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20

21   **Impact statement:** Electron-tomography and biochemical approaches demonstrate a

22 direct role for WDR35, beyond integrity of the IFT-A holocomplex, in formation and fusion of

23 electron-dense coated vesicles to the ciliary sheath and pocket for delivery of cargos

24 necessary for axoneme elongation.

Abstract

Intraflagellar transport (IFT) is a highly conserved mechanism for motor-driven transport of cargo within cilia, but how this cargo is selectively transported to cilia is unclear. WDR35/IFT121 is a component of the IFT-A complex best known for its role in ciliary retrograde transport. In the absence of WDR35, small mutant cilia form but fail to enrich in diverse classes of ciliary membrane proteins. In *Wdr35* mouse mutants, the non-core IFT-A components are degraded and core components accumulate at the ciliary base. We reveal deep sequence homology of WDR35 and other IFT-A subunits to α and ß' COPI coatomer subunits, and demonstrate an accumulation of 'coat-less' vesicles which fail to fuse with *Wdr35* mutant cilia. We determine that recombinant non-core IFT-As can bind directly to lipids and provide the first *in-situ* evidence of a novel coat function for WDR35, likely with other IFT-A proteins, in delivering ciliary membrane cargo necessary for cilia elongation.

Introduction

The primary cilium is a highly specialized sensory organelle and signaling hub compartmentalized from the rest of the cell and positioned with a unique interface towards the extracellular environment. Analogous to a cell's antenna, many roles for cilia have emerged in development, disease and homeostasis (Reiter and Leroux, 2017). Enrichment of signaling receptors and effectors in ciliary membranes is critical for cilia function, yet all biosynthesis of cilia-localised membrane proteins occurs in the endoplasmic reticulum and is sorted by the Golgi and vesicular membrane traffic system to efficiently route cargo-laden vesicles for incorporation into the elongating ciliary membrane. The details of this highly efficient, directed transport process for the delivery of diverse cargos to cilia remains unclear.

In mammalian cells, electron microscopy (EM) studies reveal the Golgi stacks closely apposed to the mother centriole (Sorokin, 1962; Wheatley, 1969). During intracellular ciliogenesis, small vesicles are recruited, most likely from the Golgi, to the mother centriole, where they fuse to form a large preciliary vesicle (PCV) attached at the distal appendages (Yee and Reiter, 2015). More secondary vesicles later fuse with the PCV, allowing elongation of cilia. Interestingly, the Golgi remains close to mature cilia, suggesting a continuous exchange of materials, enabling cilia maintenance (Sorokin, 1962; Wheatley, 1969). Several proteins essential for ciliogenesis localize to both the Golgi and the mother centriole and are implicated in this early stage of ciliogenesis including CCDC41 (CEP83), IFT20, HOOK2, and CEP164 (Baron Gaillard et al., 2011; Follit et al., 2006; Graser et al., 2007; Joo et al., 2013; Schmidt et al., 2012; Tanos et al., 2013). In some cases, including HOOK2 and CEP164, these components recruit Rab8a and Rabin-8, which facilitate membrane transport to cilia (Baron Gaillard et al., 2011; Moritz et al., 2001; Nachury et al., 2007). For some specific ciliary cargos, including rhodopsin (Wang and Deretic, 2014) and PKD2 (Follit et al., 2008, 2006; Hoffmeister et al., 2011; Kim et al., 2014; Noda et al., 2016), Golgi-to-cilia transport mechanisms have been described. However, these processes seem to involve cargo-specific traffic modules. A more universal Golgi-to-cilia transport machinery, if one exists, has yet to be identified.

In contrast to traffic to cilia, movement of cargos within cilia requires highly conserved motor-driven macromolecular cargo binding complexes that traffic along axonemal microtubules closely apposed against the ciliary membrane, in a process known as intraflagellar transport (IFT) (Cole, 2009; Kozminski et al., 1993; Pazour et al., 1998; Pigino et al., 2009; Rogowski et al., 2013; Rosenbaum and Witman, 2002). Bidirectional movement of

71 IFT complexes regulates cilia content; the IFT-B complex aids in kinesin-dependent
72 anterograde transport of cargo, whilst the IFT-A complex is required for retrograde transport
73 driven by dynein motors (Blacque et al., 2006; Efimenko et al., 2006; Jonassen et al., 2012;
74 Lee et al., 2008; Piperno et al., 1998; Tran et al., 2008; Tsao and Gorovsky, 2008). The IFT-A
75 complex is composed of three core (IFT144/WDR19, IFT140/WDTC2, IFT122/WDR10) and
76 three non-core proteins (IFT139/TTC21B/THM1, IFT121/WDR35, and IFT43)(Behal et al.,
77 2012; Hirano et al., 2017; Piperno et al., 1998). However, beyond classical retrograde ciliary
78 traffic defects (an inappropriate accumulation of cargos within the cilium) mutations in
79 *IFT144, IFT140, IFT122, IFT121/WDR35*, and *IFT43* result in either severe reduction in cilia
80 length or complete loss of cilia, implying they also have critical roles in transport of cargo to
81 cilia (Avidor-Reiss et al., 2004; Caparrós-Martín et al., 2015; Duran et al., 2017; Hirano et al.,
82 2017; Liem et al., 2012; Mill et al., 2011; Takahara et al., 2018; Zhu et al., 2017). Indeed,
83 several IFT-A mutants fail to localize a range of ciliary membrane proteins including EVC1/2,
84 SMO, ARL13B, INPP5E, and SSTR3 to cilia (Brear et al., 2014; Caparrós-Martín et al., 2015; Fu
85 et al., 2016; Hirano et al., 2017; Jensen et al., 2010; Lee et al., 2008; Liem et al., 2012;
86 Mukhopadhyay et al., 2010; Takahara et al., 2018). However, the mechanism of transport
87 and the location of any IFT-A extra-ciliary function remains unclear.

88   The movement of cargos between membranes of spatially separated organelles in the
89 cytoplasm involves vesicular traffic. Indeed, IFT proteins have been observed to localize to
90 various endomembranes and vesicular compartments outside cilia. For example, the IFT-B
91 protein IFT20 localizes to the Golgi (Follit et al., 2006; Noda et al., 2016), whereas both IFT-B
92 (IFT20, IFT27, IFT46, IFT52, IFT57, IFT88 and IFT172) and IFT-A proteins (IFT139, IFT140)
93 cluster around periciliary vesicles, shown by immuno-EM and light microscopy (Sedmak and
94 Wolfrum, 2010; Wood et al., 2012; Wood and Rosenbaum, 2014). Direct interaction of IFTs
95 with membranes *in vitro* has also been described where the adaptor TULP3 and
96 phosphoinositides mediate the membrane association of IFT-As (Mukhopadhyay et al., 2010).
97 More recently, purified IFT172 was shown to bind to lipids and pinch off smaller vesicles,
98 similar in size to classic COPI vesicles (Wang et al., 2018). It has been postulated that IFT
99 proteins have evolved from membrane traffic coat complexes: soluble multimeric protein
100 complexes which 'coat' donor membranes, facilitating cargo enrichment and membrane
101 remodeling prior to traffic and fusion with target membranes (Jékely and Arendt, 2006; van
102 Dam et al., 2013). Nonetheless, a functional requirement for an IFT-dependent vesicle-to-cilia
103 traffic module and what its dynamic architecture may resemble is currently unknown.

104   To dissect how traffic of newly synthesized ciliary membrane proteins to the cilium

105   occurs, we undertook a series of biochemical and imaging experiments in *Wdr35* null mouse

106   embryonic fibroblasts (MEFs) (Caparrós-Martín et al., 2015; Mill et al., 2011). To distinguish

107   extra-ciliary functions from canonical retrograde traffic defects, we compared $Wdr35^{-/-}$

108   phenotypes with those of the retrograde IFT dynein $Dync2h1^{-/-}$ (Criswell et al., 1996; Huangfu

109   and Anderson, 2005; Porter et al., 1999; Signor et al., 1999). Whilst accumulations of intact

110   IFT-B proteins were observed inside cilia in both mutants, only in the absence of WDR35 does

111   the IFT-A holocomplex fragment and fail to enter $Wdr35^{-/-}$ cilia. Without intact IFT-A, we

112   observe broad defects in the ciliary import of diverse membrane and lipidated proteins, as

113   well an accumulation of 'coat-less' vesicles around the base of *Wdr35* mutants, which fail to

114   fuse with the ciliary sheath. We demonstrate that together recombinant non-core IFT-A

115   proteins (WDR35, IFT43 and IFT139) are sufficient to specifically bind lipids *in vitro*. Together

116   with our localization data, our results provide the first *in situ* evidence of a WDR35-

117   dependent coat required to deliver essential cargo from vesicles to cilia.

Results

Wdr35 null cells have rudimentary, short cilia with intact transition zones

We utilized primary MEFs carrying null mutations in two components of the retrograde IFT machinery (*Figure 1E*), one part of the motor complex that moves cargos (the retrograde dynein heavy chain *Dync2h1)*, and the non-core IFT-A component *Wdr35,* in order to dissect the stage at which ciliogenesis defects occurred (Caparrós-Martín et al., 2015; Mill et al., 2011). Cilia length measured by acetylated α tubulin staining was drastically reduced in both $Wdr35^{-/-}$ (0.48 µm mean ± 0.35 SD) and $Dync2h1^{-/-}$ (0.76 µm mean ± 0.35 SD) mutants, compared to wild type (WT) (2 µm mean ± 0.45 SD) MEFs (*Figure 1A, B*). Given there was no reduction in cilia number (*Figure 1C*), as previously shown (Fu et al., 2016; Liem et al., 2012; Mukhopadhyay et al., 2010), our results suggest that DYNC2H1 and WDR35 are needed for cilia elongation at later stages of ciliogenesis. Defects in centriolar satellite traffic, implicated in ciliogenesis, were previously reported for *WDR35* mutant RPE-1 cells (Fu et al., 2016), however we saw no difference in levels or localization of endogenously tagged PCM1 protein (PCM1-SNAP) which marks centriolar satellites in MEFs (*Figure 1- figure supplement 1*). In *C. elegans* non-core IFT-A mutants, extension of the MKS module into the axoneme from the transition zone due to failure of cargo retrieval had been reported (Scheidel and Blacque, 2018). However, we observed intact transition zone modules as shown by NPHP1 and MKS1 localization in both mammalian mutants (*Figure 1D, E*). We noted that $Wdr35^{-/-}$ axonemes, while acetylated, were not polyglutamylated suggesting differences in tubulin post-translational modifications (PTMs) and stability (*Figure 1D*). Together these data suggest that the initial steps of ciliogenesis occur in both $Dync2h1^{-/-}$ and $Wdr35^{-/-}$ mutants, however, subsequent axoneme elongation may be differentially affected.

Wdr35 null cells have intact IFT-B complexes with a retrograde defect and unstable IFT-A holocomplexes which fail to enter cilia

Axoneme elongation during cilia assembly requires the import of key building blocks from their place of synthesis in the cell body into the cilium across the transition zone via IFT. We focused first on the anterograde, IFT-B machinery, monitoring two subunits IFT81 and IFT88. We found that IFT-B complex proteins have similar retrograde traffic defects in both $Wdr35^{-/-}$ and $Dync2h1^{-/-}$ cells (*Figure 2A, B*)**,** accumulating beyond the length of the acetylated axoneme. We next looked to see if IFT-B complex assembly is disturbed in the absence of WDR35 by immunoprecipitation (IP) of endogenous IFT88, followed by mass spectrometry (MS) to identify co-purifying subunits. IFT88 is the link between IFT-B1 and IFT-B2 complexes

151 (*Figure 1E*), interacting with IFT38 on the IFT-B2 side and IFT52 on the IFT-B1 side (Katoh et

152 al., 2016; Mourão et al., 2016; Taschner et al., 2016). MS analysis of immunoprecipitates

153 from E11.5 *Wdr35^{+/+}* and *Wdr35^{-/-}* embryo lysates revealed no statistically significant

154 differences in stoichiometric composition of IFT-B complexes (*Figure 2C, D*). We were able to

155 isolate almost the entire IFT-B complex (14 out of 16 IFT-B components) aside from IFT70,

156 which is not yet reported in mouse as well as IFT25, which binds IFT27 to form a heterodimer

157 (Bhogaraju et al., 2011; Funabashi et al., 2017; Katoh et al., 2016; Wang et al., 2009) and is

158 necessary for Hh signaling (Keady et al., 2012). Because the composition of the IFT-B complex

159 and its ability to enter cilia each appear unaltered, we conclude that exit from cilia is

160 impaired in the absence of WDR35.

161 We next examined the composition of the IFT-A holocomplex in WT vs *Wdr35^{-/-}*

162 embryos by immunoprecipitation of endogenous IFT-A core protein IFT-140 and its

163 interactors. Whilst IFT140 immunoprecipitated all six components of the IFT-A complex from

164 *Wdr35^{+/+}* embryo lysates, in *Wdr35^{-/-}* samples both non-core components IFT139 and IFT43

165 were missing from our MS datasets (*Figure 3A*). Their absence was confirmed by

166 immunoblotting (*Figure 3B*). Moreover, core components IFT122 and IFT144 were also

167 significantly reduced in the purified mutant complex (*Figure 3A*), suggesting that WDR35 is

168 critical for the stability of the IFT-A complex and its components. We also compared total

169 cellular levels of IFT-A component proteins and found IFT139 and IFT43 levels were also

170 undetectable on blots with lysates from both *Wdr35^{-/-}* embryos (*Figure 3C, D*) and MEFs

171 (*Figure 3- figure supplement 1A, B*). This suggests WDR35 is not only critical for the

172 formation of stable IFT-A holocomplex but is also required for stability of its non-core

173 components.

174 In contrast, the individual core components of the IFT-A complex were nearly equally

175 expressed in WT and *Wdr35^{-/-}* lysates, except for IFT122 which had higher expression levels in

176 *Wdr35^{-/-}* MEFs (*Figure 3C, D* and *Figure 3- figure supplement 1A, B*). Other core components

177 have been shown to have higher levels in the absence of WDR35 in human fibroblasts (Duran

178 et al., 2017). Thus, our work also supports previous studies demonstrating a level of

179 interdependence in the levels of IFT-A subunits, which might be required for their

180 coordinated function (Behal and Cole, 2013; Duran et al., 2017; Fu et al., 2016; Picariello et

181 al., 2019; Zhu et al., 2017).

182 These results suggest WDR35 might be a link between IFT-A core and non-core

183 proteins, that when absent results in the decreased abundance of IFT-A non-core subunits.

184 To further distinguish between increased protein degradation from transcriptional changes,
185 control and mutant MEFs were treated with the proteasome inhibitor MG-132 (20 µM)
186 (*Figure 3E*). Treated cells displayed increased levels of IFT43, which suggests that in the
187 absence of WDR35, non-core proteins may be targeted by the proteasomal degradation
188 pathway. Interestingly IFT139 and IFT121 are degraded in *IFT43* null cells and both are
189 rescued similarly by MG-132 treatment (Zhu et al., 2017), confirming that the stability of IFT-
190 A complex proteins is interdependent.

191   We next looked at the localization and levels of the IFT-A components by
192 immunofluorescence. IFT-A components were present in *Dync2h1^-/-* cilia suggesting entry of
193 IFT-A holocomplexes is not affected, but return from the distal tip is compromised in the
194 absence of the dynein motor (*Figure 3F, Figure 3- figure supplement 1C*). In contrast, in
195 *Wdr35^-/-* MEFs, IFT-A core components fail to enter cilia and remain restricted at the ciliary
196 base (*Figure 3F*), as shown by the difference in length covered by IFT-A components relative
197 to cilia length measured by acetylated tubulin staining (*Figure 3- figure supplement 1C*),
198 whereas non-core proteins were undetectable, consistent with degradation (*Figure 3C-F*).
199 These results are consistent with previous reports of the inter-dependence of IFT-A
200 components for ciliary localization. IFT140 is decreased in cilia of *IFT122* mutants in mouse
201 and fly (Lee et al., 2008; Qin et al., 2011), IFT139 is reduced in the flagella of *Chlamydomonas*
202 with *IFT144* mutation (Iomini et al., 2009), and IFT144 fails to get recruited into cilia in
203 *WDR35^-/-* RPE cells (Fu et al., 2016). IFT-A proteins require CPLANE chaperones for
204 holocomplex assembly and cilia entry (Toriyama et al., 2016). In all cases, failure of IFT-A
205 holocomplex integrity impairs its recruitment into the cilia axoneme. Recent cryo-EM work
206 had suggested IFT-A is being carried by IFT-B trains inside the *Chlamydomonas* flagella in WT
207 cells and these structures are missing in the *IFT139* mutant (Jordan et al., 2018). Our work in
208 the mammalian system in the absence of WDR35 has a similar effect with IFT-B proteins
209 accumulating inside the cilium whilst IFT-A core proteins accumulate proximal to the cilia
210 base, and the non-core components are degraded.

211

212 **Membrane proteins fail to be recruited into *Wdr35^-/-* cilia**

213   Cilia membrane protein cargos are synthesized in the cell body (rough ER) and traffic
214 into cilia through a variety of direct and indirect routes. These include lateral diffusion from
215 the plasma membrane (Leaf and Von Zastrow, 2015; Milenkovic et al., 2009), recycling of

216 plasma membrane proteins via the endocytic pathway (Boehlke et al., 2010) as well as more

217 directly from Golgi-derived vesicles (Follit et al., 2008, 2006; Kim et al., 2014; Witzgall, 2018).

218 Moreover, ciliary membrane content is dynamically regulated in response to external signals.

219 First, we tested appropriate dynamic localization of the GPCR Smoothened (SMO), which is

220 recruited to the cilia in response to Hh ligand (*Figure 4A*). SMO is already present in *Dync2h1*[-]

221 [/-] mutant cilia, even in the absence of Hh. In contrast, even in the presence of Hh activation,

222 SMO fails to enter *Wdr35*^{-/-} cilia. We investigated endogenous levels and localizations of

223 membrane associated GTPases ARL13B and ARL3, which are enriched in cilia in control cells

224 (*Figure 4A*). We saw that while they accumulate in excess in *Dync2h1*^{-/-} mutants as per a

225 retrograde defect, strikingly they fail to be recruited into *Wdr35*^{-/-} cilia. Detecting low levels

226 of endogenous protein localization and their mislocalization in *Wdr35* mutants by

227 immunofluorescence can be challenging. To overcome this, we transiently expressed

228 membrane cargos, including fluorescently-tagged SMO and ARL13B (*Figure 4B and Video 2*),

229 which effectively traffic into the cilia of WT cells. However, they fail to localize to *Wdr35*^{-/-}

230 cilia, with some accumulation at the cilia base. Interestingly, in our *Wdr35*^{-/-} cells, SMO was

231 predominantly localized to vesicles in the cytoplasm of mutant cells, whereas overexpressed

232 ARL13B when not transported into cilia, is concentrated on other membranes, particularly

233 the plasma membrane and pericentrosomal vesicles (*Figure 4B and Video 2*).

234   In trypanosomes, localization of flagellar membrane proteins was shown to be

235 dependent on lipid rafts highly enriched in axonemes (Tyler et al., 2009). Here, dual acylation

236 was shown to direct potential association with lipid rafts, membrane microdomains that

237 function as specialized platforms for protein/lipid transport and signaling. Indeed, ARL13B

238 requires palmitoylation for its cilia membrane targeting and ciliogenesis in worms and

239 mammals (Cevik et al., 2010; Li et al., 2010; Roy et al., 2017), where it acts as the cilia-

240 localized GEF for ARL3 driving it to release lipid modified cargos from carriers UNC119 and

241 PDE6**δ** within cilia membranes (Gotthardt et al., 2015; Kapoor et al., 2015)*.* As ARL13B and

242 ARL3 fail to localize to mutant cilia, we next asked about the ability to recruit general

243 lipidated cargo in *Wdr35*^{-/-} MEFs. We examined the localization of lipidated motifs tagged to

244 EGFP (Williams et al., 2014) to look at specialized membrane microdomains. In WT MEFs,

245 untagged EGFP is present in the cell, but not in the cilium. When tagged with either

246 myristoylation and palmitoylation (MyrPalm) or dual palmitoylation (PalmPalm) motifs, EGFP

247 robustly enriches within cilia (*Figure 4C*). We observed no enrichment of dual geranylation

248 (GerGer) modified EGFP within control fibroblast primary cilia (data not shown), in contrast

249   to the low level expression previously reported in the most proximal portions of highly

250   specialized olfactory sensory cilia (Williams et al., 2014). This suggests that cell-type and cilia-

251   specific differences exist. In marked contrast to WT cells, in *Wdr35$^{-/-}$* MEFs, both the

252   myristoylation and palmitoylation (MyrPalm) or dual palmitoylation (PalmPalm) EGFP failed

253   to concentrate in mutant cilia (***Figure 4C***). This failure to recruit lipidated cargoes into *Wdr35*

254   mutant cilia is consistent with a more general traffic disruption of ciliary-destined membrane-

255   microdomains, containing broad categories of the membrane and membrane-associated

256   cargos.

257

258   **WDR35 and other IFT-A complex proteins share close sequence and structural**

259   **similarity to COPI complex proteins α and ß'**

260   It has previously been suggested that IFTs evolved from a protocoatomer (Jékely and

261   Arendt, 2006; Taschner et al., 2012; van Dam et al., 2013). Three classic coat complexes

262   (COPI, COPII and clathrin) exist and perform similar functions but on different membranes

263   and follow different routes through the cell. They are made of different protein components,

264   which share a similar division of labour, characterized functionally as either adaptors or cage

265   forming proteins. Although components like the cage proteins share significant structural

266   homology in organization of protein domains, they do not share detectable sequence

267   homology (Field et al., 2011). Given the defects in ciliary membrane content observed in the

268   *Wdr35* mutant cilia, we hypothesized that WDR35, in collaboration with other IFT-A complex

269   proteins, may be required for moving ciliary membrane cargos between donor membranes,

270   such as the Golgi or endosomes, to their destination ciliary membrane, in a manner

271   comparable to coat complexes. WD40 repeat (WDR) and tetratricopeptide repeat (TPR)

272   motifs are common throughout cellular proteomes and are involved in a wide range of

273   biological processes. Agnostic of structure, we used deep sequence analysis of the whole

274   human proteome and homology modeling to ask which proteins were most similar to IFT-A

275   components. Simple alignment strategies with proteins such as IFT subunits, which contain

276   tandem repeat motifs, could erroneously align with other repeat proteins to suggest a close

277   evolutionary relationship where none exists (i.e., false positives). To address this, we used

278   four IFT-A subunits (IFT144, IFT140, IFT122 and IFT121) and two of IFT-B (IFT80 and IFT172) as

279   seed sequences for multiple iterative rounds of homology searches via profile-HMM

280   alignment (Remmert et al., 2011). We then clustered the resulting proteins based on

281 sequence similarity, as previously described (Wells et al., 2017; Wells and Marsh, 2019). This

282 was repeated using the COP protein subunits as seeds for reverse analysis. Together, these

283 reciprocal analyses revealed that out of the entire proteome, COPI α and β' cluster most

284 closely with 6 IFT proteins (two IFT-B and four IFT-A components), both having TPR and

285 WD40 repeats (*Figure 5A*). In contrast, homology searches with COPI β and COPI Υ1/2, which

286 have HEAT/ARM repeats, did not yield any hits with IFT components, as was the case with

287 COPI ε, which has TPR repeats but no WD40 domains. COPI δ and COPI ζ1/2, which have no

288 identifiable repeat domains, are most closely related to adaptor protein complex subunits

289 AP2 and AP3. In summary, using multiple rounds of sequence homology searches, we

290 generated a broad set of putatively related repeat proteins, clustering of which reveals clear

291 relationships between coatomers and IFT-A/B complex components.

292   Next, we used SWISS-MODEL (Waterhouse et al., 2018) to predict the structures of

293 IFT-A proteins. COPI α (COPA) and β' (COPB2) structures were top hits with 12 %-15 %

294 sequence identity and 26 %-27 % sequence similarity to four IFT-A complex proteins (IFT144,

295 IFT140, IFT122, and WDR35). Based on the target-template alignment models, built using

296 ProMod3, ribbon diagrams of all 4 IFT-A subunits modeled structures with two N-terminal

297 seven-bladed WD40 β propellers and C-terminal extended TPR repeats, also found in α and β'

298 COPI proteins (*Figure 5 - figure supplement 1A*), as previously modeled for WDR35 (Mill et

299 al., 2011). The remaining 2 IFT-A subunits were not possible to model accurately. IFT139

300 contains only TPR repeats with limited sequence similarity to the ε subunit of COPI coatomer

301 (van Dam et al., 2013). IFT43 is the smallest and unstructured protein and could not be

302 modeled and is presumed to be made of α-helices (Taschner et al., 2012). While undertaking

303 this work, a crystal structure for IFT80 was published, highlighting that despite the same

304 domain organization IFT80 adopted a distinctive 3D conformation of the second β-propeller

305 domain from β'-COP and also formed a dimer unlike the triskelion COPI cage (Taschner et al.,

306 2018). However, purified IFT172 adopted two configurations by negative stain electron

307 microscopy (EM) when incubated with and without lipids (Wang et al., 2018). Thus,

308 respecting the limitations of homology modeling without solved structures, we found 4 IFT-A

309 proteins (IFT144, IFT140, IFT122, and IFT121) to have very high sequence and structural

310 similarities to COPI α and β' subunits with N-terminal WD40 repeats and C-terminal TPR

311 region (*Figure 5 - figure supplement 1A*). Given the structural homology of WDR35 and IFT-

312 As to COPI proteins, which derive vesicles from the Golgi, we asked whether WDR35 and IFT-

313 As were sufficient to directly bind membranes.

314

Purified non-core IFT-A can bind directly to specific lipids *in vitro*

316   To test if the IFT-A complex directly associates with lipids *in vitro*, we purified recombinantly

317   expressed IFT-A non-core complex (IFT139/121/43) as well as the dimeric IFT121/43 and the

318   isolated IFT43 subunit of the unicellular organisms *Chlamydomonas reinhardtii* (Cr) using

319   eukaryotic expression systems (***Figure 5B, C*** and ***Figure 5- figure supplement 1B-D***). All three

320   samples were soluble, eluted as stoichiometric proteins from size-exclusion chromatography

321   and were positively identified by mass-spectrometry. The hetero-trimeric IFT-A complex

322   purified from mammalian cells was assessed for lipid-binding using membrane lipid strips,

323   detecting any bound protein complex using antibodies against the His-Tag on IFT43. From the

324   protein-lipid overlay results in ***Figure 5C***, the His-GFP tagged IFT-A trimeric complex displays

325   strong binding to phosphatidic acid (PA) as well as weaker binding to phosphatidylserine (PS).

326   Thus the IFT-A trimeric complex binds to these negatively charged (anionic)

327   phosphoglycerates exclusively, without binding to neutral or inositol-based lipids as had been

328   reported for the IFT-A adaptor TULP3 (Mukhopadhyay et al., 2010). Although there are no

329   reports that PA is a constitutive phospholipid of Golgi apparatus in *Chlamydomonas*

330   *reinhardtii*, it was shown to be the third most abundant phospholipid in cilia (Lechtreck et al.,

331   2013). As a low abundance phospholipid, PA is known to play both important structural roles

332   facilitating membrane curvature during vesicle fusion and fission events (Arisz and Munnik,

333   2011; Zhukovsky et al., 2019) as well signaling functions such as flagellar excision in response

334   to environmental stresses (Goedhart and Gadella, 2004; Lechtreck et al., 2013; Quarmby et

335   al., 1992). To further investigate which subunit of the IFT-A non-core complex is responsible

336   for lipid binding, His-tagged IFT121/43 and IFT43 were also tested in the lipid-strip

337   assay (***Figure 5 - figure supplement 1B-D***). Neither the IFT121/43 dimer nor IFT43 alone

338   showed detectable lipid binding demonstrating that the IFT139 subunit is essential for lipid

339   interaction by the non-core IFT-A complex. In order to further test whether the trimeric IFT-A

340   complex was capable of specifically bind to PA-containing liposomes, we performed negative

341   stain electron microscopy of purified proteins incubated with liposomes composed of

342   PE/PG/PA or POPC (control) (**Figure 5D**). The IFT-A trimer was observed to associate with

343   PE/PG/PA liposomes but not to control liposomes (**Figure 5D**). Consistent with the lipid

344   overlay assay (***Figure 5 - figure supplement 1B-D***), the IFT121/IFT43 dimer display only weak

345   association with PE/PG/PA liposomes (**Figure 5D**). The structural homology of IFT-As to COPI

346   proteins and the ability of the non-core IFT-A complex to bind directly to lipids *in vitro* led us

347 to ask whether IFT-A complex may function similarly to COPI vesicle coats assisting vesicular

348 transport between the Golgi and cilia *in vivo*.

349

350 **Distinct ultrastructural ciliary defects are observed between disruption of IFT-**

351 **A versus the retrograde IFT motor**

352 We undertook ultrastructural studies to examine traffic phenotypes with higher

353 resolution around cilia in MEFs. In all genotypes, ciliation was observed to start very close to

354 the nucleus and remain close to the Golgi stacks throughout cilia elongation (*Figure 6A,*

355 *Video 3; Figure 6B, Video 4; Figure 6C, Video 5; Figure 6 - figure supplement 2, Video 6*). In

356 control MEFs, even after 24 h of serum starvation, very few (~1 %) cilia were observed to

357 emerge from the cell, highlighting the deep-seated ciliary pocket in MEFs (*Figure 6B, Video 4;*

358 *Figure 6 - figure supplement 1 and 4A*), and as described for RPE-1 cells (Molla-Herman et al.,

359 2010). In control MEFs, polymerised microtubules formed a well-structured axoneme (*Figure*

360 *6B, Video 4; Figure 6 - figure supplement 1 and 4A*) as previously described in MEFs

361 (Rogowski et al., 2013) and reported in other primary cilia (Kiesel et al., 2020; Molla-Herman

362 et al., 2010). Additionally, microtubules can be seen attached at the cilia base and radiating in

363 different directions in the cell (*Figure 6 - figure supplement 1*). In contrast to the well-defined

364 ciliary membrane and well polymerized microtubules of the control axoneme, *Wdr35*$^{-/-}$ cilia

365 have 'wavy' membranes and disorganized microtubules (*Figure 6C, Video 5 and Figure 6 -*

366 *figure supplement 4B*). Mammalian *Dync2h1*$^{-/-}$ mutants retained a well-defined ciliary

367 membrane and an apparently well-structured axoneme present throughout (*Figure 6 - figure*

368 *supplement 4C, Video 7*), similar to previous reports of the *fla14* dynein mutant in

369 *Chlamydomonas* (Pigino et al., 2009). Stacked standing trains with a periodicity of 40 nm

370 were reported in *fla-14* mutants (Pigino et al., 2009; Stepanek and Pigino, 2016) and in our

371 *Dync2h1*$^{-/-}$ mutant axonemes, we observed similar stacking of stalled IFT trains with a

372 periodicity of 40 nm, irrespective of the length of mutant cilia (*Figure 6 - figure supplement*

373 *4C, Video 7* and (Liem et al., 2012)). Although IFT-Bs also accumulated in *Wdr35*$^{-/-}$ cilia (*Figure*

374 *2A, B*), these stripes were not observed (*Figure 6C, Video 5; Figure 6 - figure supplement 4B*)

375 suggesting that both IFT-B and IFT-A are required to form the higher ordered IFT trains which

376 stall in *Dync2h1* mutants.

377

378 **WDR35 facilitates formation of coated vesicles containing membrane proteins**

379   **destined for cilia**

380   We further tested our hypothesis that IFT-A acts as a coat-like complex for vesicles

381   targeted to cilia by transmission electron microscopy (TEM) analysis of ciliated MEFs. We

382   observed electron-dense coated vesicles between the Golgi and cilia in WT MEFs (*Figure 6A,*

383   *Video 3*). We also observed these coated vesicles clustering at the cilia base (*Figure 6B, Video*

384   *4*) and bulging from ciliary pockets and ciliary sheaths in WT MEFs (*Figure 6 - figure*

385   *supplement 1*). These electron-dense vesicles around control cilia were more prominent at

386   the early stage of ciliogenesis in EM (*Figure 6A, Video 3*).

387   In contrast, in *Wdr35*$^{-/-}$ mutant cells, there is a ten-fold increase in the average

388   number of vesicles around the ciliary base (*Figure 6C, Video 5; Figure 6 - figure supplement*

389   *2, Video 6; Figure 6 - figure supplement 4B; Figure 7 - figure supplement 1A;* quantified in

390   *Figure 7B*)**.** Importantly, virtually all of these mutant vesicles lack the electron-dense coats

391   observed in control cells (*Figure 6C, Video 5; Figure 6 - figure supplement 2, Video 6; Figure*

392   *6 - figure supplement 4B; Figure 7E; Figure 7 - figure supplement 1B;* quantified in *Figure*

393   *7D*). Notably, we did observe other electron-dense coats, likely clathrin, on budding vesicles

394   at the plasma membrane in these same *Wdr35* mutant cells, emphasizing that other coats

395   are preserved in these conditions (*Figure 6 - figure supplement 2, Video 6; Figure 7E*).

396   Moreover, no difference in the density or distribution of periciliary clathrin-positive vesicles is

397   observed around the base of *Wdr35*$^{-/-}$ mutant cilia (*Figure 7 - figure supplement 1C, D*). In

398   contrast, the accumulation of coatless vesicles spreads in a volume ~2 μm^3 around the

399   *Wdr35*$^{-/-}$ ciliary base (*Figure 6C, Video 5, Figure 6- figure supplement 2, Video 6,* quantified in

400   *Figure 7B, Figure 7 - figure supplement 1A*). In spite of their proximity to the ciliary sheath

401   and their abundance, fusion events were not observed in *Wdr35*$^{-/-}$ mutants (*Figure 6C, Video*

402   *5; Figure 6 - figure supplement 2, Video 6;* quantified in *Figure 8D*). We do not believe this

403   periciliary vesicle accumulation phenotype is a general defect in global membrane traffic as

404   the accumulation of vesicles lacking electron densities occurs specifically around mutant cilia,

405   and not at other regions of *Wdr35* mutant cells (*Figure 6 - figure supplement 3, Video 7*).

406   Clathrin-mediated endocytosis at the ciliary pocket is proposed to regulate

407   internalization of ligand/receptor complexes or membrane content at the base of cilia (Molla-

408   Herman et al., 2010). To test whether these vesicles might be important for the import or

409   export of cargo directed to cilia, we analyzed *Dync2h1*$^{-/-}$ cilia, which we showed to contain

410   increases in IFTs (*Figure 2 and Figure 3*) and membrane protein cargo (*Figure 4*) in the

411   absence of retrograde transport. Consistent with the redistribution of IFT pools from the base

412   into the ciliary compartment (*Figure 2A, and Figure 3F*), we observed no vesicles at the base

413   of *Dync2h1*^{-/-} cilia (*Figure 7A and Video 8;* quantified in *Figure 8D*). Interestingly ectosomes,

414   which are previously reported to regulate the content of cilia in a variety of systems (Cao et

415   al., 2015; Nager et al., 2017; Wood and Rosenbaum, 2014), budding from the tip were much

416   more prevalent in *Dync2h1*^{-/-} cilia than in WT cells (*Figure 6 – figure supplement 4C, Video 8*).

417   We interpret these data as evidence that the coated vesicles around the WT cilia function to

418   transport cargo possibly from the Golgi or via an endosomal intermediate to the cilia. In the

419   absence of WDR35, non-coated vesicles accumulate around the ciliary base marking a failure

420   in this process in either the formation and/or maintenance of this coat and subsequent

421   fusion at the target ciliary pocket.

422   To further confirm our hypothesis that these electron dense vesicles directed to cilia

423   contain WDR35 and IFT-A proteins, we performed correlative light and electron microscopy

424   (CLEM) imaging in *Wdr35*^{-/-} MEFs expressing WDR35-EmGFP, which we had previously shown

425   to completely rescue cilia phenotypes (*Figure 1A, B; Figure 8A*). Expressing WDR35-EmGFP in

426   *Wdr35*^{-/-} ensures that every WDR35 particle was labelled with EmGFP, minimizing

427   competition with non-labeled species. Using Airyscan confocal imaging of WDR35-EmGFP

428   MEFs grown on grids for subsequent TEM, we saw WDR35-EmGFP enriched at the ciliary base

429   of rescued mutant cilia. Moreover, we observed that this signal coincided with the re-

430   appearance of electron-dense vesicles in the TEM images (*Figure 8A, B*). We also observed

431   recovery of fusion events of coated vesicles at the cilia base in cells expressing Wdr35-EmGFP

432   as well as rescue of the periciliary vesicle accumulation phenotype (*Figure 8B, C,* quantified in

433   *Figure 8D, Figure 8 - figure supplement 1, Video 9*). Next, we performed immunogold

434   labelling directly on 70 nm sections and observed sparse but specific labelling of GFP-positive

435   particles at the cilia base, within the axoneme and around putative vesicles at the cilia base

436   and ciliary sheath (*Figure 8 - figure supplement 2, 3*). Together, these results demonstrate

437   that WDR35 is required for the formation of these coated vesicles and that these coated

438   vesicles coincided with WDR35-EmGFP signal, confirming WDR35 supports the assembly of a

439   novel coat on vesicles destined to deliver membrane cargos to cilia.

440

441   ## Discussion

442   **WDR35 is a component of a novel coat-like complex required for entry of**

443   **cargos into cilia**

444   Vesicle coat proteins, with the archetypal members clathrin and the coat protein
445 complexes I and II (COPI and COPII, respectively), are macromolecular machines that play two
446 central roles in the homeostasis of the cell's endomembrane system. They enable vesicle
447 formation and select protein and lipid cargo packaged for delivery from a specific donor to
448 functionally segregated compartments. Given the deep sequence structural similarities
449 between IFT-A and COPI subunits and the ability of the non-core IFT-A to bind directly to
450 lipids *in vitro*, coupled to the phenotypic defects in *Wdr35^{-/-}* cells (including lack of ciliary
451 enrichment of a broad range of membrane cargos and the absence of electron-densities on
452 accumulated periciliary vesicles), we propose a novel function for WDR35 and other IFT-A
453 proteins to act as a coat-like complex that is critical for the transport of ciliary membrane
454 cargo into cilia. Two other macromolecular complexes have been proposed to form vesicle
455 associated coats involved in ciliary traffic: clathrin (Kaplan et al., 2010; Molla-Herman et al.,
456 2010) and the BBSome complex (Jin et al., 2010).

457   Clathrin is a classical vesicle coating protein with some documented activity at the
458 ciliary pocket (Clement et al., 2013; Pedersen et al., 2016). From static images, the
459 directionality of events is difficult to resolve: fission (endocytosis) or fusion (exocytosis).
460 Clathrin vesicles can be both endocytic, where they concentrate cargos and curve off donor
461 membranes for selective transport into the cytoplasm, or exocytic where they can use fuse to
462 release their contents. For example, a subset of AP-1 clathrin vesicles were shown to traffic
463 between the trans Golgi and basolateral membranes of polarized epithelial cells (Fölsch et al.,
464 1999), via the recycling endosome compartment (Futter et al., 1998). Indeed, in both *C.*
465 *elegans* (Bae et al., 2006; Dwyer et al., 1998; Kaplan et al., 2010; Ou et al., 2007) and
466 trypanosomes (Vince et al., 2008) deletion or depletion of AP-1 leads to defects in cilia
467 assembly and protein traffic into cilia. However, in mammalian cells, depletion of clathrin and
468 clathrin-associated proteins results in a normal number of cilia with normal lengths (Kaplan
469 et al., 2010; Molla-Herman et al., 2010), as opposed to the drastically reduced size of *Wdr35^{-}*
470 *[/-]* cilia (Caparrós-Martín et al., 2015; Fu et al., 2016; Mill et al., 2011). This suggests that
471 clathrin is dispensable for vesicular transport into mammalian cilia. Although electron-dense
472 vesicles were observed invaginating from the mammalian ciliary pocket, the electron-density
473 on these vesicular invaginations was unchanged in the absence of clathrin (Molla-Herman et
474 al., 2010). Using live cell-imaging, the directionality of clathrin-mediated traffic was reported
475 to be largely away from cilia (Molla-Herman et al., 2010). Importantly, we still observe
476 clathrin-coated endocytic structures on the plasma membrane of *Wdr35^{-/-}* cells (***Figure 6-***

477 *figure supplement 2; Video 6*), and we found no difference in the distribution of clathrin

478 intensity in a volume of ~2 μm³ around the ciliary base in *Wdr35^{-/-}* cilia compared to controls

479 (*Figure 7- figure supplement 1 C,D*). Moreover, studies on clathrin-mediated exocytosis

480 demonstrated that depletion of human clathrin heavy or light chains results in increased total

481 fusion events with complete release of membrane cargos from vesicles in fibrosarcoma cells

482 (Jaiswal et al., 2009), the opposite to what is observed in *Wdr35* mutants where vesicles stack

483 up adjacent to the ciliary sheath but do not fuse.

484   The BBSome is a macromolecular machine of Bardet-Biedl syndrome (BBS) proteins

485 which is also postulated to have evolved from an early ancestral coat complex (Jékely and

486 Arendt, 2006; van Dam et al., 2013). The BBSome shares similar structural elements to the

487 archetypal coats and plays a role in cilia function (Nachury, 2018). In contrast to IFT,

488 mutations in BBSome components, including ARL6/BBS3, do not affect cilia assembly and

489 length regulation (Domire et al., 2011; Eguether et al., 2014; Lechtreck et al., 2013, 2009;

490 Liew et al., 2014; Nager et al., 2017; Shinde et al., 2020; Xu et al., 2015; Ye et al., 2018).

491 Instead, they generally are required for regulating cilia content, mostly for the export of

492 ciliary membrane proteins. Although this suggests that BBSomes regulate movement of

493 ciliary components between compartments, endogenous localization of the BBSome remains

494 unclear, without evidence supporting endomembrane or plasma membrane localization. In

495 contrast, IFT20 localizes to the Golgi (Follit et al., 2006; Noda et al., 2016). Moreover, whilst

496 there is *in vitro* evidence that BBSomes can cluster on liposomes, they do not deform

497 membranes, a key step in vesicle formation by coatomers (Jin et al., 2010). In contrast,

498 purified IFT172, an IFT-B component that is also homologous to COPI α and ß' like WDR35,

499 can not only assemble on liposomes with high affinity but can also bud 50 nm vesicles

500 consistent with coatomer sized products (Wang et al., 2018). We report here that the

501 purified trimer of non-core IFT-A (WDR35, IFT43 and IFT139) can also directly and specifically

502 bind to lipids, notably phosphatidic acid (PA) which is involved in membrane deformation in

503 COPI maturation and exocytosis (Yang et al., 2008; Zeniou-Meyer et al., 2007). We are

504 currently testing whether non-core IFT-A can also pinch off vesicles. Together the evidence,

505 including its evolutionary conservation of the BBSome with more classical coat proteins

506 (Jékely and Arendt, 2006; van Dam et al., 2013), interaction with *in vitro* membranes in

507 presence of the ARF-like GTPase ARL-6, interaction with phospholipids (Jin et al., 2010;

508 Nachury et al., 2007) and recent cryo-EM structures of the complex (Chou et al., 2019; Klink

509 et al., 2020; Singh et al., 2020; Yang et al., 2020), suggests the BBSome may be working as an

510   adaptor for IFT-A mediated cage formation, similar to other coat adaptors for clathrin (i.e.,

511   AP1/AP2) or COP (i.e. β-, γ-, δ-, and ζ-COP for COPI). Our data suggests that the electron

512   density observed on vesicles around the ciliary base in control cells is neither clathrin nor

513   BBSome in nature, and is likely composed of WDR35/IFT-A.

514

Mechanism of WDR35/IFT-A-assisted vesicle coat function; regulators of vesicular fusion and fission

517   Our study demonstrates a requirement for IFT-A to deliver ciliary membrane cargo

518   into cilia, potentially by acting as a vesicle coat operating between the Golgi and the ciliary

519   base. Archetypal coatomer protein complexes, including COPII, COPI, and clathrin,

520   concentrate cargo within donor membranes and pinch off vesicles (fission), which then travel

521   to their target organelle membranes, where SNARE and Rab GTPases assist their fusion

522   (Bonifacino and Glick, 2004). In these cases, the electron-dense coats are progressively

523   dismantled such that uncoated vesicles can fuse with acceptor membranes, presumably to

524   facilitate access to the fusion machinery, such as SNAREs, on the surface of the vesicle. As a

525   result of interactions with cargo and lipids with the vesicles, there is evidence that the COPI

526   coat can remain stable on membranes after fission. Moreover, this suggests that COPI vesicle

527   uncoating may be incomplete, such that residual COPI on the vesicle surface enables vesicle

528   recognition and tethering necessary for fusion to the correct acceptor membrane (Orci et al.,

529   1998). In contrast to the trail of electron-dense vesicles between the Golgi and the base of

530   cilia in control cells, we observed ten times more vesicles stalled around the cilia base of

531    $Wdr35^{-/-}$ MEFs. These all lack an electron-dense coat suggesting that these transport vesicles

532   are formed but fail to fuse at the ciliary target membrane in the absence of WDR35.

533   This raises a question as to why a protein like WDR35, which shares structural

534   homology to fission-inducing proteins, gives phenotypes consistent with a fusion-facilitating

535   protein. One possibility is that while $Wdr35^{-/-}$ MEFs are missing one COPI α/β'- homolog, the

536   other three core IFT-As (IFT144, IFT140, and IFT122) may be sufficient to compensate by

537   providing interaction motifs necessary for the fission of vesicles from donor membranes such

538   as the Golgi. Indeed, we show IFT122 to be upregulated in $Wdr35^{-/-}$ mutant cells, similar to

539   previous reports in $WDR35$ patient cells (Duran et al., 2017). However, we and others have

540   demonstrated that in the absence of WDR35, the IFT-A complex is unstable (Zhu et al., 2017)

541   such that any core IFT-A coat on the vesicles from donor membranes such as the Golgi may

542 be easily disassembled. It is interesting to note that non-core IFT139 and IFT43 are helical

543 (Taschner et al., 2012) similar to SNARE proteins that mediate vesicle fusion with target

544 membranes. Importantly, we show here that these components, which are degraded in the

545 absence of WDR35, could help mediate the fusion of vesicles with the ciliary pocket or base

546 to transfer membrane cargos into the growing cilia sheath. Indeed, we show that purified

547 non-core IFT-A complex is sufficient to specifically bind phosphatidic acid (PA), which is

548 present in ciliary membranes, as well the Golgi and the recycling endosome compartment

549 (Farmer et al., 2020; Lechtreck et al., 2013; Yang et al., 2008). The lipid composition of

550 membranes is known to determine their curvature (McMahon and Boucrot, 2015); PA being

551 conical in shape, concentrates on more curved regions of membranes resulting in nanoscopic

552 negative curvature such is found in the ciliary pocket (Zhukovsky et al., 2019). Moreover, with

553 a small head group, negative charge, and a phosphomonoester group, PA interacts with

554 proteins and lipids in several subcellular compartments that facilitate fission and fusion of

555 membranes (Zhukovsky et al., 2019). From our liposome assay, we speculate that IFT139

556 binding to the IFT121/43 dimer increases the binding affinity to lipids. Indeed, on its own, the

557 IFT-A dimer signal is below the threshold of detection in the protein-lipid overlay assays but

558 observed to weakly associate to PA-containing liposomes. In our purification of the non-core

559 IFT-A complex with the affinity tag on IFT43, only IFT43/121, rather than IFT43/139, was co-

560 purified together with the trimeric complex, indicating that IFT121 interacts with both IFT43

561 and IFT139, and is responsible for mediating the interactions between IFT43 and IFT139,

562 which is consistent with what has been previously reported (Behal et al., 2012; Zhu et al.,

563 2017). In the *Wdr35*^{-/-} mutant, and likely IFT139 or IFT43 KO strains, the non-core IFT-A

564 complex will not form, which leads to non-coated vesicles (*Figure 9*). Important next steps

565 will be to systematically investigate vesicular traffic defects in other IFT-A mutants, as well as

566 identify the GTPase which acts to drive formation, uncoating and fusion of these vesicles.

567   Recruitment, remodeling, and regulation of protein coats involve cycles of GTP

568 hydrolysis, for example ARF1 regulates recruitment to membranes of the COPI coatomer

569 (Dodonova et al., 2017). It is interesting to note that we and others have been unable to

570 purify IFT-A complex with any GTPases (Mukhopadhyay et al., 2010), suggesting that any

571 interaction is transient. This is even in conditions where we can purify endogenous IFT-B

572 complexes with its associated GTPases IFT22/RABL5 and IFT27/RABL4. In COPI, recruitment

573 of coat components to donor membranes starts with the insertion of small GTPase ARF1 into

574 membranes (Dodonova et al., 2017). So far only one ARF, ARF4 acting at the TGN (Mazelova

575   et al., 2009; Wang et al., 2017) has been implicated in ciliary traffic. However, it plays non-

576   ciliary roles, and shows early lethality in mouse knock-outs without affecting cilia assembly

577   (Follit et al., 2014). Mutations in several related ARLs have defects in cilia structure and/or

578   content, including ARL3, ARL6 and ARL13B (Alkanderi et al., 2018; Cantagrel et al., 2008; Fan

579   et al., 2004). At least in the case of ARL13B and ARL3, they fail to accumulate and/or enter

580   mutant cilia, even when overexpressed in the absence of WDR35, although periciliary

581   vesicular staining can be observed. Rab GTPases have been implicated in the ciliary targeting

582   of vesicular cargos (Blacque et al., 2018). Notably, expression of dominant negative RAB8 in

583   *Xenopus* photoreceptors (Moritz et al., 2001) results in a strikingly similar accumulation of

584   vesicles to our *Wdr35* mutants which fail to fuse with the ciliary base. Similarly, in RPE-1 cells,

585   dominant negative RAB8 impairs traffic of ciliary membrane cargos (Nachury et al., 2007).

586   However, functional redundancy between RABs may exist as neither single nor *Rab8a;Rab8b*

587   double mutant mice have defects in cilia formation. On the other hand, defects in ciliation

588   were observed when *Rab10* was additionally knocked down in *Rab8a;Rab8b* double mutant

589   cells (Sato et al., 2014). Excitingly, our work demonstrates IFT-As to be important for the later

590   stage of ciliogenesis, similar to GTPases like RAB23 (Gerondopoulos et al., 2019) or RSG-1

591   (Agbu et al., 2018; Toriyama et al., 2016). Given that these GTPases have also been shown to

592   sequentially interact with CPLANE subunits INTU and FUZ, which are also required for IFT-A

593   holocomplex assembly (Gerondopoulos et al., 2019; Toriyama et al., 2016), they will be

594   priorities for future investigations.

595   We have demonstrated that an IFT-A-dependent coat for membrane vesicles exists

596   and is necessary for their fusion with the ciliary sheath and ciliary pocket, which is continuous

597   with the ciliary membrane. We also showed that this coat is necessary to efficiently deliver

598   cilia-destined signaling molecules into the elongating axoneme of the cilium. This raises the

599   possibility that some of this IFT-A dependent coat may remain upon vesicle fusion as a now

600   linear 'train' carrying membrane cargos to be picked up by cytosolically assembling IFT-B

601   particles allowing import across the transition zone and then anterograde IFT within the

602   cilium (**Figure 9, insert B**). Excitingly, we show that purified non-core IFT-A including WDR35

603   is sufficient to bind selectively to phosphatidic acid (PA). This low abundance signalling lipid

604   has well-described roles in vesicle traffic through where it promotes COPI vesicle fission in

605   the Golgi (Yang et al., 2008), maintenance of the endosome recycling compartment (ERC)

606   (Farmer et al., 2020) as well as promoting exocytosis through formation of fusion competent

607   granules (Zeniou-Meyer et al., 2007). Defining at which points in vesicular traffic IFT-A

608 dependent coats act, both fission and fusion, within cells as well as the biochemical nature of

609 lipids and cargos these vesicles carry will be required. Given its efficacy, this IFT-dependent

610 'targeted delivery' module may also be repurposed for other non-ciliary membrane targeting

611 events via polarized exocytosis. Notably in the immune synapse of T cells, where IFT20 is

612 required for rapid clustering of TCRs necessary for T cell activation (Finetti et al., 2009) as well

613 as photoreceptor dendrites in which IFT localization to vesicles tracking towards the

614 postsynaptic membranes was observed (Sedmak and Wolfrum, 2010), where dendritic

615 exocytosis is implicated synaptic plasticity and neuronal morphology (Kennedy and Ehlers,

616 2011). Future studies into this IFT-dependent coat complex and the membrane traffic

617 processes it controls may expand our phenotypic understanding of the ciliopathies beyond

618 the cilium.

619

Material and Methods

Preparation of primary MEFs, cell culture, ciliation and genotyping

622 Primary MEFs were harvested from E11.5 embryos and cultured in complete media [Opti

623 MEM-I (Gibco, 31985-047) supplemented with 10 % foetal calf serum (FCS) and 1 % penicillin-

624 streptomycin (P/S) and 0.026 µl β-mercaptoethanol] and incubated at 37 ℃ in a hypoxic

625 incubator (3 % O_2 and 5 % CO_2). To induce ciliogenesis, 70-80 % confluent cells were serum-

626 starved for 24 h. Genotyping was done as described before for the *Wdr35* line (Mill et al.,

627 2011) and *Dync2h1* line (Caparrós-Martín et al., 2015). *Pcm1-SNAP* mouse line was made by

628 Dr. Emma Hall (Hall E. et al., unpublished) by endogenous tagging of PCM1 by CRISPR.

629 $Pcm1^{SNAP}$ mouse line was crossed with $Wdr35^{-/+}$ and genotyped to screen E11.5 embryos

630 homozygous for both $Wdr35^{-/-}$ and $Pcm1^{SNAP/SNAP}$. MEFs prepared from these embryos were

631 used to image PCM1 localization in WT and $Wdr35^{-/-}$ using antibodies and other reagents

632 listed in **Table 1**.

633

Electroporation of MEFs

635 Cells were trypsinized to a single-cell suspension and resuspended in 10 µl Resuspension

636 Buffer R per 0.5×10^5 cells/transfection reaction, mixed with plasmid DNA (0.75

637 µg/transfection) **Table 3** and electroporated (voltage 1350 V, width 30 ms, one pulse) using a

638 Neon Nucleofection kit (Thermo Fisher Scientific MPK-1096), according to the manufacturer's

639 protocol. Transfected cells are harvested or visualized 24-48 h post electroporation.

640

Live Cell Imaging

641

Primary MEFs (0.5 x 10^5 cells/transfection) were electroporated with *ARL13B-EGFP* or

642

Smoothened-GFP using the Neon™ Transfection System, 10 µL Kit (Thermo Fisher Scientific,

643

MPK-1096) and seeded in 24 well glass-bottomed plates (Greiner Sensoplates, 662892) with

644

pre-warmed media (Opti-MEMI ® (1X) (Gibco, 31985-047), 10 % foetal calf serum (FCS) and

645

0.026 µl β-mercaptoethanol). Samples were incubated in antibiotic-free media 37 ℃/ 5% CO_2

646

/ 3% O_2 overnight and then serum-starved for 24 h. SiR-tubulin kit (Spirochrome, SC002), a 1

647

mM stock solution, was prepared in anhydrous DMSO and stored at -20 ℃, without

648

aliquoting. For staining, 1:5000 (200 nmol) of SiR-tubulin stock was diluted in serum-free

649

media, and added to cells for 1 h in the hypoxic incubator, then live imaged without washing.

650

For live-cell PCM1 imaging, MEFs electroporated with *ARL13B-EGFP* were incubated with

651

1:1500 TMR-SNAP (New England BioLabs, S9105S, stock 30 nmol) in low serum media in the

652

hypoxic incubator for 30 min. Cells were washed twice with low serum media for 1 h each in

653

the incubator. Samples were then incubated for 1 h in 1:5000 SiR-tubulin (200 nmol). Hoechst

654

344442 (Thermo Fisher Scientific, H1399) was added 10 min before imaging. Plates were

655

allowed to equilibrate in the Okolabs stage top incubator before confocal imaging on the

656

Leica SP5 using the LAS-AF software, 405 nm diode, Argon and 561 and 648 nm laser lines,

657

three Photomultiplier tubes, and one HyD GaSP detector, as per the requirement of the

658

experiment. Images were scanned using a 63X 1.4 NA oil immersion objective and processed

659

using ImageJ and Imaris software.

660

661

662

Endogenous IFT IPs

663

Embryos were lysed and homogenized in IP lysis buffer (10 µl/mg) at 4 ℃ on a rotator for 30

664

min. Composition of IP lysis buffer is (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 % glycerol,

665

0.5 mM EDTA, 0.5 % IGEPAL, and 1/100 Halt protease and phosphatase inhibitor (Thermo

666

Fisher Scientific, 78443) and a tablet of protease inhibitor tablet - 1 tablet per 10 ml

667

(Complete Mini, Roche, 11836170001). The lysate was cleared by spinning at 4 ℃, 14,000

668

rpm, for 20 min. The protein concentration was determined using the BCA Protein Assay Kit

669

as per manufacturer's instruction (Thermo Fisher Scientific, 23225). For each IP, 500 µg of

670

protein was incubated with 3 µg of each antibody overnight at 4 ℃ (**Table 1**) with mild

671

agitation (side-to-side). Immunoprecipitation of immunocomplexes was done using

672

673   PureProteome™ Protein G magnetic beads (Millipore LSKMAGG10). 30 µl beads/IP were

674   equilibrated with 500 µl IP lysis buffer by gentle agitation for 5 min at 4 ℃. Tubes were

675   placed on a magnet for 2 min, and the buffer was aspirated off with the fine pipette. 200 µl

676   antibody-lysate mix was added to each tube of 30 µl equilibrated beads and incubated for 45

677   min with agitation, to concentrate immunoglobulin complexes on beads at 4 ℃. Washes (8

678   times) were performed, each lasting 5 min. Washes were as follows: 2x washes in Wash

679   Buffer-1 (same as IP lysis buffer), followed by 2x washes with Wash Buffer-2 (IP lysis buffer

680   with reduced 0.2 % IGEPAL), finally 4x washes with Wash Buffer-3 (IP lysis buffer without any

681   IGEPAL detergent). All wash buffer is aspirated, and dry beads were stored at -80 ℃, or

682   samples were sent immediately for mass spec.

683

684   **Mass spectrometry**

685   All mass spectrometry experiments were done at the IGMM Mass Spectrometry facility, as

686   per their published protocol (Turriziani et al., 2014). Briefly, the immunocomplexes collected

687   on magnetic beads were processed to generate tryptic peptides. Proteins were eluted from

688   beads by incubating at 27 ℃ for 30 min in elution buffer (2 M urea, 50 mM Tris-HCl pH 7.5

689   and 5 µg/mL trypsin). The sample was centrifuged, bead pellets washed twice and the

690   supernatant from samples digested overnight at room temperature. Iodoacetamide was

691   added to the samples to inhibit disulfide bond formation and incubated for 30 min in the

692   dark. Followed this, trifluoroacetic acid (TFA) was added to stop tryptic digestion. Desalting

693   and pre-fractionation of the digested peptides were done by manually using C18 pipette

694   stage-tips filled with 3M Empore disc activated with 50 % acetonitrile and 0.1 % TFA and then

695   washed once with 0.1 % TFA. The peptide mixtures were passed manually along to the

696   column with a syringe to concentrate and purify the analytes. Peptides were subsequently

697   eluted twice in 50 % acetonitrile and 0.1 % TFA and both eluates were combined. Samples

698   were concentrated and resuspended in 0.1 % TFA. This was followed by chromatographic

699   separation on a Reprosil column along a 3-32 % acetonitrile gradient. The LC setup was

700   attached to a Q-Exactive mass spectrometer, and ion mass spectra were obtained following

701   HPLC during a tandem MS run. Mass spectra were analyzed using MaxQuant software. Label-

702   free quantification intensity (LFQ) values were obtained for analysis by identifying

703   mass/charge ratio, and their intensities at a specific elution time for individual peptides. The

704   data was collected for both control (GFP) and specific proteins IPs (i.e., IFT88, IFT140 - **Table**

705   **1**). LFQ values for the proteins were obtained by summing the ion intensities corresponding

706   to peptides after assigning the unique peptides to proteins. The ratio of LFQ intensities of

707   test: control was taken, where higher the ratio better corresponds to a better enrichment of

708   protein in complex. Complete mass spec data is available on ProteomeXchange (*identifier*

709   *PXD022652*). The relative concentration of IFTs was calculated after normalizing the

710   individual test values with respective GFP- LFQs, as shown in the figures.

711

712   **Western Blots**

713   Cells or tissues were lysed in 1X Cell Lysis Buffer with the addition of 1/100 Halt protease and

714   phosphatase inhibitor (Thermo Fisher Scientific, 78443) and a Complete protease inhibitor

715   tablet, 1 tablet per 10 ml (Complete Mini, Roche, 11836170001). Prepare 1X Cell Lysis Buffer

716   by diluting 10X stock in ddH$_2$0 (Cell Signalling Technology (10x #9803): 20 mM Tris-HCl (pH

717   7.5), 150 mM NaCl, 1 mM Na$_2$ EDTA, 1 mM EGTA, 1 % Triton-X100, 2.5 mM sodium

718   pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na$_3$VO$_4$, 1 μg/ml leupeptin). The lysate

719   from embryos was homogenized at 4 ℃ for 30 min and from cells was sonicated briefly (5x,

720   10-sec pulses, Bioruptor Diagenode) to lyse the tissue or cells. The lysate was centrifuged at

721   14,000 g at 4 ℃ for 30 min and the supernatant transferred to a fresh tube. Ready-to-use

722   SDS-PAGE gels (NuPage Novex precast gels, Thermo Fisher Scientific) were used to separate

723   proteins.

724   The resolved proteins on the gel were transferred to PVDF (Hybond P, GE HealthCare) using

725   the XCell II Blot module as per manufacturer's instruction. The membrane was then blocked

726   with a 10 % solution of dried skimmed milk (Marvel Premier Foods) made in 1X TBST (0.05 %

727   Tween-20 in TBS) for 1 h RT, washed with PBS and incubated with primary antibody (**Table 1**)

728   diluted in 1 % skimmed milk solution in 1X TBST overnight at 4 ℃ on shaker/roller.

729   Membranes were washed in 1X TBST 3X, 10 min followed by a 1X wash with PBS, and

730   incubated in HRP-conjugated secondary antibody from appropriate species (**Table 2**) for 1 h

731   at RT, diluted in a solution of 1X TBST and 1 % milk. Blot was then washed with 1X TBST, three

732   times and with PBS twice. After the washes, signals were detected by the Super Signal ELISA

733   Femto kit (Thermo Fisher Scientific, 37074) or Super Signal ELISA Pico kit (Thermo Fisher

734   Scientific, 37069). Protein bands were visualized digitally by transmission light imaging on

735   ImageQuant LAS 4000 (GE HealthCare) and analyzed using ImageQuant TL software. Protein

736   bands on blots were quantified with ImageJ/FIJI software by measuring individual bands

737   intensity and normalizing intensities with loading control bands on the same blot.

738

Immunofluorescence

Cells were washed two times with warm PBS, then fixed in either 4 % PFA in 1X PHEM/PBS 15 min at room temperature, 2 % fresh glutaraldehyde in 1X PHEM for 15 min, or pre-extracted for 30 s on ice in PEM (0.1 M PIPES pH 6.8, 2 mM EGTA, 1 mM $MgSO_4$) prior to fixing in ice cold methanol on ice for 10 minutes according to **Table 1**, then washed twice with PBS. 1X PHEM (pH 6.9) contains: 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 4 mM $MgSO_4 \cdot 7H_2 0$). The cells were treated twice with 50 mM NH_3Cl for 15 min each for PFA fixed cells, or 0.01 mg of $NaBH_4$ in 1X PBS for 7 min for glutaraldehyde fixed cells to quench autofluorescence. Cells were then washed twice with PBS. Cells were permeabilized with 0.25 % Triton-X 100/TBS for 10 min at room temperature. Cells were rinsed twice in 1X TBS for 5 min. Blocking for non-specific binding was done by incubating samples in 10 % donkey serum in 0.2 % Tween-20/TBS for 60 min at room temperature. Samples were washed twice with PBS. Primary antibodies (**Table 1**) were added to samples and incubated for 60 min at room temperature or 4 ℃ overnight, in dilutant made of 1 % donkey serum in 0.025 % Triton X-100/TBS. Samples were washed in 0.25 % Triton-X 100/TBS 4-6 times, 10 min each. Secondary antibodies diluted in 1 % donkey serum and 0.025 % Triton X-100/TBS were incubated on samples for 60 min room temperature. Samples were washed with 0.25 % Triton-X 100/TBS 4-6 times 10 min, stained with DAPI (1:1000) in PBS for 5 min at room temperature, again washed with PBS and directly imaged or coverslips were added on slides using ProLong Gold antifade (ThermoFisher Scientific), according to the manufacturer's instructions. Confocal imaging for was done on a Leica SP5 using the LAS-AF software, 405 nm diode, Argon and 561 and 648 nm laser lines, three Photomultiplier tubes, and one HyD GaSP detector, as per the requirement of the experiment. Images were scanned using a 63X 1.4NA oil immersion objective and latter processed using ImageJ and Imaris software.

IFT-A sequence homology search and structural modelling

The sequence match of IFT-A proteins was found by iterative rounds of homology searches via alignment for sequence proximity-based clustering as described before (Wells et al., 2017; Wells and Marsh, 2019). Further Swiss Model server was used to model IFT-A complex protein structures as described on the server (Waterhouse et al., 2018). Briefly, a template search with BLAST and HHblits was performed against the SWISS-MODEL template library. The target sequence was searched with BLAST against the primary amino acid sequence contained in the SMTL. An initial HHblits profile, followed by one iteration of HHblits against

772   NR20, was run and the obtained profile then searched against all profiles of the SMTL. The

773   top hit in all of IFTA searches was 3mkqA (Lee and Goldberg, 2010), a coatomer β' subunit 2.5

774   Å X-ray structure with 14 % - 20 % sequence identity and 25 % - 30 % sequence similarity with

775   different IFT-A proteins. A coatomer α subunit was also found within these top matches.

776   Models were built on the target-template alignment using ProMod3. Coordinates that are

777   conserved between the target and the template were copied from the template to the

778   model. Insertions and deletions were remodelled using a fragment library. Side chains were

779   then rebuilt. Finally, the geometry of the resulting model was regularized by using a force

780   field. In case loop modeling with ProMod3 fails, an alternative model was built with

781   PROMOD-II. The global and per-residue model quality has been assessed using the QMEAN

782   scoring function. The obtained model was processed later in Pymol software for structural

783   analysis.

784

785   **Cloning, expression and purification of the _Cr_ IFT-A trimeric (IFT139/121/43)**

786   **complex from mammalian cells**

787   The codon-optimized sequences for _Chlamydomonas reinhardtii_ IFT-A trimeric complex

788   (UniProt accession codes: IFT43_A8HYP5, IFT121_A8JFR3 and IFT139_A9XPA6) were

789   assembled into a single construct for expression in mammalian cells. The _IFT43_ gene was

790   fused to TEV cleavable His-GFP-tag at the N-terminus for affinity purification and inserted

791   into pAceCMV vector while the _IFT139_ and _IFT121_ subunits were untagged. The

792   pAceCMV_His-GFP-IFT43, pIDC_IFT121 and pIDK_IFT139 were fused using an _in vitro_ Cre

793   recombinase (New England Biolabs) by the LoxP sites in the vectors to form the IFT-A trimer

794   construction.

795

796   Large-scale transient expression of the IFT-A trimeric complex in mammalian HEK293S cells

797   was carried out by transfection of the IFT-A trimer construct using PEI (40 kDa linear

798   polyethylenimine, 1 mg/ml stock in water). Before transfection, sterile and high-quality DNA

799   was prepared using a NucleoBond Maxiprep Kit (MACHEREY-NAGEL) with 200 ml overnight

800   culture of DH5α cells containing the construct. HEK293S cells were cultivated one day before

801   the transfection in medium (FreeStyle™ 293 Expression Medium, Thermo-Fisher) with 1 %

802   FBS and 1 % penicillin/streptomycin. Cultures were incubated in a humidified incubator with

803   5 % CO_2 at 37 °C with 130 rpm shaking to let the cells grow. The cells were diluted to 1.1 x 10^6

804   cells/ml before transfection with fresh and warm medium. The transfection mixtures were

805   prepared using a sterile flow bench. For expression in 1 L of HEK293S cells, 1000 µg IFT-A

806   trimer DNA were diluted into 25 ml medium without antibiotics or FBS. In another tube, 3000

807   µg PEI were diluted in 20 ml medium and added to the diluted DNA dropwise. The mixture

808   was incubated at room temperature for 5 min to let the PEI-DNA transfection complex form.

809   The mixture was added dropwise to cells and mixed gently by swirling the flask. Cells were

810   incubated at 37 °C in a CO_2 incubator for 48 h. The cells were harvested by centrifugation at

811   800 x g for 10 min at 4 °C, and the cell pellet was flash-frozen in liquid nitrogen and stored at

812   -80 °C until use.

813

814   The IFT-A trimeric complex was purified using the His-tag on the IFT43 for affinity

815   chromatography. Briefly, a frozen pellet from 1.5 L of HEK293S cell culture was thawed on ice

816   and resuspended in lysis buffer (50 mM HEPES pH 7.4, 250 mM NaCl, 2 mM $MgCl_2$, 10 % (v/v)

817   glycerol and 5 mM β-mercaptoethanol) supplemented with 1 µl DNase and one Complete

818   Protease Inhibitor tablet (Complete-EDTA Free Protease Inhibitor tablet, Roche Applied

819   Science) to a final volume of 20 ml. Cells were lysed in a dounce-type tissue grinder

820   (Wheaton) using 30 strokes. The cell lysate was cleared by centrifugation at 48,000 x g for 45

821   min at 4 °C. The clarified supernatant was loaded onto a 1 ml TALON column (HiTrap, Cytiva)

822   pre-equilibrated with lysis buffer at 4 °C. The bound protein was washed with 15 mM

823   imidazole in Q_A buffer (20 mM Tris–HCl pH 7.5, 10 % glycerol, 50 mM NaCl and 5 mM β-

824   mercaptoethanol), followed by elution with 150 mM imidazole in Q_A buffer. The elution

825   containing the IFT-A proteins was loaded onto a 5 ml Q column (HiTrap Q FF, Merck-

826   Millipore), and the bound IFT-A proteins were eluted in Q_A buffer with a 50-500 mM gradient

827   of NaCl. The elution fractions containing the IFT-A proteins were concentrated to 500 µl in a

828   100 kDa molecular weight cut-off concentrator (Amicon Ultracel, Merck-Millipore) for

829   subsequent Size Exclusion Chromatography (SEC) on a pre-equilibrated Superose 6 Increase

830   column (10/300 GL, Merck-Millipore) in SEC Buffer (10 mM HEPES pH 7.5, 150 mM NaCl, 2

831   mM $MgCl_2$, 1 mM DTT, 5 % glycerol). The SEC peak fractions were analyzed by SDS-PAGE and

832   resulted in the sample used in *Figure 5B* and *C*.

833

834   **Cloning, expression, and purification of *Cr* His-IFT43, His-IFT43/121 and His-**

835   **IFT43/121/139 from insect cells**

836   DNA sequences encoding for the *IFT43* with an N-terminal TEV cleavable hexa-histidine tag

837   and untagged *IFT121* were cloned into the two expression cassettes of the pFL vector. The

838   gene encoding for *IFT139* was cloned in another pFL vector.

839

840   The expression and purification of His-IFT43, His-IFT43/121 complex and His-IFT43/121/139

841   complex was performed as previously described for the CrODA16 protein (Taschner et al.,

842   2017) with the following modifications. Two recombinant baculoviruses for IFT139 and

843   IFT43/121 were generated from separate constructs. The expression was carried out in sf21

844   suspension cells by co-infection with these two recombinant baculoviruses. After 3 days of

845   incubation at 27 °C, the cells were harvested by centrifugation.

846

847   The His-43, His-IFT43/121 and His-IFT43/121/139 were purified using a similar purification

848   procedure to that for the His-GFP-tagged IFT-A trimer describe above, and were purified by

849   Ni-NTA affinity, ion-exchange and size exclusion chromatography. The SEC fractions

850   containing His-tagged IFT-A proteins were used for the lipid overlay assays in *Figure 5- figure*

851   *supplement 1B-D*.

852

853   The purified His-IFT43/121 and His-IFT43/121/139 were digested overnight using TEV

854   protease for removal of His-tag. The resulting IFT-A proteins was loaded onto SEC, and

855   fractions containing untagged IFT-A proteins were further used for the binding assay with

856   liposomes in *Figure 5D.*

857

858   **Lipid overlay assay**

859   To detect the direct binding between non-core IFT-A complexes and lipids, the His-GFP

860   tagged IFT-A trimeric complex or His-tagged proteins purified from insect cells and

861   Membrane Lipid Strips (Echelon Biosciences, P-6002) with 100 pmol of fifteen different lipids

862   were used following the manufacturer's protocol. The strips were blocked in 3 % (w/v) BSA in

863   TBS-T buffer (50 mM Tris (pH 7.4), 150 mM NaCl, and 0.1 % (v/v) Tween 20) at 4 °C overnight

864   in dark with gentle agitation. After blocking, they were washed in TBS-T buffer three times

865   and 5 min each, followed by incubation at room temperature for 1 h with IFT-A proteins in

866   SEC buffer supplemented with 3 % (w/v) BSA. The strips were washed three times in TBS-T as

867   before and soaked in 3 % (w/v) BSA in TBS-T with primary antibody against His-tag (THE His

868   Tag Antibody, Mouse, GenScript) at a 1: 2,500 dilution for 1 h at room temperature. Strips

869   were washed three times and incubated with Horseradish peroxidase (HRP)-conjugated

870   polyclonal rabbit anti-mouse immunoglobulins (1: 1,000 dilution, Dako) for 1 h followed by

871   three TBS-T washes. An ECL Prime Western Blotting reagent (Amersham) was used as the

872   substrate for the horseradish peroxidase, and the binding of IFT-A proteins onto spotted

873   lipids was recorded with the ChemiDoc imaging system (BioRAD).

874

875   **Negative stain EM**

876   The POPC-liposomes and PA containing liposomes (PE/PG/PA) were purchased from T&T

877   SCIENTIFIC CORP. The liposomes (PE/PG/PA) have a similar phospholipids composition to that

878   of *Chlamydomonas* ciliary membrane as reported previously (Lechtreck et al., 2013). The

879   percentage of PA was 11.36 % while the ratios of PE and PG as the framework of liposomes

880   were re-quantified to 63.18 % and 25.46 % respectively.

881

882   To observe the binding between IFT-A complexes with liposomes, the liposomes (PE/PG/PA,

883   0.20 mM) were applied to homemade carbon grids directly or after incubation with IFT-A

884   complexes (untagged IFT139/121/43 trimer or IFT121/43 dimer, 0.25 µM) at 25 °C for 10 min.

885   3 µl of the sample were applied to the plasma-cleaned grids for 30 sec before it was blotted,

886   and the sample was stained with 2 % (w/v) uranyl-format staining by applying 3 µl of stain

887   three times on the grids. The negative stain grids were imaged on an FEI Tecnai G2 Spirit

888   Transmission Electron Microscope (TEM) operated at 120 kV with a 67,000 X nominal

889   magnification corresponding to the digital pixel size of 1.59 Å/pixel. The electron micrographs

890   were recorded on a water-cooling 4k CMOS CaMeRa (TemCam-F416). The mixture of IFT-A

891   trimer (0.10 µM) with POPC liposomes (0.20 mM), as a negative control, was checked using

892   negative staining EM by following the same procedure.

893

894   **Transmission electron microscopy**

895   <u>TEM sample preparation:</u> 24 h serum-starved MEFs were chemically fixed for flat embedding

896   using the following protocol: (1) Cells were grown on 60 mm dishes, and ciliogenesis was

897   induced by serum starvation for 24 h. (2) For prefixation under culture conditions, 25 %

898   glutaraldehyde was added to the growth medium to a final concentration of 1 %, mixed

899   gently, and incubated for a few min at 37 ℃. (3) The growth medium (containing the

900   glutaraldehyde) was replaced with a sample buffer (0.1 M HEPES, 4 mM $CaCl_2$, pH 7.2)

901   containing 2 % glutaraldehyde and incubated 1 h at room temperature (replacing the fixation

902   buffer with fresh one after 20 min). All prefixation solutions were pre-warmed to 37 ℃, and

903   all steps were done at 37 ℃, to preserve the cytoskeleton. (4) The fixation buffer was

904   replaced with fresh fixation buffer and incubated for 4 h at 4 ℃. (5) After that, the sample

905   was washed once in sample buffer and 2–3 times in distilled water, each for 5–10 min, gently

906   removing and replacing the buffer. (6) Samples were incubated in 1 % OsO_4 (EMS) (in distilled

907   water) for 1 h at 4 ℃, (7) washed 3–4 times for 10 min each in distilled water, and (8)

908   incubated in 1 % uranyl acetate (EMS) in distilled water overnight at 4 ℃. (9) Then, samples

909   were rinsed 3–4 times for 10 min each in distilled water and (10) dehydrated using a

910   graduated series of ethanol: 30 %, 50 %, 70 %, 80 %, 90 %, 96 % ethanol, 5 min each step at 4

911   ℃, followed by twice rinsed in anhydrous 100 % ethanol 10 min each at RT. (11) Infiltration

912   was performed using a 1:1 mixture of LX112 (Ladd Research, USA; EMS) and ethanol 2 h,

913   followed by pure LX112 overnight and another 2 h pure LX112, where all steps were

914   performed at room temperature. (12) Flat embedding: For flat embedding, the caps of the

915   BEEM embedding capsule (size, #3, EMS) were cut off and capsules filled with LX112. The

916   capsules were inverted over a selected area of the cell monolayer in the dish, and the resin

917   cured at 60 °C oven for 48 h. The capsule was then removed by breaking off from the dish,

918   leaving the monolayer cells embedded in the surface of the block. (13) Sectioning and post-

919   staining: For sectioning and post-staining, 300 nm thick serial sections were cut by Leica

920   Ultracut UCT (Leica microsystem, Wetzlar, Germany) with a diamond knife and sections

921   picked up with a Formvar (EMS) coated 1x2 mm slot copper grid (EMS). Sections were post-

922   stained with 2 % uranyl acetate for 10 min, then with lead citrate for 5 min. Imaging: Sections

923   were stained on the grid with fiducials (15 nm gold nanoparticles, Sigma-Aldrich). 70 nm thick

924   sections were cut for regular TEM imaging, and 300 nm thick sections were prepared for

925   tomographic acquisition.

926   Tilt series were acquired on a Tecnai F30 (FEI) transmission electron microscope, operated at

927   300 kV, and equipped with 2048x2048 Gatan CCD camera and FEI Titan Halo transmission

928   electron microscope operated at 300 kV equipped with a field emission gun (FEG) and a

929   Gatan K2 direct detector. The SerialEM software (Mastronarde, 2005) was used for automatic

930   acquisition of double tilt series. Tomographic tilt series were recorded with a pixel size of

931   1.235 nm on Titan Halo and 1.178 nm on F30, a maximum tilt range of about 60°, and tilt

932   steps of 1°. Tomographic reconstruction, joining of tomograms from consecutive sections,

933   segmentation, and visualization of the tomograms was done using the IMOD software

934   package (Kremer et al., 1996). In wild type cells, very few vesicles are observed around the

935   base of the cilia which mostly have electron-dense coats with a range of intensities, including

936   those observed fusing with the ciliary pocket or sheath. For simplicity, we have labeled these

937   as 'coated' and coloured them magenta. By comparison, the accumulation of vesicles around

938   the base of *Wdr35* mutant cilia generally lack prominent densities around them. For

939   simplicity, we have labeled these as 'coatless' and coloured them cyan, as whether coats

940   previously existed but disassembled or whether they failed to form in the first place is not

941   clear from our study.

942   24 h serum-starved WT, *Wdr35*^{-/-}, and *Dync2h1*^{-/-} cells were serially sectioned parallel

943   to the adherent surface. Two to four 300nm parallel serial sections are required to get the

944   whole 3D volume ultrastructural view covering full cilia and their cellular surroundings. We

945   reconstructed 45 tomograms to get a minimum of 3-4 whole cell volumes for each genotype.

946   We took micrographs of 30 WT, 20 *Wdr35*^{-/-}, and 30 *Dync2h1*^{-/-} cells for this study.

947

948   **CLEM (correlative light and electron microscopy)**

949   WDR35-EmGFP and ARL13B-mKate expressing *Wdr35*^{-/-} MEFs were serum-starved for 24 h,

950   stained with Hoechst 33342 (R37605) for 10 min in culture condition, fixed with 4 % PFA and

951   0.1 % GA in 1X PHEM and imaged on Zeiss LSM 880 upright single photon point scanning

952   confocal system with Quasar detector (32 spectral detection channels in the GaAsP detector

953   plus 2PMTs) and transmitted light detector, Airy scan detector for high-resolution imaging.

954   Cells were grown on 35mm glass bottom dishes with grids (Cat. No. P35G-1.5- 1.4-C- GRID)

955   and firstly brightfield images were made with Plan-Apochromat 10X/0.45 M27 objective to

956   save the coordinates of cells needed for the correlation with the respective TEM data.

957   Confocal and airy scan imaging was done using Plan-Apochromat 63x/1.4 oil DMC M27

958   objective, 405 nm laser diode, 458, 477, 488, 514 nm multiline integrated Argon laser and

959   594 nm integrated HeNe laser. Z-stack was acquired sequentially to get the whole 3D volume

960   of the cell and the image was further deconvolved using the inbuilt software. After Airy scan

961   imaging the sample was processed for TEM as described above. 70 nm sections were made

962   for the regions of saved coordinates from brightfield imaging, mounted on grids and imaged

963   on FEI Morgagni TEM (100kV) microscope.

964

965   **Immunogold labeling**

966   *Wdr35*^{-/-} MEFs expressing WDR35-EmGFP and ARL13B-mKate2 (**Table 2**) were serum-starved

967   for 4 h. MEF cells were grown on 6 mm sapphire disks (Wohlwend GmbH, Switzerland, 1292)

968   and high pressure frozen (EM ICE, Leica Microsystems, Germany). The frozen samples were

969   processed by freeze substitution in a Leica AFS2 temperature-controlling machine (Leica

970   Microsystems) using 0.01 % uranyl acetate (Polyscience Europe GmbH, 21446) and 4 % water

971 in glass distilled acetone (EMS, E10015) as freeze substitution medium and then embedded in

972 Lowicryl HM-20(Polysciences, 15924-1). 70 nm thick serial sections were sectioned on a Leica

973 Ultracut UCT ultramicrotome (Leica Microscopy systems). Sections were labelled with anti

974 GFP antibody, 1:20 (Abcam, ab6556- **Table 1**) followed by secondary goat anti rabbit

975 antibody coupled to 10-nm gold, 1:30 (BBI Solutions, Batch 008721 -Table 2). Before antibody

976 staining, grids were incubated twice section side for 10 min each on blocking buffer PBG (0.5

977 % BSA/ 0.1 % fish skin gelatin in PBS). Following blocking, grids were incubated for 1 h in

978 primary-Ab/PBG in a wet chamber, given five 2 min washes with PBG and incubate for 1 h in

979 secondary-Ab/PBG. Grids were washed five times for 2 min with PBG, followed by five 2 min

980 washes with PBS. Antibodies were subsequently fixed for 1 min 0.1 % glutaraldehyde/PBS,

981 followed by five 2 min washes with PBS and five 2 min washes with H_2O. After immunogold

982 labelling, the sections were stained with 1 % uranyl acetate (Polyscience Europe GmbH,

983 21446) in water for 8 min and 0.04 % lead citrate (EMS, 17800) for 5 min. The sections were

984 imaged using Tecnai 12 (Thermo Fisher Scientific, formerly FEI/ Philips) at 100kV with TVIPS

985 F214 and F416 cameras (TVIPS, Gauting, Germany).

986

987 **Image analysis and measurements**

988 All image processing was performed using FIJI (Schindelin et al., 2012). Macros for

989 quantification of PCM1 (RadialIntensityFromCentrosomes.ijm) and clathrin

990 (3DMeanIntensityfromUserDirectedPoints.ijm) can be found on GitHub

991 (https://github.com/IGMM-ImagingFacility/Quidwai2020_WDR35paper). To measure PCM1

992 intensity radially from the centrosomes, an average intensity projection of the z-stack was

993 obtained, and the gamma-tubulin signal was segmented using RenyiEntropy threshold and

994 the Analyze Particles tool to obtain masks of the centrosomes. The selections obtained from

995 the masks were enlarged using the "Make Band" function to create a band region of interest

996 (ROI). This was done by increasing in 1 µm increments until there were five bands. The

997 centrosome masks and the surrounding bands were measured on the PCM1 channel of the

998 average intensity projection image. To quantify clathrin intensity around the cilia base, a

999 point was manually selected as the center of the basal point. The user was blinded to file

1000 name and condition while quantification took place. This point was expanded 1 µm in each

1001 direction to create a shall of 2 µm diameter in x,y, and z. This shell was then measured using

1002 the 3D image suite in ImageJ (Ollion et al., 2013). Etomo and IMOD (Kremer et al., 1996) were

1003 used to reconstruct tomograms and manually segment tomograms respectively. These

1004   segmentations were used to create objects using the 3D Image suite in FIJI. The 3D centroids

1005   were obtained and the manually segmented ROI on the 2D slice that the 3D centroid was on

1006   was selected to move forward with. A 20 nm width band around this ROI was measured using

1007   the "Make Band" function. The integrated density of this band ROI was quantified as an

1008   indication of how electron dense the region around the user segmented vesicle is. 3D objects

1009   were measured using the 3D Image Suite. Statistical analyses were carried out in GraphPad

1010   Prism8.

1011

1012 **Table 1. List of primary antibodies**

1013

Antibody	Species	Source	Dilution	Fixation
ARL3	Rabbit	Proteintech 10961-1-AP	1:100 IF	PFA
ARL13B	Rabbit	Proteintech 17711-1-AP	1:100 IF	PFA
Ac α-tubulin	Mouse	Sigma T6793	1:1000 IF	PFA/GA
Ac α-tubulin	Rabbit	Abcam ab179484	1:1000 IF	PFA/GA
γ-tubulin	Mouse	Sigma T6557	1:100 IF	PFA
γ-tubulin	Rabbit	Abcam ab11317	1:100 IF	PFA
GFP	Mouse	Roche 11814460001	1:1000 IF 1:1000 WB	PFA
GFP	Rabbit	Abcam ab6556	1:1000 IF 1:1000 WB 1:20 ImEM	PFA
GFP	Rabbit	Santa Cruz sc-8334 Stock- 0.2µg/µl	1:5000 WB 3µg IP	-
His-tag	Mouse	GenScript A00186	1:2500 WB	-
IFT43	Rabbit	From Victor L. Ruiz-Perez, University of Madrid, Spain	1:200 IF 1:2000 WB	PFA
IFT81	Rabbit	Proteintech 11744-1-AP	1:200 IF 1:1000 WB	PFA
IFT88	Rabbit	Proteintech 13967-1AP Stock 0.23µg/µl	1:200 IF 1:1000 WB 3µg IP	PFA
IFT121	Rabbit	Custom made from Proteintech	1:50 WB Not for IF	-
IFT122	Rabbit	Proteintech 19304-1-AP	1:200 IF 1:1000 WB	PFA
IFT122	Rabbit	Aviva ARP 53817_P050	Not for IF 1:1000 WB	-
IFT139	Rabbit	Novus-NBP1-90416	1:1000 WB 1:200 IF	PFA
IFT140	Rabbit	Proteintech 17460-1-AP	1:200 IF 1:1000 WB 3µg IP	PFA
IFT144	Rabbit	Proteintech 13647-1-AP	1:200 IF 1:1000WB	PFA
MKS1	Rabbit	Proteintech 16206-1-AP	1:100 IF	MeOH
NPHP1	Mouse	640 from Greg Pazour, University of Massachusetts	1:100 IF	MeOH
PCM1	Rabbit	Proteintech 19856-1-AP	1:100 IF	MeOH
Rootletin	Goat	Santa Cruz sc-67828	1:100	MeOH
SNAP	Rabbit	New England Biolabs P9310S	1:300 IF	MeOH
Other reagents				
SiR-Tubulin	-	Spirochrome (SC002)	200 nM	Live cell imaging
SNAP-TMR	-	New England Biolabs SNAP-Cell TMR-STAR (S9105S)	1 µM	Live cell imaging

1014 **PFA: Paraformaldehyde, GA: Glutaraldehyde, ImEM: Immuno Electron Microscopy, IF:**

Immunofluorescence, WB: Western Blot, IP: Immunoprecipitation, MeOH: Methanol.

Table 2. List of secondary antibodies

Antibody	Host	Source	Dilution	Application
ECL α-Mouse IgG, HRP-conjugated	Sheep	GE Healthcare NA931-1ML	1:10000	WB
ECL α-Mouse IgG, HRP-conjugated	Rabbit	Dako P0260	1:1000	SO
ECL α-Rabbit IgG, HRP-conjugated	Goat	GE Healthcare RPN4301	1:10000	WB
α-Rabbit Light-Chain specific HRP conjugated	Mouse	Millipore MAB201P	1:10000	WB
α-Rabbit IgG Light-Chain Specific mAb	Mouse	Cell Signalling Technology L57A3	1:10000	WB
Alexa 488, 594, 647 conjugated-α-Mouse	Donkey	Molecular Probes	1:500	IF
Alexa 488, 594, 647 conjugated-α-Rabbit	Donkey	Molecular Probes	1:500	IF
Alexa 488, 594, 647 conjugated-α-Goat	Donkey	Molecular Probes	1:500	IF
10nm gold conjugated- α-Rabbit (Batch 008721)	Goat	BBI Solutions EM GAR10/0.25	1:30	ImEM

ImEM: Immuno Electron Microscopy, IF: Immunofluorescence, SO: Lipid Strip Overlay WB: Western Blot,

Table 3. List of plasmids

Plasmid	Source
Arl13b-mKate2	(Diggle et al., 2014)
ARL13B-EGFP	(Hori et al., 2008)
pEGFP-N1	Clontech 6085-1
Ift122-EGFP	(Qin et al., 2011)
PalmPalm-EGFP	(Williams et al., 2014)
MyrPalm-EGFP	(Williams et al., 2014)
pEGFP-mSmo	(Chen et al., 2002)
Wdr35-EmGFP	(Mill et al., 2011)

1026 **Figure Legends**
1027

1028 **Figure 1. *Wdr35*^{-/-} and *Dync2h1*^{-/-} mutant cells have a drastic reduction in cilia length but have no**
1029 **difference in the number of cilia.** (A) WT and mutant MEFs and those rescued by transiently
1030 expressing WDR35-EmGFP serum starved for 24 h, fixed and stained with acetylated α tubulin (green)
1031 and γ tubulin (magenta), nuclei (blue). Boxed regions are enlarged below, and arrows point at ciliary
1032 axoneme stained for acetylated α tubulin. (B) Quantification of cilia length for acetylated α tubulin. n=
1033 total number of cells from three different biological replicates (represented by different shapes).
1034 Asterisk denotes significant p-value from t-test: (*, $P < 0.05$), (**, $P < 0.01$), (***, $P < 0.001$). (C)
1035 Percentage of acetylated α tubulin positive ciliated cells. (D) 24 h serum starved WT and mutant MEFs
1036 stained for nuclei (blue), acetylated α tubulin/ polyglutamylated tubulin (green), rootletin (cyan) and
1037 transition zone proteins MKS1/NPHP-1 (magenta) show no difference in the localization of transition
1038 zone protein MKS1 and NPHP-1. Grey scale enlarged regions are labeled green (G), magenta (M), and
1039 cyan (C). (E) Schematic of intraflagellar transport (IFT) pathway in cilia.

1040
1041 **Figure 1- figure supplement 1. The organisation of centriolar satellites (CS) around *Wdr35*^{-/-} cilia is**
1042 **not changed.** CS marker PCM1 intensity and localisation are unchanged in *Wdr35*^{+/+} and *Wdr35*^{-/-}
1043 MEFs serum starved for 24 h to induce ciliogenesis and imaged (A) fixed after staining with
1044 antibodies; PCM1 (magenta) and γ-tubulin (green). Nuclei are in blue. (B) Quantification of PCM1
1045 intensity around the centrosome in concentric rings of 1 μm around the basal body (γ-tubulin). n=50
1046 cells (3 biological replicates each). (C) Imaged live after staining with SNAP-TMR dye for endogenous
1047 SNAP tagged PCM1 (magenta) and microtubule marker SiR-tubulin (grey). These cells are also
1048 expressing ARL13B-EGFP (green) (*Video 1*).

1049
1050 **Figure 2. *Wdr35*^{-/-} cilia exhibit retrograde transport defects of IFT-B, similar to *Dync2h1*^{-/-}, although**
1051 **IFT-B complex assembly is unaffected.** (A) IFT-B (green) accumulates beyond the axoneme (Ac-α
1052 tubulin, magenta) in *Wdr35* and *Dync2h1* mutant cilia from 24 h serum-starved and fixed MEFs. (B)
1053 Length quantification shows IFT-B accumulates beyond acetylated α tubulin in significantly shorter
1054 mutant cilia. n= total number of cells from three different biological replicates represented by
1055 different shapes. Asterisk denotes significant p-value from t-test: (*, $P < 0.05$), (**, $P < 0.01$), (***, $P <$
1056 0.001). Scale bars = 5 μm. (C-D) Despite differences in localization, IFT88-IP/MS analysis of E11.5 WT
1057 and *Wdr35*^{-/-} littermate embryos reveal no difference in the composition of the IFT-B complex.
1058 Antibody highlights bait (IFT88) for IP. (C) Normalized LFQs to IFT88 intensity reveals no difference
1059 between WT and *Wdr35*^{-/-} IFT-B complex composition. N= 4 embryos/genotype. (D) The number of
1060 unique peptides identified in IP/MS.

1061
1062 **Figure 3. WDR35 is essential for the stability and recruitment of the IFT-A complex into cilia.** (A)
1063 IP/MS data shows the stability of the IFT-A complex is disrupted in *Wdr35*^{-/-} lysates. N= 6

1064 embryos/genotype. Antibody highlights bait (IFT140) for IP. (B) Immunoblots confirm the non-core

1065 IFT-A complex is unstable in *Wdr35* mutants. IFT43 runs close to the molecular weight of IgG, is shown

1066 by an arrow as IFT43 band over the IgG band from IFT140 IP in WT. The corresponding band is absent

1067 in *Wdr35* null samples. (C-D) Immunoblots for the total level of IFT-A subunits in E11.5 embryo lysates

1068 show non-core components IFT139 and IFT43 to be missing in *Wdr35* mutants (C), quantified by

1069 densitometry (D). N= biological replicates. Asterisk denotes significant p-value from t-test: (*, P <

1070 0.05), (**, P < 0.01), (***, P < 0.001). (E) Inhibition of the proteasome by treatment with MG-132

1071 rescues IFT43 stability in *Wdr35$^{-/-}$* MEFs. (F) MEFs serum starved for 24 h reveal a retrograde transport

1072 defect in *Dync2h1$^{-/-}$* versus a failed recruitment of IFT-A proteins into *Wdr35$^{-/-}$* cilia. Cells are fixed and

1073 stained for respective IFT-A (green) and γ and acetylated α tubulin (magenta). Arrowheads point at

1074 cilia. Scale bars = 5 μm. Due to a lack of specific immunoreagents, IFT122 signal is from transiently

1075 expressed Ift122-GFP. All other panels represent endogenous signal detected by IF.

1076

1077 **Figure 3- figure supplement 1. WDR35 is essential for the stability and recruitment of the IFT-A**

1078 **complex into cilia.** (A,B) Immunoblots for the total level of IFT-A subunits in MEF lysates show non-

1079 core components IFT139 and IFT43 to be missing in *Wdr35* mutant cells (A), quantified by

1080 densitometry (B). N= biological replicates. Asterisk denotes significant p-value from t-test: (*, P <

1081 0.05), (**, P < 0.01), (***, P < 0.001). (C) Quantification of cilia length for acetylated α tubulin, and

1082 IFT-As. n= total number of cells from three different biological replicates (represented by different

1083 shapes). Asterisk denotes significant p-value from t-test: (*, P < 0.05), (**, P < 0.01), (***, P < 0.001).

1084

1085 **Figure 4. Membrane proteins fail to localise to *Wdr35$^{-/-}$* cilia.** (A) 24 h serum-starved WT, *Wdr35$^{-/-}$*

1086 and *Dync2h1$^{-/-}$* MEFs stained for Smoothened (SMO), ARL13B and ARL3 (green), and acetylated α

1087 tubulin (magenta) show failed localization of membrane proteins in *Wdr35$^{-/-}$* and retrograde transport

1088 defect in *Dync2h1$^{-/-}$*. (B) Smoothened-EGFP and ARL13B-EGFP (green) expressing ciliated cells stained

1089 with SiR-tubulin (magenta) show failed localization of exogenously expressed membrane proteins

1090 inside mutant cilia (**Video 2**). Dashed arrows point at the enrichment of ARL13B on the membrane in

1091 the mutant. (C) 24 h serum-starved cells expressing respective general lipidated GFP cargos (green)

1092 and stained for SiR-tubulin show enrichment of lipidated GFP in WT cilia and failed localization in the

1093 mutant. Arrowheads point at cilia in all the images. Scale bars = 5 μm.

1094

1095 **Figure 5. IFT-A subunits have close sequence and structural similarity to α and β' COPI subunits and**

1096 **can directly bind to phosphatidic acid *in vitro*. (A)** Clusters of IFT and COPI subunits generated from

1097 the results of reciprocal sequence similarity searches with HHBlits using IFT144, IF140, IF122, and

1098 WDR35 as initial search queries, suggest a very close similarity between a subset of IFT proteins and

1099 the COPI α (COPA) and β' (COPB2) subunits. Clusters are color-coded according to protein structural

1100 motifs with TPR repeat proteins (blue) and dual WD40 repeat and TPR repeat-containing proteins

1101   (magenta). Lines between clusters indicate sequence-based proximity. (B) The SDS-PAGE analysis of

1102   the purified IFT139/121/His-GFP-43 after purification by Size Exclusion Chromatography (SEC). (C)

1103   Lipid-strip overlay assay to detect binding between the IFT-A trimer shown in panel A and various

1104   lipids as indicated in the schematics on the left-hand side of panel C. The IFT-A trimer displays strong

1105   binding to phosphatidic acid (PA) and weaker binding to phosphatidylserine (PS) in the protein-lipid

1106   overlay assay. Both are negatively charged (anionic) phosphoglycerates, whereas the trimer shows no

1107   binding to neutral or inositol-based lipids. (D) Negative stain micrographs show that the IFT-A trimer

1108   (IFT139/121/43) complex associates with liposomes (PE/PG/PA) but not with POPC-liposomes lacking

1109   PA. The IFT121/43 dimer associates weakly with liposomes (PE/PG/PA). The particles of liposomes

1110   with smooth surfaces are highlighted in black arrows, and liposomes with rough surface displaying

1111   protein binding are highlighted in magenta arrows. Scale bar: 100 nm.

1112

1113   **Figure 5- figure supplement 1. IFT-A subunits have close sequence and structural similarity to α and**

1114   **β' COPI subunits and can directly bind to phosphatidic acid *in vitro*.** (A) Structure prediction showed

1115   IFT144, IFT140, IFT122, and WDR35 to have close structural similarity to COPI complex proteins α and

1116   β'. 2.5 Å X-ray structure of β' (PDB:3mkq) and IFT-A proteins are shown with N-terminal WD40 repeat

1117   (blue) and C-terminal TPR repeats (magenta). Sequence identity, similarity, and coverage between

1118   COPI -β' and respective IFT-A proteins are shown in the table below. (B, C) Purified His-IFT43 or

1119   hetero-dimeric His-IFT43/121 (right hand side) show no binding to any of the lipids spotted on the

1120   strips (left hand side). Schematic (left panel B) outlines distribution of different lipids as indicated. As

1121   a positive control, 1 μl of His-IFT43 was spotted directly on the dry membranes presented in panels B

1122   and C before blocking in 3 % BSA solution. (D) IFT-A trimer (His-IFT43/121/139) display binding to PA

1123   demonstrating the requirement of IFT139 for lipid binding. IFT139 on its own is unstable *in vitro*.

1124

1125   **Figure 6. Electron-dense vesicles are observed tracking between the Golgi and cilia base**

1126   **in WT fibroblasts whereas a 'coat-less' vesicles accumulate around *Wdr35* mutant cilia.** The tilt

1127   series of TEM samples were made from 24 h serum starved MEFs. Reconstructed tomograms are

1128   color-coded to highlight the ciliary membrane (brown), ciliary sheath (orange), ciliary pocket (yellow),

1129   basal body (purple), Golgi (green), electron-dense coated vesicles (magenta), and vesicles lacking

1130   electron cloud (cyan). (A) Z-projections from 600 nm TEM serial tomograms of WT MEFs show a track

1131   of electron-dense vesicles between the Golgi and cilia (**Video 3**). Arrows point at the path of vesicles

1132   between the Golgi and cilia. The image in the left panel is segmented in the right panel. (B) Z-

1133   projections from 300 nm tomograms from WT MEFs show electron-dense coated vesicles close to the

1134   cilia base and along the length of the cilium (**Video 4**). Arrows point at coated vesicles near the cilium.

1135   (C) Z-projections from 600 nm serial tomogram from *Wdr35*^{-/-} MEFs has a massive accumulation of

1136   vesicles in a 2 μm radius of the cilia base (cyan), and these vesicles lack a visible coat, or electron-

1137   dense cloud on them (**Video 5**). The length of cilia is drastically reduced, the ciliary membrane is

1138   wavy, and axoneme microtubules are broken in the mutant. (B and C) On left is the same Z-projection

1139   in the upper panel segmented in the lower panel, and on the right is another Z-projection from the

1140   same tomogram. Asterisk shows a coatless vesicle which fails to fuse with the ciliary sheath (see lower

1141   left panel, 6C). Scale bars = 1 μm.

1142

1143   **Figure 6- figure supplement 1. Vesicles with electron-dense coats are observed protruding/fusing**

1144   **with the ciliary sheath in WT MEFs.** 24 h serum-starved WT MEFs are processed for TEM imaging.

1145   TEM micrographs of 70 nm sections show vesicles fusing with or protruding from the ciliary sheath,

1146   mostly at the ciliary pocket and less along the length. Vesicles are enlarged in the middle panel. Other

1147   structures pointed by straight lines are actin filaments (Ac), microtubules (Mt), axoneme (Ax), ciliary

1148   sheath (csh), ciliary membrane (cm), ciliary pocket (cp), basal body (bb), daughter centriole (dc). Scale

1149   bars = 1 μm in the side panels and 100 nm in the middle panel.

1150

1151   **Figure 6- figure supplement 2. Vesicles around cilia in *Wdr35*$^{-/-}$ MEFs fail to fuse with ciliary pocket**

1152   **or ciliary sheath.** After 24 h of serum starvation, the tilt series was made for 300 nm *Wdr35*$^{-/-}$ TEM

1153   samples. Z-projection from 900 nm serial tomograms is color-coded, highlighting the daughter

1154   centriole (dark blue), basal body (purple), ciliary membrane (brown), ciliary sheath (orange), ciliary

1155   pocket (yellow), basal foot (red), transition fibres (periwinkle), Y-links (white), axonemal microtubules

1156   (magenta), Golgi (green), and vesicles around the cilia (cyan) (**Video 6**). Images in the left panel are

1157   segmented in the right panel. Coatless vesicles (cyan) accumulate around mutant cilia but fail to fuse

1158   with it. Transition zone (TZ) appeared intact in *Wdr35* mutants. Enlarged TZ in the last panel show no

1159   disturbance in (9+0) microtubule doublet arrangement and Y-links connecting axoneme to cilia

1160   membrane. The clathrin-coated vesicles that can be seen invaginating from the plasma membrane are

1161   shown by arrows in the upper two left panels. Asterisk shows coatless vesicle which fails to fuse with

1162   ciliary sheath. Scale bars = 1 μm.

1163

1164   **Figure 6- figure supplement 3. Vesicle accumulation/fusion defect around cilia in *Wdr35*$^{-/-}$ MEFs is**

1165   **observed focally suggesting it is not a global membrane traffic defect.** Zoomed-out field of view (9.3

1166   μm x 9.3 μm x 900 nm XYZ – **Video 7**) centered on the ciliated cell in the *Wdr35*$^{-/-}$ TEM sample shown

1167   in **Figure 6- figure supplement 2**, **Video 6**-, which also captures two adjacent *Wdr35*$^{-/-}$ MEFs (yellow

1168   and cyan). In all three cells, clathrin-like densely coated vesicles can be seen invaginating from the

1169   plasma membrane are shown by arrows in **Video 7** (white arrows (central cell), black arrows (yellow

1170   cell), cyan arrows (cyan cell)). In contrast, vesicles accumulating around mutant cilia are largely

1171   coatless. Importantly, accumulation of coatless vesicles is not observed at a distance beyond 2 μm

1172   from mutant cilia or close to or budding from cell membranes, suggesting this is not a defect in global

1173   membrane traffic. Scale bars = 1 μm

1174

1175

1176 **Figure 6- figure supplement 4. Retrograde dynein motor mutant has a different ciliary structure**

1177 **defect than *Wdr35* mutants.** (A) 70 nm (cell 1) TEM micrograph and a Z-projection from a tomogram

1178 of 300 nm WT MEF showing cilia ultrastructure; basal body (BB), transition zone (TZ), axoneme (Ax),

1179 transition fibres (TF), and basal foot (BF). The arrowhead points at the IFT train entering cilia at the

1180 ciliary pocket stacked between the axoneme and the ciliary membrane. (B) Z-projection from a serial

1181 tomogram reconstructed from 600 nm thick section of *Wdr35$^{-/-}$* MEFs, the ciliary membrane is less

1182 well-defined, and microtubules in the axoneme are disrupted, and periciliary vesicles accumulate

1183 around cilia. (C) Z-projection from a serial tomogram of a 900 nm thick section (cell 1-**Video 8**) and

1184 TEM micrograph of 70 nm section (cell 2) of *Dync2h1$^{-/-}$* MEFs has a striped pattern with a periodicity

1185 of 40 nm apparent throughout the length of the cilium. Cell 2 is enlarged to show the same striped

1186 pattern (magenta lines). The arrowhead points at the exosome budding from the tip of *Dync2h1$^{-/-}$*

1187 cilium in cell1 (**Video 8**). Scale bars= 250 nm, except the bottom panel which is 500 nm.

1188

1189 **Figure 7. Vesicles clustering around *Wdr35$^{-/-}$* cilia lack electron dense decorations although electron-**

1190 **dense clathrin coated vesicles are still observed budding from the mutant plasma membrane.** (A)

1191 Zoomed-in views of periciliary vesicles observed in WT (**zoomed- Figure 7B, Video 4**), *Wdr35$^{-/-}$*

1192 (**zoomed- Figure 7C, Video 5**), *Dync2h1$^{-/-}$* MEFs 24 h post-serum starvation show vesicles around WT

1193 cilia are coated (magenta) and around *Wdr35$^{-/-}$* are coatless (blue). Very rare vesicles are observed

1194 surrounding *Dync2h1$^{-/-}$* mutant cilia. (B) The average number of vesicles around cilia in control and

1195 *Wdr35$^{-/-}$* cells, counted in a volume of 2 µm radius around cilia in TEM tomograms show ten times

1196 more vesicles in *Wdr35$^{-/-}$* cells. N= number of whole-cell volume tomograms per genotype. (C) The

1197 diameter of the periciliary vesicles shows a small, but significant increase in size between control and

1198 *Wdr35$^{-/-}$*. n= number of vesicles. The paucity of vesicles around *Dync2h1$^{-/-}$* cilia prohibited

1199 quantification. (D) 2D quantification of electron density around vesicles shows signal for control

1200 vesicles is lower (darker) than mutant median (lighter) as determined by 20 nm ring outside all

1201 annotated objects. (E) Zoomed-in images to highlight the difference in the electron dense cloud

1202 surrrounding periciliary vesicles in WT (**Video 4**) which are largely missing in *Wdr35$^{-/-}$* (**Video 6, 7**)

1203 MEFs. Clathrin vesicles from the same mutant (**Video 6**) maintain their coat confirming missing

1204 electron density on *Wdr35$^{-/-}$* periciliary vesicles is not a fixation artefact. Scale bars, A= 1 µm and E =

1205 50 nm. N= number of cells examined. n= number of vesicles scored. Asterisk denotes significant p-

1206 value from t-test: (*, $P < 0.05$), (**, $P < 0.001$), (***, $P < 0.0001$).

1207

1208 **Figure 7- figure supplement 1. Increased periciliary vesicles in *Wdr35* mutant cells are unlikely to be**

1209 **clathrin-based as number and distribution of clathrin-positive foci remains unchanged.**

1210 (A) 3D projections of segmented vesicles from tomograms (top and side views) highlights the

1211 accumulation of vesicles in mutants. (B) Examples of automated 20 nm band around segmented

1212 objects for quantification in Figure 7D. (C) 24 h serum-starved cells stained for clathrin antibody

1213   (green) and acetylated α tubulin (left panel) and γ-tubulin (right panel) antibodies (magenta) do not

1214   show any difference in the distribution of clathrin around cilia. Scale bars = 5 μm. (D) No difference in

1215   the mean intensity of clathrin foci quantified in a volume of 2 μm radius around the base of cilia. n=

1216   30 cells (3 biological replicates shown by different shapes each). Asterisk denotes significant p-value

1217   from t-test: (*, $P < 0.05$), (**, $P < 0.001$), (***, $P < 0.0001$).

1218

1219   **Figure 8. WDR35 is sufficient to rescue cilia elongation and restore traffic of coated vesicles, which**

1220   **are GFP-positive by correlative light and electron microscopy.** 4 h serum-starved *Wdr35^{-/-}* cells

1221   rescued for ciliogenesis by expressing WDR35-EmGFP (green) and imaged first with Airyscan confocal

1222   imaging followed by TEM imaging. ARL13B-mKATE (magenta) is used as a cilia marker. A1 and A2

1223   represent two sequential Z-stacks from Airyscan confocal imaging. B1 and B2 represent TEM

1224   sequential images of 70 nm sections of the same cell. Arrows point at WDR35 localizing close to the

1225   cilia base, as shown by LM imaging, whilst arrowheads correspond to electron-dense vesicles shown

1226   in Z=9 and Z=10 TEM images (B) The same two sections Z = 9 and Z = 10 enlarged in the last panel

1227   show two rescued coated vesicles close to cilia. (C) Zoomed-out Z-section from 1200 nm thick TEM

1228   tomogram of a different cell expressing Wdr35-EmGFP showing coated vesicle fusing with ciliary

1229   pocket (arrowhead) left. Bottom, zoomed in view of two sections showing electron density on the

1230   fusing vesicle (full series shown in **Figure 8 – figure supplement 1, Video 9**). (D) Quantification of

1231   fusion figures observed between genotypes. N= number of cells. See **Figure 8- figure supplement 1,**

1232   **Video 9**. Scale bars: A2 and B1 are 5 μm, B2 and B are 500 nm and C is 500 nm (upper panel) and 100

1233   nm (lower panel).

1234

1235   **Figure 8- figure supplement 1. WDR35 is sufficient to rescue cilia elongation and restore coated**

1236   **vesicles fusion with the ciliary pocket.** (A) Zoomed out and (B) zoomed in select sections from 4 h

1237   serum-starved *Wdr35^{-/-}* cell rescued for ciliogenesis by expressing WDR35-EmGFP and ARL13B-

1238   mKATE, and processed for TEM. Arrowheads correspond to electron-dense vesicle fusing with the

1239   ciliary pocket. Sections from 1200 nm thick TEM tomogram created from stitching together 300 nm

1240   serial sections. Restoring WDR35 to mutant cells rescues ciliogenesis and the electron density on

1241   vesicles in the periciliary region, and restores the fusion of these coated vesicles to the ciliary pocket

1242   (arrowhead). See **Figure 8C** which illustrate sections and **Video 9** showing the tomogram through the

1243   entire cilia, quantified in **Figure 8D**. Scale bars: A, B = 500 nm.

1244

1245   **Figure 8- figure supplement 2. WDR35 localizes on vesicles around the cilia and concentrates at the**

1246   **ciliary pocket before entering the cilia by immunogold EM labeling.** (A, B) *Wdr35^{-/-}* cells transfected

1247   with WDR35-EmGFP and Arl13b-mKate2, then serum starved for 4 h and processed for TEM. 70 nm

1248   serial sections were subsequently stained with immunogold-tagged antibodies against GFP (anti-GFP).

1249   Snapshots from 70 nm serial sections show WDR35 accumulating at the ciliary pocket (A''', B'''').

1250   Staining is also seen along the axoneme (A", B""), at the vesicles at the ciliary base (B') and what looks

1251   like fusing or in close proximity to the ciliary sheath (A', B", B"'). WDR35 epitopes were exposed to

1252   antibodies directly on the surface 70 nm thick sections, which results in sparse but specific labeling of

1253   GFP. Arrows point to GFP-gold particles. Magenta outline ROIs highlight putative vesicles. ImmunoEM

1254   control shown in **Figure 8- figure supplement 3**. Scale bars = 500 nm.

1255

1256   **Figure 8- figure supplement 3. WDR35 localization to vesicles around the cilia and ciliary pocket by**

1257   **immunogold EM labelling is specific.** ImmunoEM control for **Figure 8- figure supplement 2** using two

1258   controls. (A) Internal control for adjacent non-transfected control *Wdr35* mutant cell from the same

1259   field of view as **Figure 8- figure supplement 2B**. In the absence of WDR35-EmGFP, *Wd35* mutant cells

1260   have rudimentary cilia (white arrowhead), coatless vesicles around cilia (magenta outlines), and no

1261   anti-GFP immunogold labeling (black arrow). (B) Negative secondary only control. Wdr35-EmGFP

1262   transfected mutant cells were grown under identical conditions and processed in parallel for

1263   immunoEM as cells in **Figure 8- figure supplement 2** without addition of primary anti-GFP antibodies

1264   demonstrating lack of immunogold labeling on any ciliary structures. Scale bars are 500 nm.

1265

1266

1267   **Figure 9. WDR35 and likely other IFT-As assist cargo transport of vesicles between the Golgi into**

1268   **cilia at the stage of cilia elongation**. Diagrammatic representation of the TEM data showing vesicles

1269   (green) with the WDR35-dependent coat (magenta halo) fusing and localizing around cilia in wild type

1270   cells (insert A) and coatless vesicles clustering around cilia in *Wdr35*^{-/-} MEFs (insert C). Vesicles follow

1271   a track between the Golgi and ciliary base in the WT cells but accumulate without fusing around cilia

1272   in *Wdr35*^{-/-} cells. Upon fusion, any remnant IFT-A dependent coat would become a linear 'train' which

1273   could assemble with cytosolic motors and IFT-B particles for ciliary import across the transition zone

1274   (insert B). Without non-core IFT-As, IFT-A core components are restricted at the base of *Wdr35*^{-/-} cilia

1275   whilst IFT-B proteins accumulate in short mutant cilia, without any enrichment of ciliary membrane

1276   proteins indicating an arrest at the later stages of ciliogenesis during cilia elongation.

1277

1278   ## Video Legends

1279

1280   **Video 1. The organization of centriolar satellites is not disrupted in *Wdr35*^{-/-} mutants.**

1281   *Wdr35*^{+/+}*; Pcm1*[SNAP/SNAP] and *Wdr35*^{-/-}*; Pcm1*[SNAP/SNAP] MEFs electroporated with ARL13B-EGFP (green),

1282   serum-starved for 24 h and stained for SiR-tubulin (grey) and SNAP-TMR (magenta) and imaged live

1283   on LEICA SP5 microscope using a 63X, 1.4 oil immersion objective. The video is compiled as 5 fps.

1284   PCM-1 density around cilia is not altered in the absence of WDR35. (**Related to Figure 1- figure**

1285   **supplement 1C**).

1286

1287 **Video 2. Cilia specific membrane-associated cargo (A) ARL13B, and membrane-integrated cargo (B)**

1288 **SMO fail to localize in *Wdr35*$^{-/-}$ cilia.** WT and *Wdr35*$^{-/-}$ MEFs expressing ARL13B-EGFP (green) and

1289 Smoothened-EGFP (green), serum-starved for 24 h, stained with SiR-tubulin and imaged live on LEICA

1290 SP5 microscope using a 63X, 1.4 oil immersion objective. The video is compiled as 5 fps. (**Related to**

1291 **Figure 4**).

1292

1293 **Video 3. Track of electron-dense vesicles are present between Golgi and cilia in control mouse**

1294 **fibroblast.** 24 h serum-starved cells are prepared for EM analysis by plastic embedding and making

1295 300 nm thick sections. Tomogram reconstructed from two 300 nm sections stitched together shows

1296 the presence of electron-dense vesicles between the Golgi and cilia. The 3D volume shown in the

1297 upper half is segmented in the lower half of the video. Daughter centriole (blue), basal body (purple),

1298 ciliary membrane (brown), ciliary sheath (orange), ciliary pocket (yellow), Golgi (green), and vesicles

1299 with dense electron clouds are shown in magenta. Arrows are pointing at the track of vesicles

1300 between the Golgi and cilia. (**Related to Figure 6A**).

1301

1302 **Video 4. Electron-dense vesicles are observed around the base of cilia in control mouse fibroblasts.**

1303 24 h serum-starved cells are prepared for EM analysis by plastic embedding and making 300 nm thick

1304 sections. Tomogram reconstructed from the 300 nm thick cell section shows electron-dense vesicles

1305 clustering at the base of cilia. The 3D volume shown in the upper half is segmented in the lower half

1306 of the Video. The basal body (purple), ciliary membrane (brown), ciliary sheath (orange), ciliary pocket

1307 (yellow), Golgi (green), and vesicles with dense electron clouds are shown in magenta. (**Related to**

1308 **Figure 6B**).

1309

1310 **Video 5. In *Wdr35*$^{-/-}$ fibroblasts, an accumulation of small coatless vesicles are present around short**

1311 **cilia.** 24 h serum-starved cells are prepared for EM analysis by plastic embedding and making 300 nm

1312 thick sections. Tomogram reconstructed from tilt series of two 300 nm thick cell sections and stitched

1313 together shows ten times more vesicles randomly clustering around cilia in the mutant. The 3D

1314 volume shown in the upper half is segmented in the lower half of the Video. The basal body (purple),

1315 ciliary membrane (brown), ciliary sheath (orange), ciliary pocket (yellow), Golgi (green), and coatless

1316 vesicles around the cilia is shown in cyan. The ciliary membrane is slack compared to WT MEFs and

1317 axonemal microtubules are poorly polymerized. (**Related to Figure 6C**).

1318

1319 **Video 6. In *Wdr35*$^{-/-}$ fibroblasts, periciliary vesicles fail to fuse with ciliary pocket or ciliary sheath.**

1320 24 h serum-starved cells are prepared for EM analysis by plastic embedding and making 300 nm thick

1321 sections. Tomogram reconstructed from tilt series of three 300 nm thick cell sections and stitched

1322 together shows the transition zone is unaltered in the mutant. Vesicles lacking any electron dense

1323 cloud cluster around mutant cilia, but fail to fuse with ciliary sheath or ciliary pocket. The 3D volume

1324   shown in the upper half is segmented in the lower half of the Video. Daughter centriole (dark blue),

1325   basal body (purple), ciliary membrane (brown), ciliary sheath (orange), ciliary pocket (yellow), basal

1326   foot/ subdistal appendage (red), transition fibres/ distal appendage (orchid), Y-Links (white), Golgi

1327   (green), and vesicles lacking any electron dense cloud present around cilia (cyan). Arrows are pointing

1328   at the clathrin-coated vesicles budding from the cell plasma membrane. (Related to **Figure 6- figure**

1329   **supplement 2)**.

1330

1331   **Video 7. In *Wdr35*$^{-/-}$ fibroblasts, preciliary vesicles fail to fuse with ciliary pocket or ciliary sheath.**

1332   Zoomed out view of tomograms from field of view in **Video 6** of 24 h serum-starved cells prepared for

1333   EM analysis by plastic embedding and making 300 nm thick sections, without segmentation. (Related

1334   to **Figure 6- figure supplement 3**). Zoomed out view shows two more mutant neighbouring cells with

1335   clathrin coated vesicles endocytosing from the cell membrane pointed by arrows. Three such vesicles

1336   are present in central cell pointed by white arrow, four in the cell at the top pointed by black arrows,

1337   and one in the cell at the right pointed by cyan arrow. This shows other type of vesicles away from

1338   cilia retain their coats. *Wdr35* mutant cilia have coatless vesicles accumulated close to the

1339   rudimentary cilia and not elsewhere in the cell showing the defect is not in the global membrane

1340   protein transport from the cell membrane.

1341

1342   **Video 8. *Dync2h1*$^{-/-}$ cilia lack both coated as well as coatless vesicles at the cilia base, whilst**

1343   **ectosomes are seen budding from the tip.** 24 h serum-starved *Dync2h1*$^{-/-}$ MEFs are prepared for EM

1344   analysis by plastic embedding and making 300 nm thick sections. Tomogram reconstructed from the

1345   tilt series of three 300 nm sections and stitched together shows a sturdy ciliary membrane, well-

1346   polymerized microtubules in the axoneme, almost no coated or coatless vesicles at the cilia base, and

1347   ectosome vesicles could be seen budding from the tip of cilia. A 40 nm striped pattern could be seen

1348   present throughout the length of cilia. Arrows point at the basal body (purple), cilia (magenta), and

1349   ectosome (green). This is also an example of the rare event of having two cilia in the same ciliary

1350   sheath. (**Related to Figure 6- figure supplement 4C- Cell1**).

1351

1352   **Video 9. WDR35 is sufficient to rescue cilia elongation and restore fusion of coated vesicles.**

1353   Zoomed in and stitched sections from 1200 nm thick TEM tomogram from 300 nm serial sections. 4 h

1354   serum-starved *Wdr35*$^{-/-}$ cell rescued for ciliogenesis by expressing WDR35-EmGFP and processed for

1355   TEM. The cells also express ARL13B-mKATE as a cilia marker to aid identification. Arrowheads

1356   correspond to electron-dense vesicle fusing with the ciliary pocket. Restoring WDR35 to mutant cells

1357   rescues vesicle fusion, electron density on some vesicles in the periciliary region and rescues

1358   ciliogenesis. See **Figure 8C** and **Figure 8- figure supplement 1,** which illustrate these features,

1359   quantified in **Figure 8D**. Scale bars: 500 nm.

1360

Acknowledgements

Source Data

Figure 1- source data 1. Full data points and stats test of cilia length for Figure 1B.
Figure 2- source data 1. Full data points and stats test IFT-B and cilia length Figure 2B.
Figure 3- source data 1. Full immunoblots labelled and unlabelled for Figure 3B, 3C and 3E.
Figure 3- figure supplement 1 source data 1. Full immunoblots labelled and unlabelled for Figure 3- figure supplement 1A as well as full data points and stats test IFT-A and cilia length for Figure 3- figure supplement 1B-D.
Figure 5- source data 1. Full immunoblots labelled and unlabelled for Figure 5B and Figure 5- figure supplement 1A.
Figure 7- source data 1. Full data points and stats test for size of vesicles Figure 7B-C, mean clathrin intensity for Figure 7- figure supplement 1D, and integrated density Figure 7D as well as ROI files used for calculations.

Declaration of interests

The authors have declared no competing interests.

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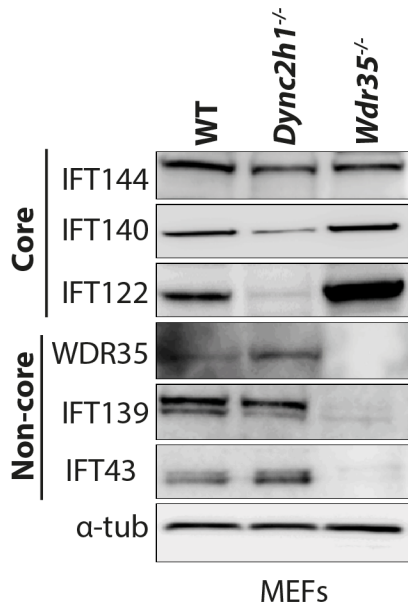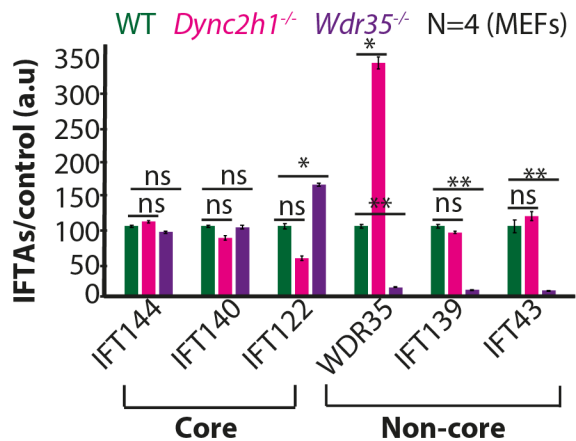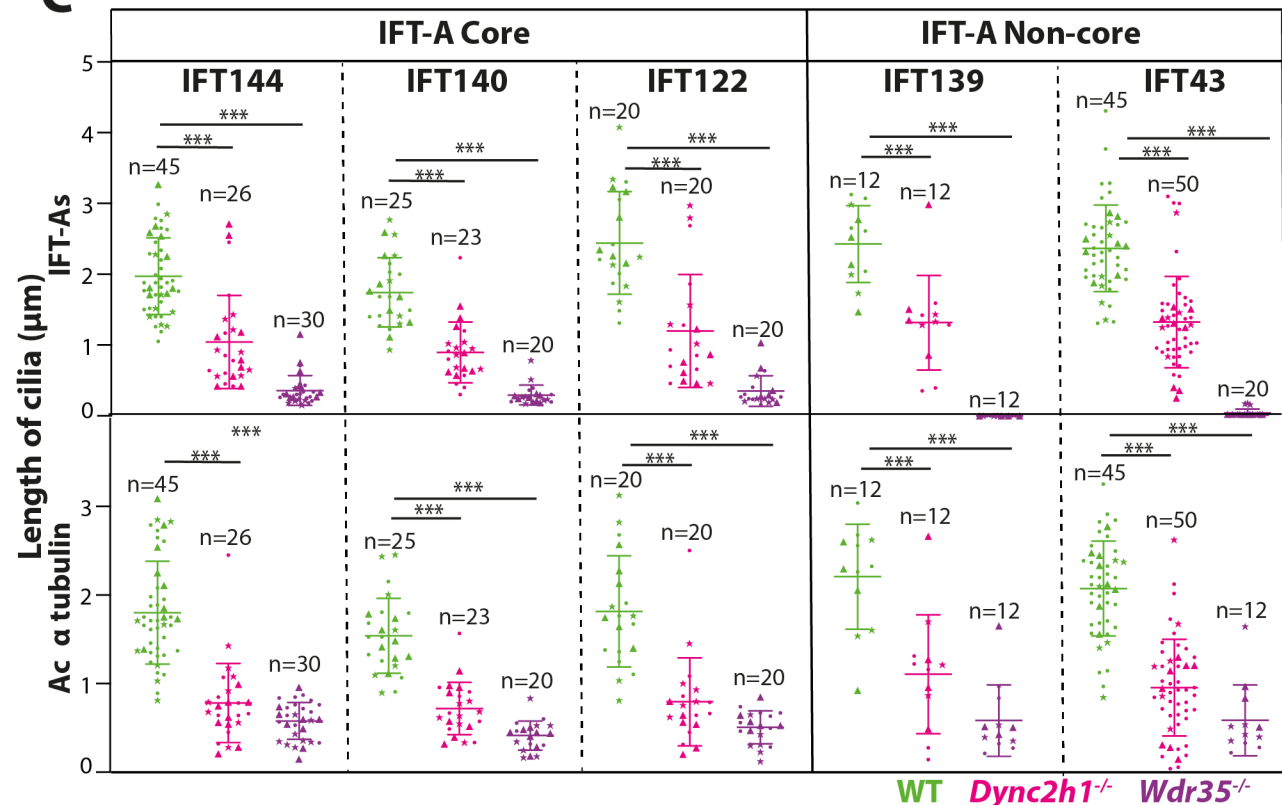1886   291. doi:10.3389/fcell.2019.00291
1887

Figure 1

A DAPI PCM1 γ-tubulin

$Wdr35^{+/+};Pcm1^{SNAP/SNAP}$ $Wdr35^{-/-};Pcm1^{SNAP/SNAP}$

5 µm

B $Wdr35^{+/+};Pcm1^{SNAP/SNAP}$
$Wdr35^{-/-};Pcm1^{SNAP/SNAP}$

PCM1 intensity (AU)

3000
2000
1000
0

Centrosome 0.0-1.0 1.0-2.0 2.0-3.0 3.0-4.0

Distance from centrosome (µm)

C ARL13B-EGFP SIR-Tubulin PCM1-SNAP-TMR

$Wdr35^{+/+};Pcm1^{SNAP/SNAP}$ $Wdr35^{-/-};Pcm1^{SNAP/SNAP}$

1 µm

A

	IFT88 Ac α Tubulin	IFT81 Ac αTubulin
WT		
Dync2h1$^{-/-}$		
Wdr35$^{-/-}$		

5 µm

M, G

B

Length of cilia (µm)

IFT-Bs / Ac α tubulin

IFT88 IFT81

WT *Dync2h1*$^{-/-}$ *Wdr35*$^{-/-}$

n=50, n=61, n=40, n=57, n=43, n=47

***

C

Mass spec — IFTBs/IFT88

WT *Wdr35*$^{-/-}$

IFT172 IFT80 IFT54 IFT20 IFT57 IFT38 IFT88 IFT52 IFT46 IFT22 IFT74 IFT81 IFT56 IFT27

IFT-B2 | IFT-B1

IFT-B

D

IFTBs	Peptides
IFT172	41
IFT80	9
IFT54	11
IFT20	6
IFT57	10
IFT38	10
IFT88	15
IFT52	5
IFT46	4
IFT22	5
IFT74	28
IFT81	29
IFT56	7
IFT27	3

5 μm

A

Core
- IFT144
- IFT140
- IFT122

Non-core
- WDR35
- IFT139
- IFT43

α-tub

WT *Dync2h1*^{-/-} *Wdr35*^{-/-}

MEFs

B

WT *Dync2h1*^{-/-} *Wdr35*^{-/-} N=4 (MEFs)

IFTAs/control (a.u)

Core: IFT144 (ns, ns), IFT140 (ns, ns), IFT122 (ns, *)
Non-core: WDR35 (*, **), IFT139 (ns, **), IFT43 (ns, **)

C

IFT-A Core | IFT-A Non-core

Length of cilia (μm)

IFT-As

IFT144 | IFT140 | IFT122 | IFT139 | IFT43

Ac α tubulin

WT *Dync2h1*^{-/-} *Wdr35*^{-/-}

A

	SMOOTHENED		ARL13B	ARL3
	-purmorphamine	+purmorphamine		
WT				
Dync2h1⁻/⁻				
Wdr35⁻/⁻				

5 μm

Ac α tubulin

B

	SMO-EGFP	ARL13B-EGFP
WT		
Wdr35⁻/⁻		

SiR- tubulin

C

	EGFP	PalmPalm-EGFP	PalmMyr-EGFP
WT			
Wdr35⁻/⁻			

SiR- tubulin

A

Miscellaneous TPR repeat proteins

CDC16, TTC25, CNOT10, TTC34, OGT, NAA15, IFIT2, CFAP70, CDC23, TOMM70, TTC6, TTC7A, TTC7B, GTF3C3, TTC26, TTC13, SPAG1, ANAPC7, TTC16, SRP72, PRPF6, TTC8, NAA16, CRNKL1, ZFC3H1, BBS4, CTR9, XAB2

Miscellaneous TPR repeat proteins

Elongator complex protein 1 (TPR repeats): ELP1

RAB6A-GEF complex partner protein (WD repeats): RIC1

IFT88

IFT-B SUBUNITS: IFT172, IFT80

IFT140, IFT122, IFT144, WDR35, IFT139

IFT-A SUBUNITS

Miscellaneous TPR repeat proteins: TTC28, TTC21A, TTC37

COP I SUBUNITS: COPA, COPB2

B

250
150 — IFT139
      IFT121
100
75 — His-GFP-IFT43
50

C

Triglyceride — Phosphatidylinositol (PI)
Diacylglycerol (DAG) — PtdIns(4)P
Phosphatidic Acid (PA) — PtdIns(4,5)P2
Phosphatidylserine (PS) — PtdIns(3,4,5)P3
Phosphatidylethanolamine (PE) — Cholesterol
Phosphatidylcholine (PC) — Sphingomyelin
Phosphatidylglycerol (PG) — 3-sulfogalactosylceramide (Sulfatide)
Cardiolipin — Blank

D

Liposomes (PE/PG/PA) control

Liposomes (PE/PG/PA) + IFT121/43

Liposomes (PE/PG/PA) + IFT139/121/43

100 nm

Liposomes (POPC) control

Liposomes (POPC) + IFT139/121/43

A

Target template COPI β′

Modeled IFT-As

IFT144 IFT140 IFT122 WDR35

	IFT144	IFT140	IFT122	WDR35
%Sequence identity	14.58	13.38	14.11	12.11
% Sequence similarity	27	27	27	26
%Sequence coverage	51	47	52	60

B

His-IFT43

Triglyceride   Phosphatidylinositol (PI)
Diacylglycerol(DAG)   PtdIns(4)P
Phosphatidic Acid (PA)   PtdIns(4,5)P2
Phosphatidylserine (PS)   PtdIns(3,4,5)P3
Phosphatidylethanolamine (PE)   Cholesterol
Phosphatidylcholine (PC)   Sphingomyelin
Phosphatidylglycerol (PG)   3-sulfogalactosylceramide (Sulfatide)
Cardiolipin   Blank

—His-IFT43
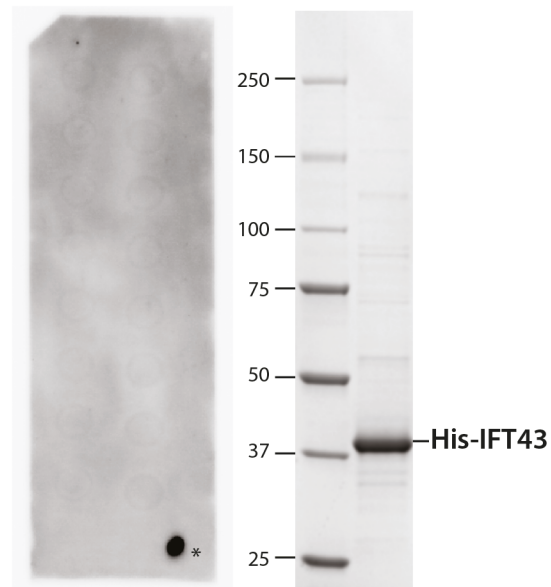
*: Pre-spotted His-IFT43 as positive control.

C His-IFT43/121
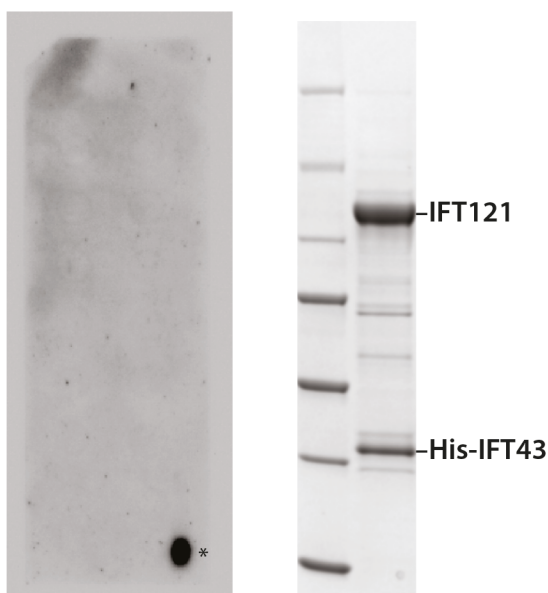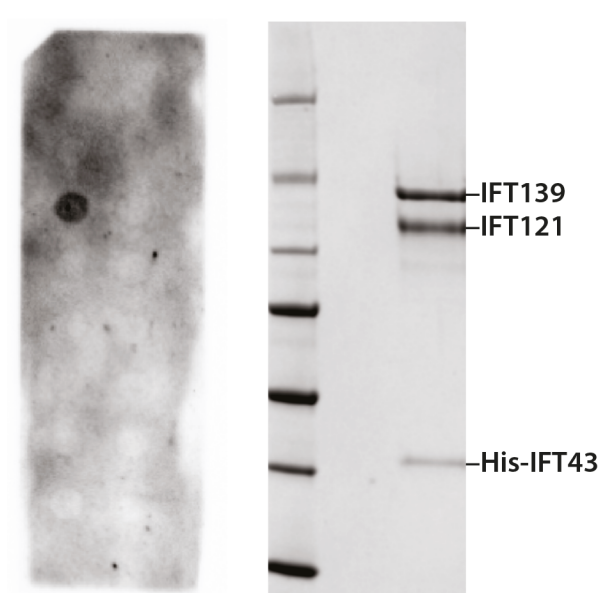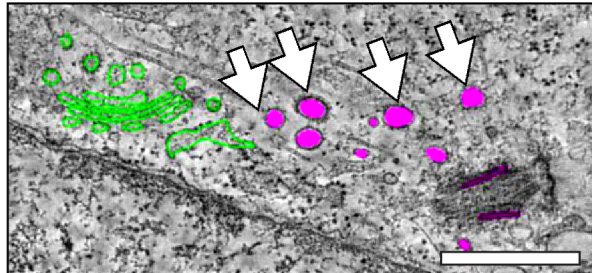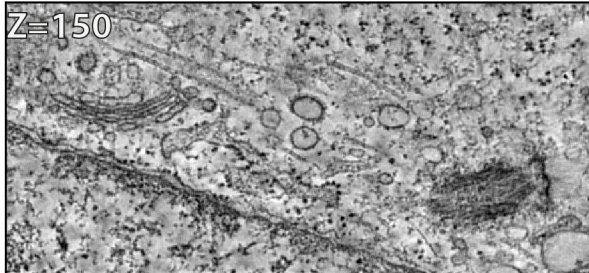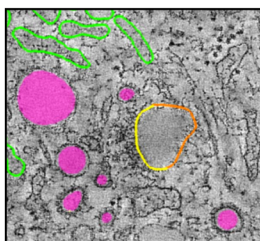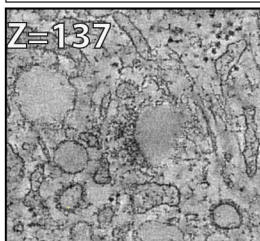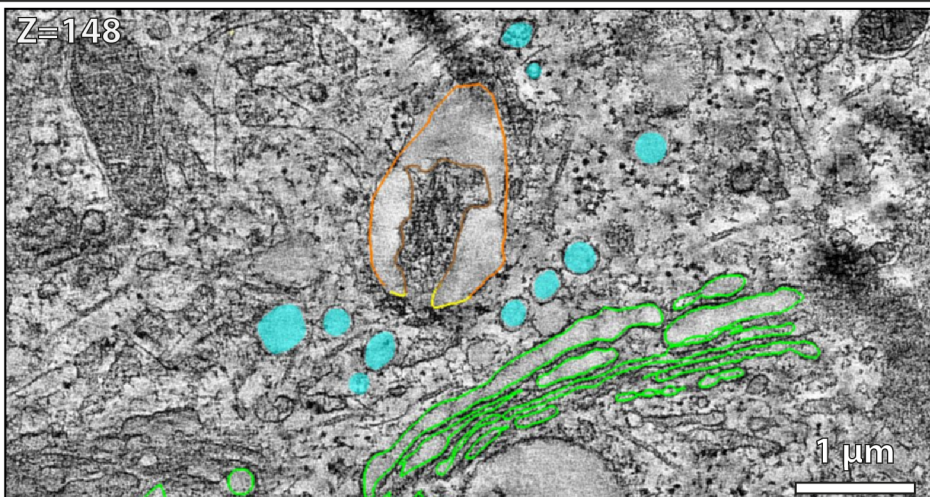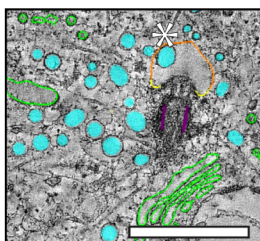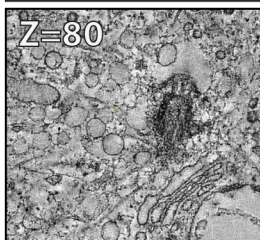
—IFT121

—His-IFT43

D His-IFT43/121/139

—IFT139
—IFT121

—His-IFT43

A WT

Z=150

B WT

Z=137   Z=70

C *Wdr35*$^{-/-}$

Z=80   Z=148

1 μm

Ciliary membrane Ciliary sheath Ciliary pocket Basal body Golgi
Coated Vesicles Coatless Vesicles

WT

Cell1

Cell2

Ac

Ax

csh

cm

cp

Mt

bb

Ac

Cell3

dc

Cell4

100 nm

1 μm

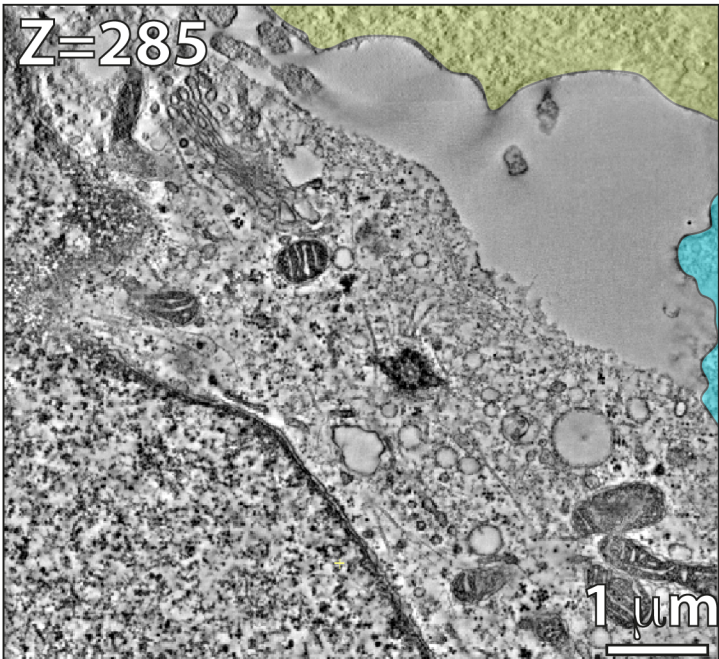**Wdr35⁻ᐟ⁻**
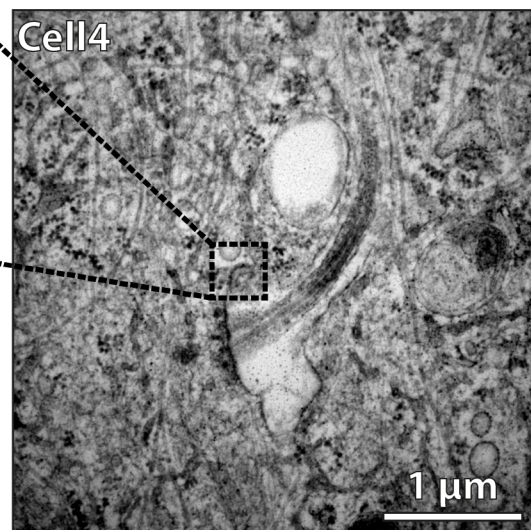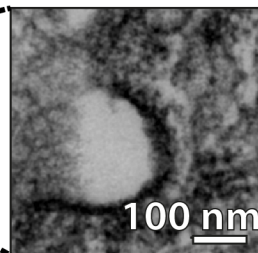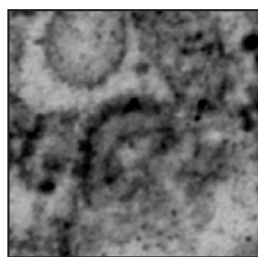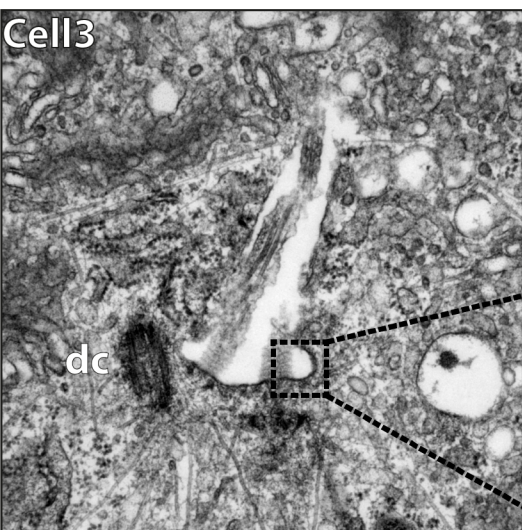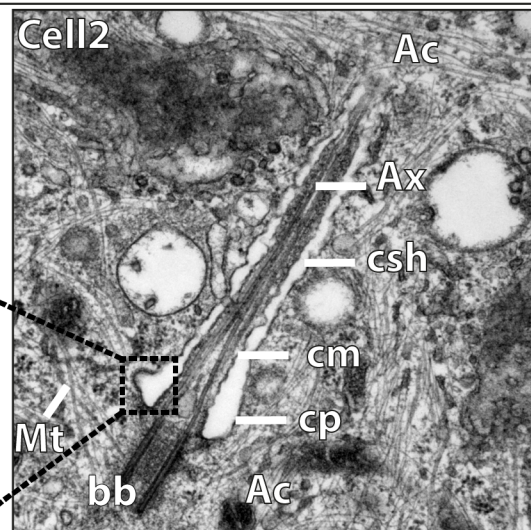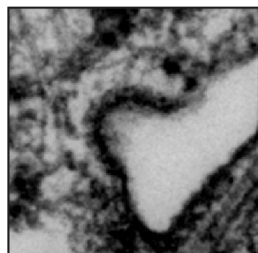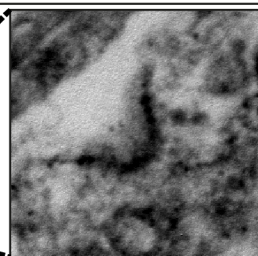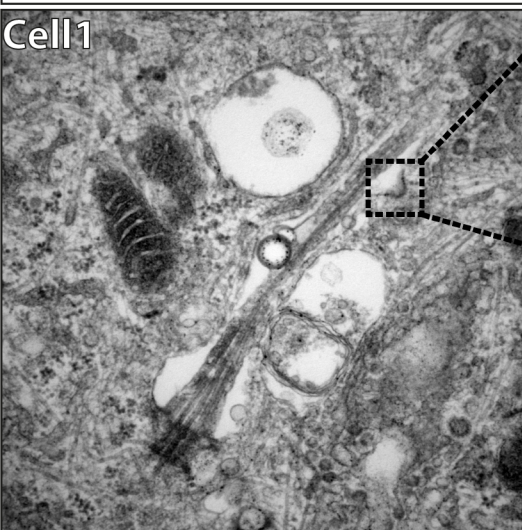
Legend (left margin):
Daughter centriole · Basal body · Ciliary membrane · Ciliary sheath · Ciliary pocket · Vesicles lacking electron dense coat · Basal foot · Transition fibres · Y-links · Axoneme · Golgi

Panels: Z=337, Z=179, Z=29, Z=368

Scale bar: 1 µm

Z=285

Z=303

Z=381

1 μm

Z=285

Z=303

Z=381

1 μm

A WT

Cell1
- IFT Train
- Ax
- BF
- TZ
- BB
- TF

Cell2

B *Wdr35*$^{-/-}$

Cell1 Z= 99

Cell1 Z= 203

C *Dync2h1*$^{-/-}$

Cell1 Z=180

Cell1 Z=411

Cell2

Cell2 Zoomed

40 nm

500 nm

A

| WT | *Dync2h1⁻ᐟ⁻* | *Wdr35⁻ᐟ⁻* |

Figure 7

1 μm

Ciliary membrane Ciliary sheath Ciliary pocket Basal Body Golgi Coated Vesicles Coatless Vesicles

B

No. of vesicles around cilia

***

WT
N=4

Wdr35⁻ᐟ⁻
N=4

C

Size of vesicles (nm)

*

WT
n=25

Wdr35⁻ᐟ⁻
n=74

D

Integrated density (AU)

***

WT
n=29

Wdr35⁻ᐟ⁻
n=509

E

Wdr35⁻ᐟ⁻

| WT | Periciliary vesicle | Clathrin vesicle |

50 nm

A

WT

Cell 4

100 nm

Cell 3

Wdr35⁻ᐟ⁻

Cell 2

Cell 5

B

WT

Wdr35⁻ᐟ⁻

200 nm

C

Clathrin   Ac α tubulin

Clathrin   γ tubulin

WT

M

G

M

G

Wdr35⁻ᐟ⁻

5 μm

M

G

M

G

D

Mean clathrin intensity
in 2 μm radius

WT   *Wdr35*⁻ᐟ⁻

1000

500

0

Figure 8

A **A1**

WDR35-EmGFP
ARL13B-mKATE-2

B1 Z=1 Z=3 Z=4

5 μm

A2

5 μm

B2 Z=8 Z=9 Z=10

500 nm

B Electron-dense vesicles

Z=9

Z=10

500 nm

C Vesicle traffic and fusion

Z=110

500 nm

Z=95 Z=115

100 nm

D

Number of vesicles Around/fusing with cilia

Wdr35⁻/⁻ N=5

WT N=14

Dync2h1⁻/⁻ N=10

Rescue N=3

A (left panel)

Z=88

Z=122

Z=305

500 nm

B (right panel)

Z=95

Z=100

Z=105

Z=110

Z=115

Z=120

Z=130

Z=140

Z=160

Z=200

Z=260

Z=300

500 nm

Wdr35::emGFP; Wdr35^{−/−}

A *Wdr35⁻ᐟ⁻ only*

Cell1 Sec13

Cell1 Sec18

Cell1 Sec22

500nm

B **Secondary only control *Wdr35-EmGFP; Wdr35⁻ᐟ⁻***

Cell1 Sec1

Cell1 Sec2

Cell1 Sec3

Cell1 Sec4

Figure 9

Key:
- transmembrane protein
- dynein
- kinesin
- IFT-B
- IFT-A
- membrane associated protein
- coatless vesicles
- IFT-A coated vesicles

WT

ciliary sheath

ciliary membrane

ciliary pocket

transition fibers

subdistal appendages

WDR35/IFT-A coated vesicles fuse around cilia base

A Fusion

B

Track of WDR35/IFT-A coated vesicles between Golgi and cilia

Golgi

Wdr35⁻ᐟ⁻

IFT-Bs accumulate in cilia

Core IFT-As restricted to ciliary base

Coatless periciliary vesicles cluster around mutant cilia

C No fusion

Key Resources Table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Gene (*M. musculus*)	*Wdr35*	Ensembl (GRCm39)	**ENSMUSG00000066643;** MGI:1921932	Also known as: *Ift121; 4930459M12Rik*
Gene (*M. musculus*)	*Dync2h1*	Ensembl (GRCm39)	**ENSMUSG00000047193;** MGI:107736	Synonyms: *4432416O06Rik, D030010H02Rik, D330044F14Rik, DHC1b, DHC2, Dnchc2, b2b414Clo, m152Asp, m407Asp*
Strain, strain background (*M. musculus*)	C57BL/6J	JAX	664	
Genetic reagent (*M. musculus*)	*Wdr35^{yeti}*	(Mill et al. 2011)	MGI:5500169	Synonyms: *Wdr35$^-$*
Genetic reagent (*M. musculus*)	*Dync2h1^{pol}*	(Caparrós-Martín et al. 2015)	MGI:6756365	Synonyms: *Dync2h1$^-$*
Genetic reagent (*M. musculus*)	*Pcm1^{SNAP}*	This paper		Non-disruptive tagging of endogenous PCM1 with SNAP-tag
Antibody	anti-ARL3 (Rabbit polyclonal)	Proteintech	Cat# 10961-1-AP, RRID:AB_2274220	IF(1:100)

Antibody	anti-ARL13B (Rabbit polyclonal)	Proteintech	Cat# 17711-1-AP, RRID:AB_2060867	IF(1:100)
Antibody	anti-Acetylated α-tubulin (Mouse monoclonal)	Sigma	6-11B-1; Cat# T6793, RRID:AB_477585	IF(1:1000)
Antibody	anti-Acetylated α-tubulin (Rabbit monoclonal)	Abcam	Cat# ab179484, RRID:AB_2890906	IF(1:1000)
Antibody	anti- γ-tubulin (Mouse monoclonal)	Sigma	GTU-88; Cat# T6557, RRID:AB_477584	IF(1:100)
Antibody	anti- γ-tubulin (Rabbit polyclonal)	Abcam	Cat# ab11317, RRID:AB_297921	IF(1:100)
Antibody	anti- GFP (Mouse monoclonal)	Roche	Cat# ab6556, RRID:AB_390913	IF(1:1000) WB (1:1000)
Antibody	anti- GFP (Rabbit polyclonal)	Abcam	Cat# ab11317, RRID:AB_305564	IF(1:1000) WB(1:1000) ImEM (1:20)
Antibody	anti- GFP (Rabbit polyclonal)	Santa Cruz	Cat# sc-8334, RRID:AB_641123	WB(1:5000)
Antibody	anti- His (Mouse monoclonal)	GenScript	Cat# A00186, RRID:AB_914704	WB(1:2500), Strip binding assay

Antibody	anti- IFT43 (Rabbit polyclonal)	Custom made, Victor Ruiz-Perez, University of Madrid	(Caparrós-Martín et al. 2015)	WB(1:2000) IF(1:200)
Antibody	anti- IFT81 (Rabbit polyclonal)	Proteintech	Cat# 11744-1-AP, RRID:AB_2121966	WB(1:1000) IF(1:200)
Antibody	anti- IFT88 (Rabbit polyclonal)	Proteintech	Cat# 13967-1AP, RRID:AB_2121979	WB(1:1000) IF(1:200)
Antibody	anti- IFT121 (Rabbit polyclonal)	Custom made, Mill lab, Proteintech	This paper, RRID:AB_2893313	WB(1:50)
Antibody	anti- IFT122 (Rabbit polyclonal)	Proteintech	Cat# 19304-1-AP, RRID:AB_2714184	WB(1:1000) IF(1:200)
Antibody	anti- IFT122 (Rabbit polyclonal)	Aviva	Cat# 19304-1-AP, RRID:AB_1294343	WB(1:1000)
Antibody	anti- IFT139 (Rabbit polyclonal)	Novus	Cat# NBP1-90416, RRID:AB_11043797	WB(1:2000) IF(1:200)
Antibody	anti- IFT140 (Rabbit polyclonal)	Proteintech	Cat# 17460-1-AP, RRID:AB_2295648	WB(1:1000) IF(1:200)

Antibody	anti- IFT144 (Rabbit polyclonal)	Proteintech	Cat# 13647-1-AP, RRID:AB_10598484	WB(1:1000) IF(1:200)
Antibody	anti- MKS1 (Rabbit polyclonal)	Proteintech	Cat# 16206-1-AP, RRID:AB_10637856	IF(1:100)
Antibody	anti- NPHP1 (Rabbit polyclonal)	Custom made, Gift from Greg Pazour, University of Massachusetts	640	IF(1:100)
Antibody	anti- PCM1 (Rabbit polyclonal)	Proteintech	Cat# 19856-1-AP, RRID:AB_2878616	IF(1:100)
Antibody	anti- Rootletin (Goat polyclonal)	Santa Cruz	Cat# sc-67828, RRID:AB_2085505	IF(1:100)
Antibody	anti- SNAP (Rabbit polyclonal)	New England Biolabs	Cat# P9310S, RRID:AB_10631145	IF(1:300)
Antibody	anti- Mouse IgG, HRP-conjugated secondary antibody (Sheep polyclonal)	GE Healthcare	Cat# NA931-1ML, RRID:AB_772210	WB(1:10000)
Antibody	anti- Mouse IgG, HRP-conjugated secondary antibody (Rabbit polyclonal)	Dako	Cat# P0260, RRID:AB_2636929	WB(1:1000), Strip overlay assays

Antibody	anti- Rabbit IgG, HRP-conjugated secondary antibody (Goat polyclonal)	GE Healthcare	Cat# RPN4301, RRID:AB_2650489	WB(1:10000)
Antibody	anti- Rabbit Light-chain specific, HRP-conjugated secondary antibody (Mouse monoclonal)	Millipore	Cat# MAB201P, RRID:AB_827270	WB(1:10000)
Antibody	anti- Rabbit IgG, Light-chain specific secondary antibody (Mouse monoclonal)	Cell Signaling Technology	Cat# 3677, RRID:AB_1549610	WB(1:10000)
Antibody	anti- Goat IgG, Alexa488, 594, 647 connjugated secondary antibodies (Donkey polyclonal)	Molecular Probes	Cat# A-11055, RRID:AB_2534102 Cat# A-11058, RRID:AB_2534105 Cat# A-21447, RRID:AB_2535864	IF(1:500)
Antibody	anti- Mouse IgG, Alexa488, 594, 647 connjugated secondary antibodies (Donkey polyclonal)	Molecular Probes	Cat# A-21202, RRID:AB_141607 Cat# R37115, RRID:AB_2556543 Cat# A32787, RRID:AB_2762830	IF(1:500)
Antibody	anti- Rabbit IgG, Alexa488, 594, 647 connjugated secondary antibodies (Donkey polyclonal)	Molecular Probes	Cat# A-21206, RRID:AB_2535792 Cat# A-21207, RRID:AB_141637 Cat# A-31573, RRID:AB_2536183	IF(1:500)
Antibody	anti- Rabbit IgG, 10 nm gold conjugated (Goat	BBI Solutions	Cat# EM GAR10/0.25, RRID:AB_1769128	IF(1:500)

	polyclonal)			
Recombinant DNA reagent	pmKate2-Arl13b (plasmid)	(Diggle et al. 2014)		Mouse *Arl13b* cDNA with a C-terminal fusion of mKate2
Recombinant DNA reagent	ARL13B-EGFP (plasmid)	(Hori et al. 2008)		Human *ARL13B* cDNA with a C-terminal fusion of EGFP
Recombinant DNA reagent	pEGFP-N1 (plasmid)	Clontech 6085-1	(Hori et al. 2008)	EGFP expression vector as control
Recombinant DNA reagent	Ift122-EGFP (plasmid)	(Qin et al. 2011)		Mouse *Ift122* cDNA with a C-terminal EGFP tag
Recombinant DNA reagent	MyrPalm-GFP (plasmid)	(Williams et al. 2014)		Lipid-anchored GFP construct generated by annealing oligonucleotides encoding the 13 amino-terminal residues from Lyn kinase (MyrPalm), into the KpnI and AgeI sites of pEGFP-N1 (Clontech).
Recombinant DNA reagent	PalmPalm-GFP (plasmid)	(Williams et al. 2014)		Lipid-anchored GFP construct generated by annealing oligonucleotides encoding the 20 N-terminal residues from GAP43 (PalmPalm) into the KpnI and AgeI sites of pEGFP-N1 (Clontech).

Recombinant DNA reagent	pEGFP-mSmo (plasmid)	(Williams et al. 2014)	Addgene plasmid # 25395 ; http://n2t.net/addgene:25395 ; RRID:Addgene_25395	Mouse *Smo* cDNA with C-terminal EGFP fusion.
Recombinant DNA reagent	pcDNA 6.2C-EmGFP-TOPO/ Wdr35 (plasmid)	(Mill et al. 2011)		Mouse *Ift121* cDNA with a C-terminal EmGFP fusion.
Recombinant DNA reagent	pACEBac1-CMV-His-eGFP-TEV-IFT43_pIDC-CMV-IFT121_ pIDK-CMV-IFT139 (plasmid)	This paper		His-eGFP version of Cr_IFT-A noncore trimer construct for expression in mammalian cells
Recombinant DNA reagent	pFL-His-TEV-IFT43 (plasmid)	This paper		His-tagged version of Cr_IFT-43 construct for the expression in insect cells
Recombinant DNA reagent	pFL-His-TEV-IFT43_IFT121 (plasmid)	This paper		His-tagged version of Cr_IFT43/121 dimer construct for the expression in insect cells
Recombinant DNA reagent	pFL-IFT139 (plasmid)	This paper		Untagged version of Cr_IFT139 construct for the expression in insect cells
Other	SNAP-Cell® TMR-Star	New England BioLabs	S9105S	1:1500
Other	SiR-tubulin kit	Spirochrome	SC002	200 nmol
Other	Hoechst 344442	Thermo Fischer Scientific	H1399	

Other	POPC Liposomes	T&T Scientific Corp.		Liposomes of POPC at 2mg/ml with a size of 50 nm
Other	PE/PG/PA Liposomes	T&T Scientific Corp.		Liposomes of DOPE, DPPG and DSPA at 2mg/ml (total lipid) with a size of 50 nm
Other	Membrane Lipid Strips	Echelon Biosciences	P-6002	
Software, algorithm	LAS AF 3.0	Leica Microsystems(Schindelin et al. 2012)		
Software, algorithm	Fiji	(Schindelin et al. 2012)	PMID: 22743772	
Software, algorithm	Imaris V9.5	Oxford Instruments		
Software, algorithm	Fiji Macro for quantification of PCM1	(RadialIntensityFromCentrosomes.ijm)	(https://github.com/IGMM-ImagingFacility/Quidwai2020_WDR35paper)	
Software, algorithm	Fiji Macro for quantification of clathrin	(3DMeanIntensityfromUserDirectedPoints.ijm)	(https://github.com/IGMM-ImagingFacility/Quidwai2020_WDR35paper)	
Software, algorithm	IMOD PMID:8742726	(Kremer et al., 1996)	PMID:8742726	
Software, algorithm	SerialEM	(Mastronarde 2005)	PMID: 16182563	
Software, algorithm	SWISS-MODEL	(Waterhouse et al. 2018)	PMID: 29788355	
Software, algorithm	HHBlits	(Remmert et al. 2011)	PMID: 22198341	

Software, algorithm	Prism8	GraphPad		
Software, algorithm	MaxQuant	(Cox and Mann 2008)	PMID: 19029910	

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