the defects of all known co-chaperones affect both ODAs and IDAs. The situation is very different in *Chlamydomonas* where



several of the algal homolog genes are important for assembly of one dynein arm type: *DNAAF1* and *HEATR2* homologs (i.e. *ODA7* and *HEATR2*, respectively) govern the assembly of ODAs only, whereas the *DNAAF4* homolog (i.e. *PF23*) contributes only to the assembly of some IDAs<sup>62</sup>.

As paramecia mimicked the human flagellar phenotype but not the ciliary one, we looked for an additional cellular model to further strengthen our conclusions on the role of *TTC12* in cilia. We developed a human differentiated cell culture model based on CRISPR-cas9 invalidation of *TTC12*. Primary AECs cultured under ALI conditions constitute a strong epithelial barrier very difficult to efficiently transfect, especially without inhibiting ciliogenesis. In this study, we show that transduction of AECs during their expansion phase is not only highly efficient, but, most importantly, it also preserves ciliogenesis, providing large amounts of ciliated cells from which DNA, RNA and protein expression can be studied. After puromycin selection, 100% of cultured cells were infected, but four populations with different genotypes might be present: no indel in *TTC12*, indels within one *TTC12* allele, distinct indels within each of the two *TTC12* alleles, or the same indel within the two *TTC12* alleles, equivalent to a wild-type genotype at the *TTC12* locus or a *TTC12* loss-of-function mutation present in the heterozygous, compound heterozygous or homozygous state, respectively. Since AECs cannot be dissociated and isolated for selection, contrary to what is done for cell lines, we had to work with a heterogeneous population of cells invalidated or not for *TTC12*. Nevertheless, our results revealed that *TTC12* CRISPR AECs perfectly mimic the phenotype observed in cilia from individuals carrying *TTC12* mutations, while *TTC12*-KD paramecia displayed a ciliary phenotype closer to the flagellar phenotype observed in human spermatozoa. From a general viewpoint, this demonstrates that CRISPR-cas9-engineered AECs represent a valuable model to study the impact of PCD mutations on ciliary axonemes.

As for clinical aspects, the diagnosis of PCD is difficult to establish in individuals with mild respiratory symptoms and no laterality defect. In such situations, low levels of nasal NO, an abnormal ciliary beat frequency and/or the presence of ciliary ultrastructural defects are essential to confirm or not the suspicion of PCD. In the current study, all the individuals were exempt of laterality defects and

displayed motile cilia with a normal beat frequency. In two of them, nasal NO values were normal. Furthermore, the ciliary ultrastructural defect (i.e. isolated absence of IDAs with an inconstant and mild axonemal disorganization) was also difficult to assert by TEM because of the usual low contrast of IDAs. The diagnosis of PCD was, however, much easier to establish in the two adult male individuals given their infertility due to a sperm immotility related to the flagellar absence of both DAs, an ultrastructural defect easily identified by TEM. It is therefore tempting to speculate that PCD could be underdiagnosed in female individuals with *TTC12* mutations, in young male individuals or in adult males in whom no TEM analyses of their spermatozoa is performed, thereby underscoring the major interest of molecular analyses in such individuals.

In conclusion, *TTC12* emerges as a cytoplasmic player in dynein arm assembly and/or transport. So far, all the mutations in genes encoding cytoplasmic pre-assembly factors cause a combined ODA and IDA defect. However, *TTC12* mutations lead to distinct ultrastructural defects in cilia (i.e. absence of single-headed IDAs) and in sperm flagella (i.e. absence of both dynein arms), which unveils its role as a possible co-chaperone in charge of monomeric IDAs in both organelles but not of ODAs in cilia. More precisely, *TTC12* is in charge of different dynein heavy chains in monomeric IDAs from cilia and from flagella. Furthermore, our findings should be of particular help to establish the diagnosis of PCD in individuals with isolated IDA defects in respiratory cilia. The development of a cellular model based on human AECs differentiated *in vitro* into ciliated cells after a CRISPR-cas9-mediated invalidation of genes of interest should open new avenues to decipher both the role of the numerous proteins expressed during ciliogenesis and the pathophysiology of diseases of the airway epithelium.

## 600 **SUPPLEMENTAL DATA**

601 Supplemental data include 12 figures, 6 tables and 7 movies.

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613

## 614 **DECLARATION OF INTERESTS**

615 The authors declare no competing interests.

616

## 617 **WEB RESSOURCES**

618 Conserved Domain Database (NCBI CDD webtool), <https://www.ncbi.nlm.nih.gov/cdd/>

619 Clustal Omega program, <http://www.ebi.ac.uk/Tools/msa/clustalo/>

620 CRISPOR TEFOR, <http://crispor.tefor.net/>

621 EMBL EBI Expression Atlas, <http://www.ebi.ac.uk/gxa/home>

622 EMBL EBI Ensembl, <http://www.ensembl.org/index.html>

623 GnomAD, <https://gnomad.broadinstitute.org/>

624 InterPro, <https://www.ebi.ac.uk/interpro/>

- 625 OMIM, <http://www.omim.org/>
- 626 *Paramecium*DB, <https://paramecium.i2bc.paris-saclay.fr>
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## FIGURE LEGENDS

### **Figure 1. Ciliary and flagellar ultrastructural defects in PCD-affected individuals with *TTC12* mutations**

Electromicrographs of cross-sections of ciliary axonemes (upper panel) from a control individual and individuals DCP153, 18GM00157, and DCP1606, compared to cross-sections of sperm flagellar axonemes (lower panel) from a control individual and from the infertile PCD-affected individuals DCP153 and DCP791. In each individual, the ciliary section shows an abnormal configuration with missing IDAs associated with a mild axonemal disorganization in individuals 18GM00157 and DCP1606, but with a clear presence of ODAs. The flagellar sections show a lack of both IDAs and ODAs. Black scale bars represent 0.1µm. NR: not relevant; NA: not available; IDAs: inner dynein arms; ODAs: outer dynein arms.

### **Figure 2. *TTC12* mutations and their putative impact at the protein level in individuals with PCD**

The mutations are shown within the exonic organization of the human *TTC12* cDNA (top) and a domain-organization model of the corresponding protein (bottom) according to SMART webtool and CDD NCBI database. TPR: tetratricopeptide repeat; ARM: armadillo repeat. The light green box represents a degenerated ARM domain as opposed to the two dark green boxes that correspond to typical ARM domains.

### **Figure 3. *TTC12* loss-of-function mutations cause defective single-headed inner dynein arm assembly in respiratory cilia**

**A)** Protein lysates extracted from AECs of a control individual and individuals 18GM00157, DCP1606 and DCP153 were resolved by SDS-PAGE and analyzed by immunoblotting with an anti-*TTC12* antibody. The expression of *TTC12* is virtually absent or dramatically reduced in PCD-affected individuals. DNAJB13, a component of radial spokes, was used as positive control.

**B)** Immunofluorescence microscopy analysis of IDA markers in respiratory epithelial cells from a control and individuals 18GM00157, DCP1606 and DCP153 homozygous for *TTC12* mutations. Cells were double-labeled with an anti-acetylated  $\alpha$ -tubulin antibody (red), a marker of ciliary axonemes, and anti-DNAH6, anti-DNAH12 or anti-DNALI1 antibodies as markers of IDAs (green). Nuclear staining was performed with DAPI (blue). In affected individuals, DNAH12 is completely absent in the cilia but seems to accumulate at the subapical part of the cytoplasm. By contrast, DNALI1 and DNAH6 appear to be strongly reduced, but partially present at the proximal part of cilia. (DNAH2 and DNAH10, two markers of the double-headed IDA-I1 were shown to be normally expressed in the individuals, see Figure S7). Scale bars, 5  $\mu$ m.

IDA: inner dynein arm.

**Figure 4. *TTC12* mutations impact the assembly of both ODAs and IDAs in spermatozoa**

**A)** Immunofluorescence microscopy analysis of ODA and IDA markers in spermatozoa from a control and the infertile individual DCP153 carrying biallelic *TTC12* mutations. Cells were double-labeled with a marker of ciliary axonemes, anti- $\alpha$ -tubulin antibody (red), together with anti-DNAH8, anti-DNAH17 or anti-DNALI1 antibodies as markers of ODAs (green), and anti-DNAH3, anti-DNAH12 or anti-DNALI1 antibodies as markers of IDAs (green). Nuclear staining was performed with DAPI (blue). In individuals DCP153, DNAH8, DNAH17, DNALI1 and DNAH3 are absent along the flagellar axoneme. By contrast, DNAH12 remains exclusively present in the proximal part of flagella, and DNALI1 appears to be present but reduced along the axoneme. Scale bars, 5  $\mu$ m.

**B)** Spermatozoa protein lysates of a control individual and individual DCP153 were resolved by SDS-PAGE and analyzed by immunoblotting with an anti-TTC12 antibody, showing the absence of TTC12 expression in the individual. Very low amounts of TTC12 were detected in spermatozoa from the infertile individual DCP153. DNAJB13, a component of radial spokes, was used as positive control.

863 C) Spermatozoa protein lysates of a control individual and individual DCP153 were resolved by SDS-  
864 PAGE and analyzed by immunoblotting with an anti-DNALI1 antibody, showing the reduced amounts  
865 of DNALI1 in flagella from the affected individual. Actin was used as positive loading control.

866 ODA: outer dynein arm; IDA: inner dynein arm.

867

868 **Figure 5. Knockdown of *TTC12a* affects the swimming velocity of paramecia**

869 A) Z projection of track recording paramecia swimming under a dark-field microscope using a 10X  
870 objective after 24h or 48h of RNAi treatment. At 24h of silencing, swimming behaviour was affected in  
871 *TTC12a*- and *TTC12a+b*- silenced cells, while the silencing of *TTC12b* did not show any swimming  
872 defect. At 48h *TTC12a* and *TTC12a+b*-silenced cells did not swim anymore and died at 72hrs.

873 B) Analysis of the swimming velocity of *TTC12a*, *TTC12b* and *TTC12a+b* KD cells as well as control  
874 paramecia (i.e. empty vector silenced paramecia). Note that *TTC12b*-depleted cells swam slightly faster  
875 (725mm/sec) than control cells (EV, 665 mm/sec). The swimming velocity of *TTC12a* (317 mm/sec) and  
876 *TTC12a+b* (282 mm/sec) KD cells was reduced by 50% of that of control cells at 24h. Data are  
877 represented as the mean  $\pm$  SEM. Statistical analyses were performed with GrahPad Prism v5 software  
878 using two-tailed unpaired t-tests, \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , (n=69-88 per experiment).

879 C) Z projection of track recording paramecia swimming under a dark-field microscope using a 10X  
880 objective. Transformed clones by *TTC12a* resistant-RNAi version (2c and 6c) as well as non-  
881 transformed paramecia were grown 36h in empty vector- or *TTC12a*-silencing media. *TTC12a*-depleted  
882 cells barely swam as shown previously, while cells expressing *TTC12a* resistant-RNAi version swam  
883 again similarly as control cells. This indicates that *TTC12a*-depleted phenotypes are rescued by *TTC12a*  
884 resistant-RNAi version.

885 D) Analysis of the swimming velocity of transformed and non-transformed cells grown in empty vector  
886 or *TTC12a*-silencing media. As expected, *TTC12a* silencing in non-transformed cells reduced  
887 dramatically the swimming velocity (52 mm/sec). However, the expression of the *TTC12* resistant-RNAi  
888 version rescues this phenotype (88% of complementation). Note that the swimming velocity of

transformed cells 2c (653 mm/sec) was similar as non-transformed paramecia (612 mm/sec), while transformed cells 6c swam slower (458 mm/sec). Data are represented as the mean  $\pm$  SEM. Statistical analyses were performed with GrahPad Prism v5 software using two-tailed unpaired *t*-tests, \*\*\**p* < 0.001, \*\**p* < 0.01, NS *p* > 0.1 (n=26-48 per experiment).

**E)** Western blot analysis on total protein extract of transformed cells 2c and 6c showing that both clones express TTC12a-GFP.

EV: empty vector, KD: knockdown.

#### **Figure 6. Knockdown of *TTC12a+b* in paramecia leads to the loss of both ODAs and IDAs**

**A)** Depletion of *TTC12a+b* in *Paramecium* does not affect cilia number and length. *TTC12a+b* silenced paramecia were fixed and immunostained for cilia (anti-polyglutamylated tubulin antibodies) after 48h of RNAi. Scale bar, 20  $\mu$ m.

**B)** Upper panel: Electromicrographs of cross-sections of cilia from *TTC12a+b* KD paramecia. Control cilia show the normal distribution of both ODAs and IDAs. By contrast, *TTC12a+b* KD cilia show a partial or total lack of ODAs and IDAs. Scale bar, 200 nm.

Lower panel: Quantitative TEM analysis of IDAs and ODAs number on 140 cross-sections from a control and *TTC12a+b* co-depleted cells. ODAs and IDAs were normally distributed in 100% of cilia from control cells, and about 50% of the co-depleted *TTC12a+b* cells. This result has to be interpreted while taking into account that, in paramecia, half of cilia is renewed at each cell division. Therefore, after the first division, paramecia display half of normal cilia and half of *TTC12a+b*- silenced cilia. The former half (generated before silencing) did not display the phenotype while the new one did (generated after silencing).

ODA: outer dynein arm; IDA: inner dynein arm; KD: knockdown.

#### **Figure 7. *TTC12* is required for the assembly of single-headed inner dynein arms**



**A)** *TTC12* was invalidated in AEC cultures with *TTC12* CRISPR-Cas9 lentiviruses, and immunostained with an anti-acetylated  $\alpha$ -tubulin antibody (red), a marker of ciliary axonemes, and anti-DNAH6, anti-DNAH12 or anti-DNALI1 antibodies as markers of single-headed IDAs (green). Immunofluorescence analysis in ciliated cells clusters shows the partial invalidation of *TTC12* in culture with a loss of DNAH6, DNAH12 and DNALI1 in cells lacking *TTC12*. Scale bars, 25  $\mu$ m. DNAH6, DNAH12, and DNALI1 labeled areas were measured with ImageJ software and normalized against tubulin labeled area. The dynein/tubulin ratios obtained in *TTC12*-CRISPR transduced cells were then normalized to ratios obtained in EV-transduced cells. Values of those final ratios are indicated on the right of the histograms, and showed the decrease of dynein chain labeling signal compared to tubulin. Data are represented as the mean  $\pm$  SEM. Statistical analyses were performed with GrahPad Prism v5 software using two-tailed unpaired *t*-tests, \*\*\**p*<0.001 (*n*=3).

**B)** Immunofluorescence images of isolated ciliated cells lacking *TTC12* showing the complete loss of DNAH12 along the cilia and its accumulation in the subapical region of the cytoplasm, while DNAH6 and DNALI1 expression appears strongly decreased but still present at the base of cilia. Nuclear staining was performed with DAPI (blue). Scale bar, 25  $\mu$ m.

**C)** Electromicrographs of cross-sections of cilia axonemes from a non-infected AEC culture and a *TTC12*-CRISPR-infected cell culture show an abnormal arrangement lacking IDAs in the *TTC12*-invalidated culture. Proportion in electromicrograph cross-sections of cilia harboring more than four IDAs, between two and four IDAs, and up to one IDA, were reported in a histogram comparing control cells to *TTC12*-invalidated cells. Scale bars, 5  $\mu$ m.

ODA: outer dynein arm; IDA: inner dynein arm; EV: empty vector.

**Figure 8. Dynein chains affected by the absence of *TTC12* and diagrammatic representation of the axonemal components impacted by *TTC12* loss-of-function mutations in respiratory cilia and sperm flagella**

939 **A)** Table summarizing the dynein chains studied by immunofluorescence in AECs of a control,  
940 individuals 18GM00157, DCP1606, DCP153, AEC cultures invalidated or not for *TTC12* by a CRISPR-cas9  
941 approach, as well as in spermatozoa from a control and individual DCP153. ODA labeling was  
942 performed with anti-DNAH5 and anti-DNAH9 antibodies in AECs, and anti-DNAH8 and anti-DNAH17  
943 antibodies in spermatozoa. Black check marks represent normal localization, minus signs represent an  
944 absence of protein, and black cross signs indicate mislocalization. NR: not relevant.

945 **B)** Schematic structure of a normal 96 nm-long axonemal unit. Each unit, which repeats along the  
946 microtubule doublets, consists of four ODAs represented in purple and IDAs shown in blue. As the  
947 exact composition of IDAs is virtually unknown in humans, the IDA representation is based on data  
948 from *Chlamydomonas* in which the seven IDA subspecies include six single-headed dynein arms (a, b, c,  
949 e, g, d) and one double-headed dynein arm (IDA-I1).

950 **C)** Phenotype of *TTC12*-deficient respiratory cilia versus *TTC12*-deficient sperm flagella. The proteins  
951 unaffected by the loss of *TTC12* are represented in full color and written in yellow and the proteins  
952 affected by the loss of *TTC12* are represented in white with red dotted line and written in red. The  
953 proteins found to be affected by *TTC12* mutations but for which the exact localization is unknown in  
954 humans, are represented in hatched red pattern and written in red. Proteins with lower but non-null  
955 expression are represented in hatched grey pattern and written in red. The proteins that could not be  
956 tested are represented and written in grey.

957 ODA: outer dynein arm; IDA: inner dynein arm; N-DRC: nexin-dynein regulatory complex; MIA:  
958 modifier of inner arms; IC/LC: intermediate chain/light chain, RS: radial spoke; MT: microtubules.

959

960 **Movie S1.** Representative movie showing immotile cilia of individual 18GM00157.

961 **Movie S2.** Representative movie beating cilia of individual 18GM00157.

962 **Movie S3.** Representative movie showing immotile cilia of individual DCP1606.

963 **Movie S4.** Representative movie showing beating cilia of individual DCP1606.

964 **Movie S5.** Representative movie showing showing immotile cilia of individual DCP153.

965     **Movie S6.** Representative movie showing beating cilia of individual DCP153.

966     **Movie S7.** Representative movie showing beating cilia of control individual.

967

## TABLES

Table 1: Phenotype of PCD-affected individuals with identified *TTC12* mutations

Individuals (Origin)	Known Consanguinity	Gender (Age at Diagnosis)	SI	Airway Disease		Fertility	Nasal NO (nL/min)	TEM defect (%)		Allele 1	Allele 2
				Lower	Upper			Cilia	Sperm		
<b>DCP791</b> (Turkey)	Yes	Male (24 years)	No	Minima	Sinusitis	Infertility	Low <sup>a</sup>	NA	IDAs+ ODAs (100%)	c.1614+3A>T p.?	c.1614+3A>T p.?
<b>18GM00157</b> (Morocco)	Yes	Female (50 years)	No	Asthma Bronchiectasis	Rhino- sinusitis	Not tested	37.6	IDAs (100%) Disorganization (22%)	NR	c.1678C>T p.Arg560*	c.1678C>T p.Arg560*
<b>DCP1606</b> (Tunisia)	Yes	Male (11 years)	No	NNRD Bronchiectasis Pneumopathies	Rhino- sinusitis Otitis	NR	146	IDAs (100%) Disorganization (24%)	NR	c.607del p.Ile203*	c.607del p.Ile203*
<b>DCP153</b> (Europe)	No	Male (33 years)	No	Bronchitis	Rhino- sinusitis	Infertility	186	IDAs (100%) Disorganization (2%)	IDAs+ODAs (100%)	c.1700T>G p.Met567Arg	c.1700T>G p.Met567Arg

SI: situs inversus; NNRD: neonatal respiratory distress; NR: not relevant; NO: nitric oxide; ppb: parts per billion; CBF: ciliary beat frequency; Hz: hertz; NA: not available; TEM: transmission electron microscopy; Spz:

spermatozoa; IDAs: inner dynein arms, ODAs: outer dynein arms. PCD nasal NO < 77 nL/min<sup>8</sup>.

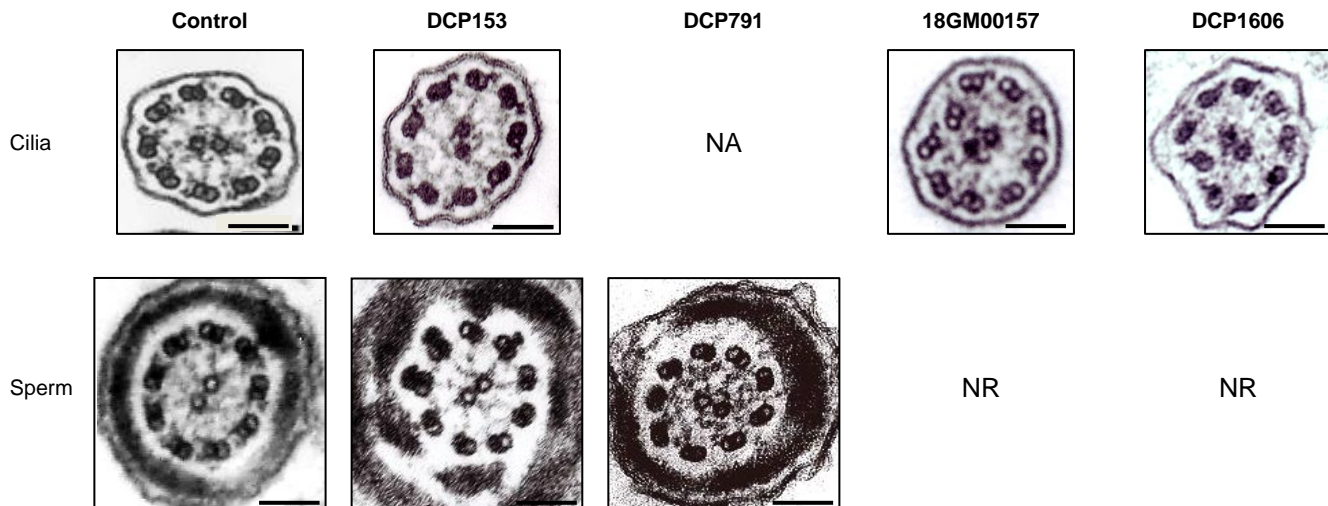
<sup>a</sup>exact value not available

Table 2. Semen characteristics of infertile individuals with identified *TTC12* mutations

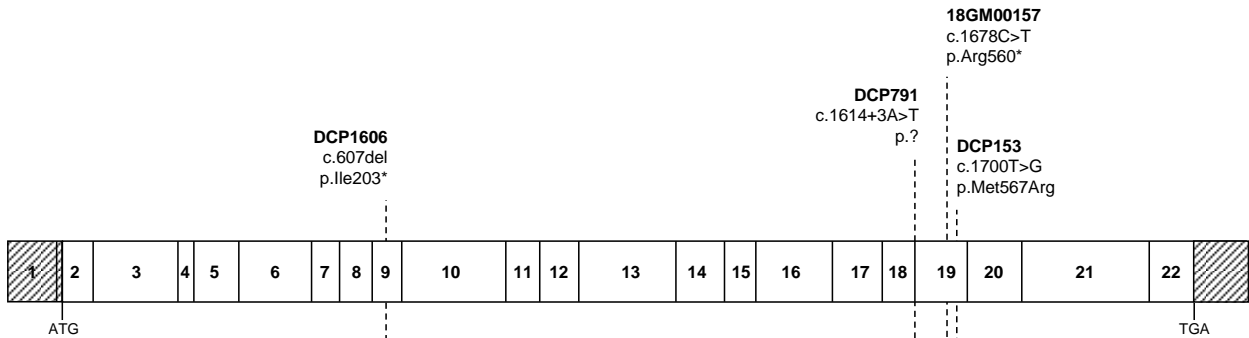
Individuals	pH	Volume (ml)	Sperm count (10 <sup>6</sup> /ml)	Total Motility (%)	Progressive Motility (%)	Vitality (%)	Typical forms (%)	Flagellum Defects				
								Absent	Short	Irregular Caliber	Coiled	Multiple
<b>DCP791</b>	ND	ND	21	<b>0</b>	<b>0</b>	75	36	7	0	0	10	0
<b>DCP153</b>	7.9	4.5	81	<b>0</b>	<b>0</b>	68	33	2	1	1	6	1
<b>Reference values <sup>a</sup></b>	7.2	1.5	15	40	32	58	23	5	1	2	17	1

Values are expressed in percent, unless otherwise specified. ND: not determined.

<sup>a</sup> According to the World Health Organization (WHO) standards<sup>23</sup> and the distribution range of morphologically abnormal spermatozoa observed in fertile individuals<sup>63</sup>; in bold characters: abnormal values



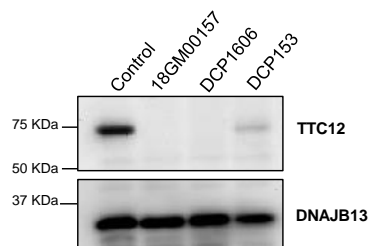
**TTC12**  
**cDNA**  
NM\_017868.4



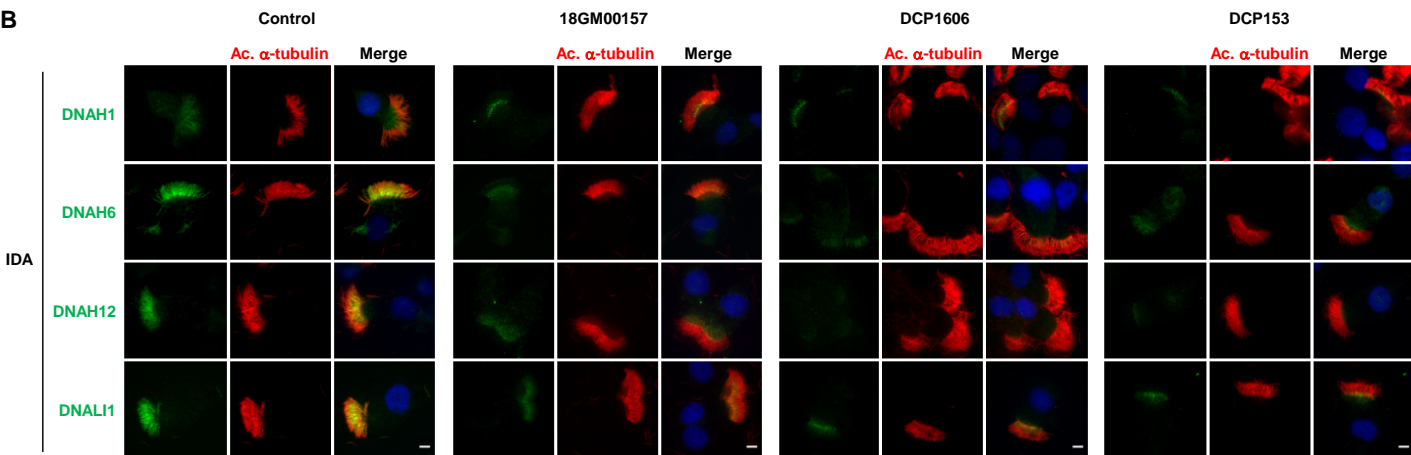
**TTC12**  
*Homo sapiens*



**A**

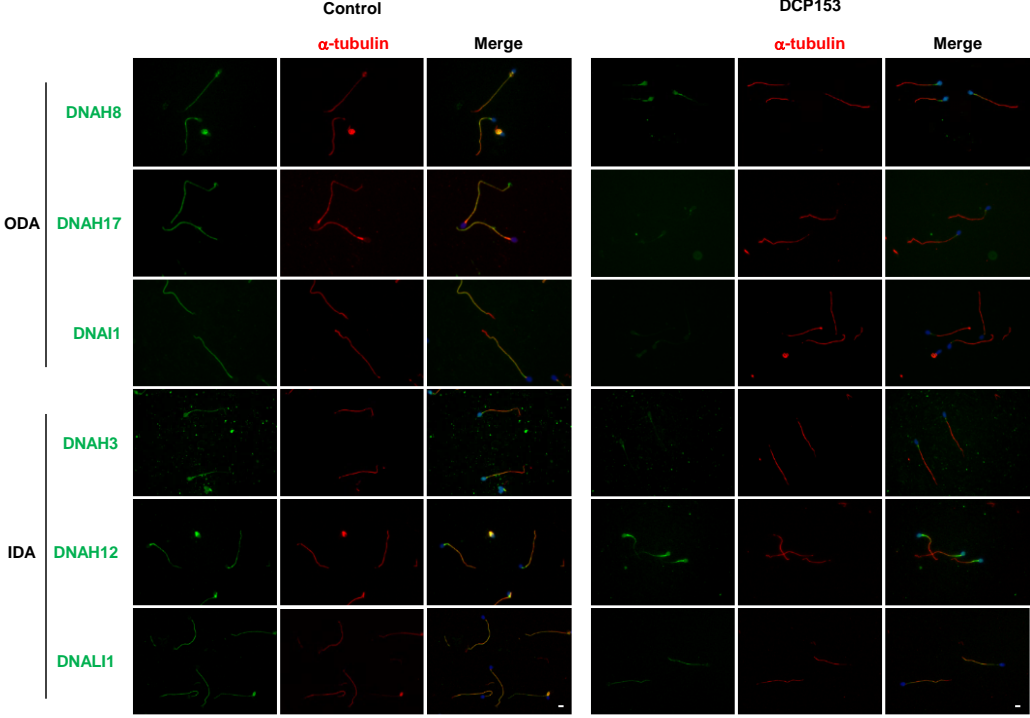


## B

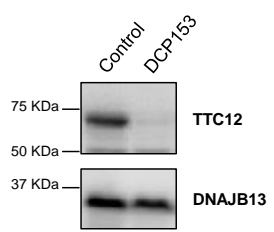




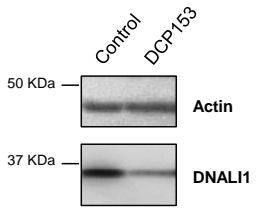
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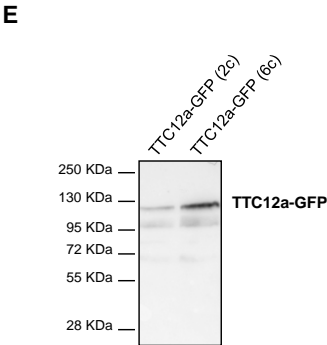
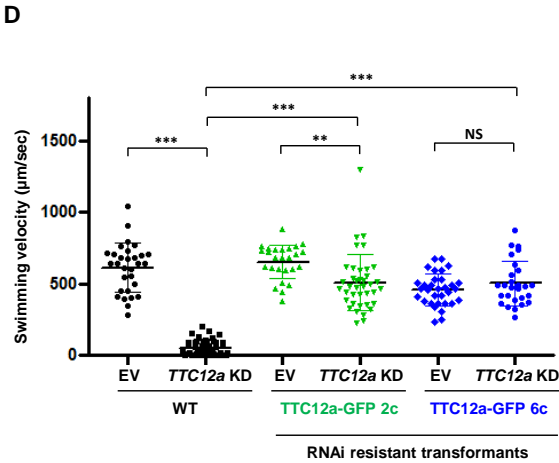
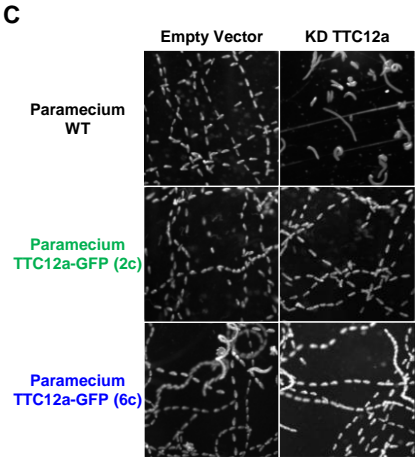
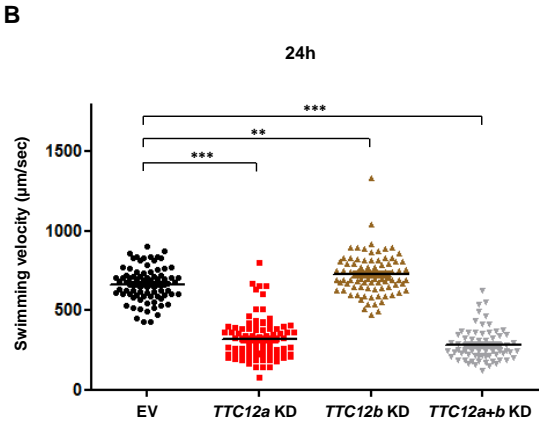
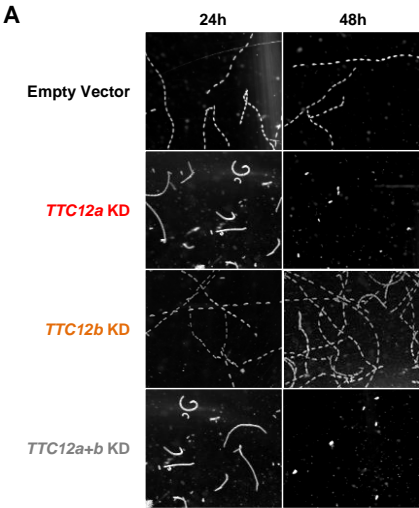


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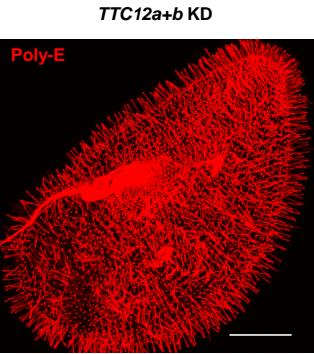


C





A



B

