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SUBUNIT COMPOSITION OF THE HUMAN CYTOPLASMIC DYNEIN-2 COMPLEX.

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3

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5 SUMMARY

6 Cytoplasmic dynein-2 is the motor for retrograde intraflagellar transport and mutations in dynein-2 are known to cause
7 skeletal ciliopathies. Here we define for the first time the composition of the human cytoplasmic dynein-2 complex. We
8 show that the ciliopathy genes WDR34 and WDR60 are bona fide dynein-2 intermediate chains and are both required
9 for dynein-2 function. In addition, we identify TCTEX1D2 as a unique dynein-2 light chain that is itself required for cilia
10 function. We define several subunits common to both dynein-1 and dynein-2 including TCTEX-1 and -3, Roadblock-1
11 and -3, and LC8-1 and -2 light chains. We also find that NudCD3 associates with dynein-2 as it does with dynein-1. In
12 contrast, the common dynein-1 regulators dynactin, LIS1, or BICD2 are not found in association with dynein-2. These
13 data explain why mutations in either WDR34 or WDR60 cause disease as well as identifying TCTEX1D2 as a candidate
14 ciliopathy gene.

15

16

17 INTRODUCTION

18 Normal cell function depends on the accurate movement and positioning of intracellular organelles and protein
19 complexes. Microtubule motor proteins are critical to these processes, with the cytoplasmic dyneins being the primary
20 minus-end directed microtubule motors. Two cytoplasmic dynein complexes exist in mammalian cells; dynein-1 is the
21 better characterized of these, being involved in membrane traffic, organelle dynamics and chromosome segregation
22 during mitosis (Paschal and Vallee, 1987). The cytoplasmic dynein-2 complex on the other hand, mediates the
23 retrograde component of intraflagellar transport (IFT), the process by which primary and motile cilia as well as flagella
24 function (Gibbons et al., 1994; Criswell et al., 1996). Retrograde IFT is essential for normal developmental signalling,
25 notably the Hedgehog pathway in which dynein-2 acts in concert with the IFT-A particle to drive transport of activated
26 components of this pathway from the cilia tip to the cell body (Huangfu and Anderson, 2005; Liu et al., 2005; May et al.,
27 2005). Furthermore, IFT-A is known to be required for the loading of key components into cilia (Mukhopadhyay et al.,
28 2010; Liem et al., 2012). Defects in the function of primary cilia lead to a cohort of human diseases known as the
29 ciliopathies (Waters and Beales, 2011); among these, mutations in components of the dynein-2 complex cause Jeune
30 syndrome, short rib polydactyly type III, and asphyxiating thoracic dystrophy (Dagoneau et al., 2009; Huber et al., 2013;
31 McInerney-Leo et al., 2013; Schmidts et al., 2013b; Schmidts et al., 2013a).

32 Dynein-1 is comprised of a dimer of heavy chain subunits (encoded by the *DYNC1H1* gene) which associate with two
33 copies of an intermediate chain (*DYNC1I1* or *DYNC1I2* depending on the tissue type), two copies of one of two light
34 intermediate chains (*DYNC1LI1* or *DYNC1LI2*), and a number of light chain subunits (which includes the dynein light
35 chain (*DYNLL1*), roadblock (*DYNLRB1* and *DYNLRB2*), and Tctex families (*DYNLT1*, *DYNLT3*) (Pfister et al., 2005;
36 Wickstead and Gull, 2007; Kardon and Vale, 2009)). The light chains are required for the correct assembly of the dynein
37 complex and have been implicated in controlling its association with cargo molecules. For the majority of its functions,
38 dynein-1 also associates with one or more binding partners (Kardon and Vale, 2009; Splinter et al., 2012). The best
39 described of these are the dynactin complex, Lissencephaly-1 protein (*LIS1*), and bicaudal gene products (notably
40 *BICD2*) (Splinter et al., 2012). In contrast, the molecular composition of the dynein-2 complex, notably in mammals, is
41 not well defined.

42 Although specific genes encoding a dynein-2-specific heavy (*DYNC2H1*, also known as *DHC1b* and *DHC2* (Criswell et al.,
43 1996)) and light intermediate chains (*DYNC2LI1* also known as *LIC3* (Grissom et al., 2002)) have been identified, the full
44 subunit composition of the motor and biochemical characterization of the subunit composition in metazoans is lacking.
45 Model organisms such as *Chlamydomonas* and *Tetrahymena* have provided further clues that equivalents to the other
46 known dynein-1 subunits are also present. *Chlamydomonas* genes encoding *FAP133* and *FAP163* (orthologues of
47 mammalian *WDR34* and *WDR60* respectively) encode functional intermediate chains of algal dynein-2 (Rompolas et al.,
48 2007; Patel-King et al., 2013). *FAP133* was shown to have putative LC8 binding motifs and to localize around the basal
49 body and within the flagellum (Rompolas et al., 2007). Furthermore, LC8 is known to play roles outside of the context of
50 the dynein-1 complex which could explain some of the *FAP133/WDR34* data to date. Our own work has shown that
51 *WDR34* localizes to the pericentrosomal region and is required for ciliogenesis and proper cilia function in vitro (Asante
52 et al., 2013). We have also previously defined a role for the dynein light chain Tctex-1 in cilia length control, presumably
53 in association with dynein-2 (Palmer et al., 2011). Others have also shown that *WDR34* localizes to a pericentrosomal
54 region and also that a fluorescent protein fusion of *WDR34* (*WDR34-tGFP*) is present in cilia (Schmidts et al., 2013a).
55 Furthermore *WDR34-tGFP* co-immunoprecipitated with FLAG-tagged LC8 consistent with a role for *WDR34* in dynein-2
56 function.

57 *FAP163* has also been shown to localize to the flagellar matrix, to co-purify with *FAP133* and LC8, and, in the planarian
58 *Schmidtea mediterranea*, to be absolutely required for ciliogenesis (Patel-King et al., 2013). Recent work from several
59 groups has shown that mutations in the human orthologues of *FAP133* (*WDR34*) and *FAP163* (*WDR60*) cause skeletal
60 ciliopathies (Huber et al., 2013; McInerney-Leo et al., 2013; Schmidts et al., 2013a). While these data are consistent
61 with *WDR34* and possibly *WDR60* being key components of human dynein-2, biochemical data validating this are
62 lacking. It also remains unclear whether dynein-2 works in cooperation with dynactin or other dynein-1 accessory
63 complexes. *LIS1*, for example, is known to function in concert with both cytoplasmic dynein-1 (Faulkner et al., 2000) and
64 the axonemal dyneins (Pedersen et al., 2007).

65 Since a metazoan dynein-2 complex has not been described in detail, we set out to define the subunit composition of
66 the human dynein-2 complex and determine whether known dynein-1 regulators also modulate the function of this
67 poorly characterized second cytoplasmic dynein motor.

68

69 RESULTS

70 We generated a human telomerase immortalized retinal pigment epithelial (hTERT-RPE1) cell line stably expressing
71 WDR34 fused to a monomeric form of GFP (mGFP). Figure 1 shows that mGFP-WDR34 localizes to centrosomes and
72 primary cilia in serum starved cells as well as showing a diffuse cytoplasmic distribution. This localization to cilia was
73 confirmed by co-labelling with antibodies directed against acetylated tubulin (Fig. 1A). mGFP-WDR34 also colocalizes at
74 the base of the cilium with known components of the basal body (γ -tubulin, ODF2, and OFD1, Fig. 1B-D). Fig. 1E shows
75 that the localization of mGFP-WDR34 within cilia differs from that of the endogenous protein which localizes to
76 pericentrosomal structures; Fig. 1F shows that the localization of mGFP-WDR34 to cilia is specific as GFP alone is not
77 localized in cilia.

78 We sought to use this cell line to define the other proteins associated with WDR34. To achieve this we took a proteomic
79 approach comparing the interactome of mGFP-WDR34 with mGFP using a GFP-nanotrap (Rothbauer et al., 2006).
80 Supplementary Table 1 shows the proteomics data for all known dynein-1 and putative dynein-2 components that were
81 identified in this experiment. Supplementary Table 1A shows the highly efficient isolation of mGFP-WDR34 using this
82 approach. Large numbers of peptides and spectra were also seen for WDR60 as well as the known dynein-2 heavy chain
83 (DYNC2H1). We also detected (albeit at low abundance) the known dynein-2 light intermediate chain subunit
84 DYNC2L1/LIC3. Listed in Supplementary Table 1B are the dynein light chains that we identified. Here, all known dynein-
85 1 light chain isoforms (LC8-1 and -2, Tctex-1 and -3, and Roadblock-1 and -2) were identified in association with mGFP-
86 WDR34. In addition we detected TCTEX1D2 (also known as Tctex-2). This subunit is not found in association with mGFP
87 alone. We also noted (Supplementary Table 1C) the association of mGFP-WDR34 with two regulators of dynein-1
88 function NudC and NudCD3 (also called NudC-like or NUDCL). Supplementary Table 1D then shows the data for the key
89 dynein accessory factors that were detected in our proteomics. These show that mGFP-WDR34 does not associate with
90 dynactin, Bicaudal 1, or LIS1 (PAFAH1B) at levels above that of mGFP alone. Bicaudal 2, Spindly, NudE, and Nudel, all of
91 which are known accessory factors for dynein-1, were not detected at all. We then used these proteomic data to guide
92 our further biochemical analyses.

93 The above data indicate that both WDR34 and WDR60 are intermediate chains of the dynein-2 complex. These findings
94 were then validated using immunoblotting. Fig. 2A shows specific isolation of mGFP-WDR34. Importantly we find that
95 the endogenous WDR34 does not co-immunoprecipitate with mGFP-WDR34 suggesting that the recombinant form
96 does not form a complex with the endogenous protein. Immunoblotting for WDR60 showed that this was highly
97 efficiently isolated in complex with mGFP-WDR34 (Fig. 2B) implying that these two putative intermediate chains might
98 be present in the same complex. As expected from the proteomic data, the canonical dynein-1 intermediate chain,
99 IC74, was not immunoprecipitated with mGFP-WDR34 (Fig. 2C) nor was a negative control protein, GAPDH (Fig. 2D).
100 Two components of the dynactin complex, p150^{Glued} (Fig. 2E) and p50^{dynamitin} (Fig. 2F) also failed to immunoprecipitate
101 with mGFP-WDR34; similarly, LIS1 was not detected (Fig. 2G). In contrast Tctex-1 (Fig. 2H) and NudCD3 (Fig. 2I) were
102 both efficiently isolated. Consistent with our identification of TCTEX1D2 in our proteomics, we were able to identify
103 TCTEX1D2 in association with mGFP-WDR34 (Fig. 2J). TCTEX1D2 is not detectable in whole cell lysates but the
104 enrichment following GFP-nanotrap isolation of mGFP-WDR34 enables its detection in immunoprecipitates (Fig. 2J).
105 These data reveal some specificity since both Tctex-1 and NudCD3, but not TCTEX1D2, associate with dynein-1 as
106 determined in five independent pull downs of dynein-1; dynactin, LIS1, and BICD2 were also always identified in these
107 dynein-1 pull-downs from both HeLa and RPE-1 cells (unpublished observations). We have been unable to analyze the
108 dynein-2 heavy chain or light intermediate chain subunits by immunoblotting owing to a lack of available antibodies.

109 Dynein-2 is predicted to be a large molecular weight complex with a high sedimentation coefficient, similar to
110 cytoplasmic dynein-1. As predicted for a large macromolecular complex, we found endogenous WDR34 in a large
111 complex that sedimented on sucrose density gradients in similar fractions to the dynein-1 intermediate chain (Fig. 3A).
112 This suggests that WDR34 functions in the context of a high molecular weight complex. As expected, mGFP-WDR34 and
113 WDR60 were both found in large complexes (Fig. 3B and C) consistent with their role as intermediate chains within the
114 same dynein-2 complex. In contrast, mGFP (Fig. 3B), like the majority of the control protein GAPDH, was found
115 predominantly in low density fractions as expected. GAPDH is known to associate with many other proteins including
116 dynein-1 (Zala et al., 2013) which likely explains the low amounts detected across the higher density gradient fractions.

117 The expression of mGFP-WDR34 had no significant effect on the distribution of WDR60 across the complex (Fig. 3C).
118 Some unincorporated mGFP-WDR34 was observed as well as some mGFP itself (Fig. 3C), most likely arising as a result of
119 proteolytic processing of the recombinant protein at the linker site between mGFP and WDR34. This could also explain
120 much of the diffuse cytoplasmic labelling seen in Fig. 1. Further to this we undertook gel filtration of cell lysates to
121 determine the apparent molecular mass of the mGFP-WDR34-containing complexes. Fig. 3D shows that using a
122 calibrated Superose 6 column, we found that all detectable mGFP-WDR34 fluorescence eluted with the void volume of
123 the column (Fig. 3D, fraction 15, 7.9 ml) corresponding to high molecular weight components ($\sim 1 \times 10^7$ Da). GFP by
124 contrast eluted within the range of $4-7 \times 10^4$ Da (Fig. 3D, fractions 33-37). As with the sucrose gradients, it is evident
125 that there is a significant fraction of mGFP-WDR34 (and also mGFP as a degradation product from this) that elutes in
126 low molecular weight and low density fractions. This complicates analysis of the exact proportion of mGFP-WDR34 that
127 is contained within the dynein-2 complex.

128 We have shown previously that defects in cilia formation and function are evident following depletion of Tctex-1
129 (Palmer et al., 2011) and WDR34 (Asante et al., 2013) *in vitro*. Specifically, siRNA-mediated depletion of either of these
130 genes results in a reduced number of cells that produce cilia but also a lengthening of those cilia that remain. To
131 determine whether this was true of the newly identified dynein-2 subunits, we first examined the role of WDR60. The
132 biochemical data shown above suggest that WDR34 and WDR60 might act within the same dynein-2 complex. We
133 found that endogenous WDR60, like WDR34, also localized around the centrosome (Fig. 4A) and that this localization
134 was lost following depletion of either WDR60 itself or of WDR34 (Fig. 4A) suggesting that they function together.
135 Consistent with this, depletion of either WDR34 or WDR60 resulted in a loss of WDR34 labelling around the centrosome
136 (Fig. 4B). Depletion of WDR60 clearly led to one of two phenotypes, an increase in cilia length (example shown in Fig. 4C
137 for WDR60 siRNA #1) or a complete loss of an extended axoneme (example shown in Fig. 4C for WDR60 siRNA #2), both
138 of which were associated with a loss of labelling of WDR60 (Fig. 4C). Quantitation of these data showed that loss of
139 WDR60 resulted in a significant reduction in the proportion of ciliated cells (Fig. 4D), with a clear increase in length of
140 the remaining cilia (Figure 4E); this is highly similar to what we observed following depletion of WDR34 (Asante et al,
141 2013). The increase in cilia length is particularly evident on a cumulative frequency plot (Fig. 4F). Immunoblotting
142 confirmed the efficacy of WDR34 and WDR60 siRNAs (Fig. 4G) as well as revealing that WDR34 and WDR60 were
143 reciprocally required for stability of the other. We then repeated our analysis using both WDR34 and WDR60 siRNAs. Of
144 note, double depletion of both WDR34 and WDR60 did not further exacerbate the cilia phenotypes seen (Fig. 4H, I, and
145 J).

146 In our previous work, we showed that the localization of WDR34 to the pericentrosomal region was dependent on the
147 function of the transmembrane Golgi matrix protein giantin (Asante et al., 2013). If WDR34 and WDR60 are indeed in
148 complex together then one would predict the same would hold true for WDR60. Fig. 5A shows that loss of giantin
149 following siRNA depletion does indeed result in a reduction of WDR60 labelling at the centrosome (quantified in Fig.
150 5B). The degree of loss of giantin also correlated with the intensity of labelling of WDR60 in the pericentrosomal area
151 (Fig. 5C, quantified in 5D) as we have seen previously for WDR34. The siRNA duplexes used to target giantin have been
152 characterized previously (Asante et al., 2013) with siRNA #1 being more effective than siRNA #2. This also is evident
153 from the data seen in Fig. 5D.

154 We then used similar assays to test the role of NudCD3 in ciliogenesis. We suppressed expression of NudCD3 using
155 siRNA (Fig. 6A) and found that this diminishes the ability of cells to generate primary cilia (Fig. 6B, quantified in 6C).
156 Furthermore, those cilia that were seen to remain were found to be longer (Fig. 6D, quantified in 6E with cumulative
157 frequency plots shown in 6F). Consistent with our other data, depletion of NudCD3 also resulted in a loss of WDR34
158 around the pericentrosomal region (Fig. 6G, quantified in 6H) without affecting the localization of OFD1 (Fig. 6I) or
159 pericentrin (Fig. 6B).

160 We then examined the role of TCTEX1D2 using siRNA transfection. Figure 7A shows the efficacy of three individual
161 siRNAs measured by quantitative PCR. As shown in Fig. 7B and quantified in Fig. 7C, depletion of TCTEX1D2 results in
162 longer cilia following serum withdrawal. In all cases we can detect a statistically significant increase in cilia length
163 following TCTEX1D2 depletion. This is more evident in cumulative frequency plots (Fig. 7D). Notably, the ability of cells
164 to form primary cilia is not obviously affected following TCTEX1D2 depletion (Fig. 7E).

166 DISCUSSION

167 Here, we provide the first molecular characterization of the human dynein-2 complex. Our data build on previous
168 reports to provide a comprehensive picture of the subunit composition of this motor as summarized in the schematic in
169 Figure 8. Our data show that human dynein-2 contains both WDR34 and WDR60 intermediate chain subunits. Individual
170 cytoplasmic dynein-1 complexes contain two copies of the same intermediate chain and same light intermediate chain
171 (typically DYNC1I2 in most cells, DYNC1I1 in neurons). Biochemical experiments using *Chlamydomonas* showed that
172 FAP133 (WDR34) co-purifies and co-immunoprecipitates with other dynein-2-specific (heavy chain and light
173 intermediate chain) subunits and with the dynein light chain, LC8. These data are consistent with FAP133 being a
174 dynein-2 intermediate chain subunit (Rompolas et al., 2007) and led the authors to propose a model in which dynein-2
175 contains two copies of FAP133, analogous to the dynein-1 complex containing two copies of the intermediate chain.
176 Our data suggest that in fact dynein-2 contains both WDR34 and WDR60 intermediate chains. This asymmetry has the
177 potential to provide more functional specialization and greater control of motor function. Furthermore, WDR60 is a
178 larger protein than either WDR34 or the IC subunits of dynein-1. It is tempting to speculate that this asymmetry is
179 related to its function. Evidence, primarily from *Chlamydomonas*, demonstrates that axonemal dyneins contain multiple
180 intermediate chains within the same complex (Mitchell and Rosenbaum, 1986; King et al., 1991). For example, Inner
181 arm dynein I1 contains three intermediate chain subunits IC140, IC138, and IC97. Dimeric outer arm dynein from
182 *Chlamydomonas* contains two intermediate chain subunits IC1 and IC2. As such cytoplasmic dynein-2 shows similarities
183 to axonemal dyneins. This could relate to the association of these motors with axonemal microtubules.

184 It is interesting to note that while mGFP-WDR34 is detectable in cilia, labelling with currently available antibodies does
185 not detect this pool (also see (Asante et al., 2013; Schmidts et al., 2013a)). These data suggest that the antibody epitope
186 is occluded within cilia. This could be due to dynein binding to the axonemal microtubules or to other factors such as
187 the IFT-A particle. The pericentrosomal labelling could indicate a different set of associated factors at the cilia base or
188 that dynein-2 is assembled at the base of primary cilia exposing a pool of WDR34. We also cannot rule out that WDR34
189 itself has some function outside of the context of the assembled dynein-2 complex; our gel filtration data are consistent
190 with the presence of mGFP-WDR34 within or in association with high molecular weight complexes. Our co-
191 immunoprecipitation data argue in favour of all WDR60 being in association with WDR34. Sucrose density gradient
192 centrifugation shows that there is a proportion of WDR34 outside of the dynein-2 complex that could act in isolation, or
193 in a heterodimeric complex with WDR60, or in some other complex. Our gel filtration experiments are also consistent
194 with this. While we have not been able to monitor the dynein-2 heavy chain by immunoblotting, our previous work with
195 dynein-1 showed that loss of the intermediate chain subunit did not result in concomitant loss of the heavy chain
196 (Palmer et al., 2011). These data also therefore do not rule out the possibility of a functional dynein-2 heavy chain
197 without associated WDR34 and WDR60. However, the importance of these intermediate chain subunits for dynein-2
198 function is underscored by their conservation through evolution (Wickstead and Gull, 2007). Mutation of either WDR34
199 (Huber et al., 2013; Schmidts et al., 2013a) or WDR60 (Rompolas et al., 2007) causes skeletal ciliopathies with a similar
200 phenotype to that seen following mutation of the dynein-2 heavy chain (Dagoneau et al., 2009; Schmidts et al., 2013b).
201 These findings that WDR34 cannot compensate for loss of function of WDR60 and vice versa are consistent with our
202 model that both act together in the same dynein-2 complex. We conclude that the full dynein-2 complex including both
203 WDR34 and WDR60 operates in IFT within cilia.

204 Analysis of dynein light chain function in the context of cilia biology provides important clues to the way in which
205 dynein-2 assembles and controls protein distribution within, and retrograde exit from, cilia. The association of Tctex-1
206 with dynein-2 complexes was predicted from our previous work (Palmer et al., 2011). Here, we now also identify the
207 TCTEX1D2 light chain (Tctex-2) as a light chain for dynein-2, not present in dynein-1. Tctex-2 has previously been found
208 to control outer arm dynein assembly in *Chlamydomonas* flagella (Patel-King et al., 1997; Pazour et al., 1999). Evidence
209 suggests that, unlike other dynein light chains, TCTEX1D2 is monomeric (DiBella et al., 2001). One possibility is that a
210 single copy of TCTEX1D2 binds WDR34 with an alternative light chain (or a functionally similar protein) binding to
211 WDR60. This could diversify the regulation of dynein-2 function and potentially influence the cargo binding capabilities
212 of the complex. Our data show that a reduction of TCTEX1D2 expression results in an increase in cilia length similar to
213 that which we observed following depletion of WDR34, WDR60, and giantin. Of note, TCTEX1D2 depleted cells still form
214 cilia. We cannot reject the idea that TCTEX1D2 is required for cilia formation as these experiments are only depletion

215 experiments and not a complete knockout. The moderate phenotype seen here in vitro might also reflect some
216 functional overlap with other dynein light chains in this system. It remains unclear whether dynein light chains act in
217 directing dynein complex assembly and/or in cargo binding. It is also important to note that our in vitro assays reporting
218 an elongation of cilia do not reflect the situation in vivo where the primary failure appears to be in ciliogenesis i.e. the
219 formation of the axoneme. Our interpretation is that our siRNA experiments reflect a defect in retrograde IFT.

220 NudCD3 has been shown to influence the assembly and stability of the dynein-1 complex (Zhou et al., 2006) and
221 localizes to centrosomes (Cai et al, 2009). Our data are consistent with it playing a similar role with dynein-2. We have
222 not been able to detect NudCD3 in cilia by immunofluorescence or using epitope tagged forms (Zhou et al., 2006)
223 arguing in favour of a role in assembly of dynein-2 within the main body of the cell. Association of NudCD3 with both
224 cytoplasmic dynein complexes makes analysis of its function more complex. Its role in ciliogenesis is most likely linked
225 to its interaction with dynein-2. However, we would not expect to find mutations in this gene associated with
226 ciliopathies as a loss-of-function would compromise dynein-1, likely leading to cell death. We also identified the related
227 NudC protein in our proteomics (Supplementary Table 1C) but as yet we have not investigated further the role of NudC
228 in cilia or dynein-2 function but we note that NudC has been shown to localize to motile cilia (Pedersen et al., 2007).

229 In our experiments we typically observe two outcomes following siRNA-mediated suppression of dynein-2 subunits:
230 fewer cilia but those that remain being longer. We interpret this as a threshold effect of our in vitro experiments. Partial
231 loss of function compromises retrograde IFT and results in longer cilia (as anterograde IFT continues to deliver
232 axonemal and other components to the tip). More effective depletion results in an inability of cells to form cilia with
233 limited or no extension of the axoneme. It is also important to note that longer cilia have not typically been described
234 for patient mutations, indeed cells from patients with WDR34 mutations show shorter cilia on average (Huber et al.,
235 2013). However, phenotypic variability, with some cells failing to extend an axoneme while others can, has been seen
236 in fibroblasts from patients with WDR60 mutations (McInerney-Leo et al., 2013). This distinction between in vitro and in
237 vivo outcomes necessitates caution in interpretation of data. Nonetheless, our data show clear roles for WDR34,
238 WDR60, and TCTEX1D2 within the context of the dynein-2 complex.

239 It is also significant to note that we do not detect any association of dynein-2 with many known regulators of
240 cytoplasmic dynein-1 and axonemal dynein, including dynactin and LIS1. It is however important to note that while our
241 work does show that dynactin and LIS1 do not stably associate with dynein-2, our data do not preclude that they do not
242 associate transiently with dynein-2. For example, LIS1 acts in the initiation of dynein-1 motility but does not remain
243 associated with it during organelle transport (Egan et al., 2012). Consistent with such a model, although we do not
244 detect LIS1 in association with dynein-2 by co-immunoprecipitation, we can localize LIS1 to primary cilia by
245 immunofluorescence (unpublished observations). LIS1 is also believed to act in generating a persistent force state of
246 dynein to aid its function in moving heavy loads (McInerney-Leo et al., 2013). While such functions are required of
247 dynein-1 (for example in nuclear migration) and axonemal dyneins (in generating the force of cilia and flagellar beating)
248 it is conceivable that such large cargo loads are not part of the cargo repertoire transported by dynein-2. LIS1, dynactin
249 and BICD family proteins likely cooperate to direct association of dynein with cargo and subsequent motility (Egan et al.,
250 2012; Splinter et al., 2012; Wang et al., 2013; Moon et al., 2014). The absence of dynactin suggests that other as yet
251 unidentified accessory factors likely act in cooperation with dynein-2 in linking the motor to its cargo. Other recent in
252 vitro reconstitution experiments have shown that dynein-1 requires the association of both dynactin and another
253 accessory factor such as BICD2 to induce a processive state (McKenney et al., 2014; Schlager et al., 2014). Dynein-2 is
254 also likely to be a processive motor and it is tempting to speculate that additional factors are required to induce
255 processivity, possibly the IFT-A complex itself. Further biochemical and biophysical analysis would undoubtedly help to
256 address such questions.

257 In conclusion, our characterization of the subunit composition of cytoplasmic dynein-2 defines a requirement for both
258 WDR34 and WDR60 in dynein-2 function, explaining why mutations in either gene cause disease. Furthermore, this
259 work identifies TCTEX1D2 as a candidate ciliopathy gene.

260

261 MATERIALS AND METHODS

262 All reagents were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated.

263 GROWTH OF CULTURE CELLS

264 Human telomerase immortalized retinal pigment epithelial cells (hTERT-RPE1) were maintained in DMEM-F12
265 supplemented with 10% FCS (Life Technologies, Paisley, UK) containing supplemented 1% L-glutamine and 1% essential
266 amino-acids. Cells were seeded onto 35 mm glass bottom dishes (MatTek, Ashland, MA).

267 CILIOGENESIS ASSAY

268 On reaching confluence, hTERT-RPE1 cells were rid of serum by washing twice with PBS and incubated at 37 °C/5% CO₂
269 for 24–48 h in serum-free medium to induce cell cycle exit and subsequent cilium assembly. Cells were fixed and
270 typically labelled with anti-acetylated tubulin to mark primary cilia. Cilia lengths were measured using the Fiji
271 implementation of Image J (Schindelin et al, 2012).

272 SOURCE OF ANTIBODIES

273 Antibodies used include: rabbit polyclonal anti-WDR60 (HPA020607), rabbit polyclonal anti-NudCD3 (HPA019136),
274 rabbit polyclonal anti-ODF2 (HPA001874), mouse monoclonal anti-acetylated tubulin (T6793) and mouse monoclonal
275 anti- γ -tubulin (T6557) (all from Sigma-Aldrich); rabbit polyclonal anti-giantin (PRB-114C), mouse monoclonal anti-GFP
276 (MMS-118P) and rabbit polyclonal anti-pericentrin (PRB-432C) (all from Covance, CA); mouse monoclonal anti-
277 p150glued (610473) and mouse monoclonal anti-p50dynamitin (BD 611003) (both from BD Biosciences, Oxford, UK);
278 rabbit polyclonal anti-WDR34 (NBP1-88805, Novus Biologicals, Cambridge, UK), rabbit polyclonal anti-Tctex1 (sc-28537,
279 Santa Cruz Biotechnology, Dallas, TX), mouse monoclonal anti-GAPDH (ab9484, Abcam, Cambridge, UK), rabbit
280 polyclonal anti-TCTEX1D2 (ab139804, Abcam), mouse monoclonal anti-DIC74 (MAB1618, Millipore, Feltham, UK), rabbit
281 polyclonal anti-Lamin A/C (2032, Cell Signaling Technologies, Hitchin UK), rabbit polyclonal anti-OFD1 (a generous gift
282 from Andrew Fry, University of Leicester, UK), and rabbit polyclonal anti-LIS1 (A300-409A, Bethyl Laboratories, from
283 Cambridge Bioscience, Cambridge, UK). Cy2-conjugated donkey anti-mouse (715-225-151), Cy3-conjugated donkey
284 anti-rabbit (711-165-152), peroxidase-conjugated donkey anti-mouse IgG (715-035-150) and peroxidase-conjugated
285 donkey anti-rabbit IgG (711-035-150) were supplied by Jackson ImmunoResearch (from Stratech Scientific, Newmarket,
286 UK). IRDye 800CW donkey anti-mouse (926-32212) and IRDye 680 donkey anti-rabbit (926-32223) were from Li-Cor
287 (Cambridge, UK).

288 CLONING OF MGFP-WDR34, GENERATION OF LENTIVIRUSES AND STABLE CELL LINES

289 The human WDR34 gene (RefSeq: NM_052844.3) was amplified from the Origene cDNA (SC319901, Cambridge
290 Bioscience) by PCR using Phusion™ high-fidelity DNA polymerase PCR kit (New England BioLabs, Hitchin, UK) with
291 forward primer (5'-GGAA-CTCGAG-ATGGCAACCCGCGCAGCC-3') and reverse primer (3'-GAAA-GAATTC-
292 TCAGGCCGCCACCTCTGCTGC-5'), containing the XhoI and EcoRI restriction sites, respectively. The PCR product was sub-
293 cloned into a modified pLVX-Puro vector (Clontech, Mountain View, CA) that includes the mGFP sequence flanked by
294 XhoI and EcoRI restriction sites. The resultant pLVX-Puro-mGFP-WDR34 CDNA was amplified in XL10-Gold
295 ultracompetent bacterial cells (Stratagene) and harvested from the bacterial cells using QIAprep Spin Miniprep Kit
296 (Qiagen, Manchester, UK). All plasmid minipreps were validated by restriction analysis and subsequently sequenced to
297 facilitate secondary validation by sequence alignment analysis.

298 Lentiviral particles of pLVX-Puro-mGFP-WDR34 were produced in HEK293T cells using the Lenti-X™ HTX Packaging
299 System (Clontech), and low passage hTERT-RPE1 cells transduced with the resultant viral supernatant, strictly according
300 to manufacturer's directives. 48 h post-transduction, confluent cells were subcultured into 10 % FBS supplemented
301 DMEM F-12 HAM (Sigma-Aldrich, Poole, UK) containing 5 μ g/ml puromycin. Cells were maintained in 0.25 μ g/ml
302 puromycin after this.

303 PROTEOMICS

304 3 x 15 cm dishes each of hTERT-RPE1-mGFP (control) and hTERT-RPE1-mGFP-WDR34 cells were serum-starved for 24 h
305 at confluence to induce ciliogenesis. Cells were briefly washed twice with 20 mL ice-cold PBS per dish and PBS drained

306 off completely by leaning the dishes at $\sim 60^\circ$ for ~ 10 s. 500 μ L of ice-cold lysis buffer 1 (10 mM Tris-HCl [pH=7.4], 50 mM
307 NaCl, 0.5 mM EDTA, 1.0 % Igepal [CA-630, Sigma], containing freshly added 1mM phenylmethane sulfonylfluoride
308 [PMSF] and 1x EDTA-free Protease Inhibitor Cocktail Set V [539137, Millipore, UK]) was added to the cells and the cells
309 scraped off the dish floor into 2 mL tubes. Cells were lysed by incubation with the buffer on a rotor at $4^\circ\text{C}/30$ min.
310 Lysates were centrifuged at $20,000\text{ g}/4^\circ\text{C}/10$ min and supernatants transferred into pre-cooled 2 mL eppendorf tubes.

311 EQUILIBRATION OF GFP NANO-TRAP BEADS AND IMMUNOPRECIPITATION

312 20 μ L of resuspended GFP nano-trap beads (Chromotek, Planegg-Martinsried, Germany) per sample was added to ice-
313 cold 500 μ L dilution buffer (10 mM Tris-HCl [pH=7.4], 50 mM NaCl, 0.5 mM EDTA, freshly added 1 mM PMSF and 1x
314 EDTA-free Protease Inhibitor Cocktail), spun down at $2700\text{ g}/2$ min/ 4°C and the supernatant discarded. This wash was
315 repeated 2 additional times. The cell lysate supernatants were added to the equilibrated beads and incubated on a
316 rotor $4^\circ\text{C}/2$ h.

317 SAMPLE PREPARATION FOR MASS SPECTROMETRY

318 At the end of the incubation the tubes were centrifuged at $2000\text{ g}/2$ min/ 4°C and the supernatants discarded. 500 μ L
319 of ice-cold dilution buffer was added to the protein-bound beads (pellets), the tubes inverted gently ~ 10 times to
320 resuspend contents and spun down at $2000\text{ g}/2$ min/ 4°C . This wash was repeated 2 more times after which the beads
321 were resuspended in 50 μ L 2X lithium dodecyl sulphate (LDS) sample buffer (NP0007, Life Technologies Ltd, Paisley, UK)
322 (106 mM Tris-HCl, 141 mM Tris-base, 2 % LDS, 10 % Glycerol, 0.51 mM EDTA, 0.22 mM SERVA® Blue G250, 0.175 mM
323 Phenol Red [pH 8.5]) containing 1x Nupage® sample reducing agent (500 mM dithiothreitol) (NP0004). The beads were
324 boiled at $95^\circ\text{C}/10$ min to denature proteins and dissociate precipitated proteins from the GFP nano-trap beads and the
325 tubes immediately placed on ice for 1 min to condense vaporised contents. The tubes were centrifuged at
326 $2700\text{ g}/2$ min/ 4°C , to sediment beads and the supernatants collected into fresh 1.5 mL eppendorf tubes. These
327 samples were submitted to the University of Bristol's proteomics facility for mass spectrometry analysis.

328 MASS SPECTROMETRY

329 Samples were run on a 10 % acrylamide gel to separate proteins. Each gel lane was cut into 6 slices and each slice put
330 through in-gel tryptic digestion in a ProGest automated digestion unit (Digilab UK). An Ultimate 3000 nano HPLC system
331 operated in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific) was employed in fractionating the
332 resulting peptides. Summarily, peptides in 1% (vol/vol) formic acid were injected onto an Acclaim PepMap C18 nano-
333 trap column (Thermo Scientific). The peptides were washed with 0.5 % (vol/vol) acetonitrile in 0.1 % (vol/vol) formic
334 acid and resolved on a 250 mm \times 75 μ m Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over
335 a 150 min organic gradient composed of 7 gradient segments. Peptides were run over the column through the
336 following sequential gradients: 1-6 % solvent B/1 min (solvent B = aqueous 80 % acetonitrile in 0.1 % formic acid;
337 solvent A = 0.1 % formic acid), 6-15 % B/58 min, 15-32 % B/58 min, 32-40 % B/5 min, 40-90 % B/1 min, 90 % B/6 min
338 and finally, 1 % B/1 min; with a flow rate of 300 nL/min. Peptides were subjected to 2.1 kV nano-electrospray ionisation
339 from a 30 μ m (internal diameter) stainless steel emitter (Thermo Scientific) with a capillary temperature of 250°C . An
340 LTQ-Orbitrap Velos mass spectrometer operated by the Xcalibur 2.1 software (Thermo Scientific) in data-dependent
341 acquisition mode was used to acquire tandem mass spectra of the peptides. Survey scans were analysed at 60,000
342 resolution (at m/z 400) within the mass range m/z 300-2000, and the top 20 multiple-charged ions in each duty cycle
343 selected for MS/MS in the LTQ linear ion trap. Charge state filtering, which excludes unassigned precursor ions from
344 fragmentation, and dynamic exclusion (repeat count, 1; repeat duration, 30 s; exclusion list size, 500) were applied. The
345 following fragmentation conditions were set in the LTQ: 40 % normalised energy of collision, 0.25 activation q, 10 ms
346 activation time, and a minimum ion selection intensity of 500 counts.

347 Proteome Discoverer software (version 1.2, Thermo Scientific) was used to process and quantify the raw data files,
348 which were then searched against the UniProt human database (122604 sequences) using the SEQUEST algorithm
349 (version 28, revision 13). Tolerances of 10 ppm and 0.8 Da were set for peptide precursor mass and MS/MS,
350 respectively. Cysteine carbamidomethylation (+57.0214) and methionine oxidation were included in the search criteria
351 as fixed modifications and variable modifications, respectively. Searches were performed with full tryptic digestion
352 allowing a maximum of 1 missed cleavage. All peptide data was filtered to satisfy a 5 % false discovery rate (FDR) by
353 enabling the reverse database search option. The Proteome Discoverer software creates a reverse "decoy" database

354 from the same protein database; peptides evading the initial filtering parameters that were derived from this decoy
355 database are badged as false positive identifications. The minimum cross-correlation factor (Xcorr) filter was separately
356 readjusted for each individual charge state to meet the predetermined target 5 % FDR, considering the number of
357 random false positive matches from the reverse decoy database. Those proteins identified in Supplementary Table 1
358 were selected on the basis of being known components or well-characterized interactors of cytoplasmic dynein-1.

359 GFP-TRAP IMMUNOPRECIPITATION

360 Cells were grown to confluence in 15 cm dishes and serum starved for 24 h. Cells were washed twice with ice-cold PBS,
361 lysed with 500 µl ice-cold buffer (10 mM Tris-HCl pH=7.4, 50 mM NaCl, 0.5 mM EDTA, 1.0% Igepal CA-630, 1 mM PMSF
362 and 1x Protease inhibitor cocktail) on rotator for 30 mins/4 °C and lysate supernatant collected after centrifuging at
363 20,000 g/10 mins/4 °C. Lysate supernatants were incubated with equilibrated GFP nano-trap beads (Chromotek) on
364 rotator for 90 mins/4 °C after which the beads were washed three times with 500 µl dilution buffer (10 mM Tris-HCl
365 pH=7.4, 50 mM NaCl, 0.5 mM EDTA, 1 mM PMSF and 1x Protease inhibitor cocktail) by centrifuging at
366 2000 g/2 mins/4 °C. Beads were resuspended in 50 µl 2x LDS sample buffer (Life Technologies) containing sample
367 reducing agent (Life Technologies) and boiled at 95 °C/10 mins, followed by SDS-PAGE and immunoblotting as
368 described below.

369 SMALL INTERFERING RNA TRANSFECTION

370 Cells were siRNA-transfected by calcium phosphate method at 3% CO₂ (Chen & Okayama, 1988). The medium was
371 changed 20 hours post-transfection and cells were washed with PBS and were incubated for 72 hours (at 37°C and 5%
372 CO₂) with fresh supplemented media. SiRNA duplexes were designed using online algorithms of, and subsequently
373 synthesized by, MWG-Eurofins. BLAST search was performed for these duplexes against the non-redundant database to
374 determine their specificity. Lamin A/C or luciferase GL2 were depleted as targeted controls.

375 Sequences used were as follows:

376 Giantin was depleted with giantin siRNA (#1) ACUUCAUGCGAAGGCCAAATT and giantin siRNA (#2)
377 AGAGAGGCUUAUGAAUCAATT. Duplexes for suppressing WDR34 were (#1) GAUGGUGUCUUGUCUGUAU and (#2)
378 GCUGUUUGAUCUCCAGAAA. WDR60 was targeted with (#1) CCAUUUGGAGAACCAUAU and (#2)
379 CAUGGUUAUAGACCAGUGA. Duplexes targeting NUDCD3 include (#1) GUGAUGCAGUGGUGUGAGA and (#2)
380 GAGAAGGCAGAACUUGAA. Luciferase GL2 (CGUACGCGGAUACUUCGAUU) and lamin A/C
381 (CUGGACUUCAGAGAACA) were used as targeted negative and positive controls, respectively. All siRNAs were
382 purchased from MWG Eurofins (Ebersberg, Germany). TCTEX1D2 was depleted with (#1)
383 AGAGGUGAAGGAGUAUUCATT, (#2) UGCUGAAUAUUCUCCAGAATT and (#3, targeting 3'UTR)
384 AGGACAUGACCAUGAAGAATT

385 QUANTITATIVE PCR

386 RNA was extracted from transfected cells using a Qiagen RNeasy purification kit according to the manufacturer's
387 instructions (Qiagen, Manchester, UK). cDNA was generated using an SuperScriptTM III First Strand Synthesis System
388 (Life Technologies). DNA was amplified under the following conditions: 95°C for 10 minutes then 30 cycles of 95°C for
389 30 seconds, 55°C for 30 seconds, 70°C for 15 seconds. Amplification was monitored by incorporation of SYBR[®] Green
390 (Finnzyme, Espoo, Finland) and analysed on a BioRad Opticon 2 PCR system (BioRad, Hemel Hempstead, UK). Gene
391 expression was quantified by the $\Delta\Delta CT$ method (Livak & Schmittgen, 2001) normalizing against GAPDH. Single product
392 amplification was verified by performing melting curve analysis and gel electrophoresis. Primers (MWG Eurofins) used
393 were as follows: TCTEX1D2 forward primer: 5'- GGAGCCCAGAACACCTATATT-3'; reverse primer: 5'-
394 GCTGAGGCATTTCTTCTGGAGA-'; GAPDH forward primer: 5'-ATCCCATCACCATCTTCCAG-3'; reverse primer: 5'-
395 CCATCACGCCACAGTTTCC-3'.

396 SUCROSE DENSITY GRADIENT CENTRIFUGATION

397 Cells were grown and lysed as described for the GFP-trap experiment and lysate supernatants layered onto 5-40% or 5-
398 20% continuous sucrose gradient columns. The former were poured using a Perspex gradient mixer, the latter using
399 Biocomp isopycnic gradient forming tube caps (10 mm isopycnic long caps, Biocomp from Wolf Laboratories,

400 Manchester, UK). Gradients were centrifuged at 23,700 rpm (96119 xg)/18 h/4 °C in a TH-641 swinging bucket rotor
401 (Thermo Scientific). Proteins were precipitated with 250 μ l or 125 μ l 100% trichloroacetic acid at 4 °C /1 h from 1 ml or
402 500 μ l column fractions respectively, pelleted by centrifuging at 13,200 rpm/5 mins/4 °C, and washed three times with
403 150 μ l ice-cold acetone. The acetone was chased off by incubation at room temperature for 30 minutes and pellets
404 resuspended in 1x LDS sample buffer containing reducing agent.

405 IMMUNOBLOTTING

406 For immunoblots to validate siRNA efficacy, cells were lysed and samples were separated by SDS-PAGE followed by
407 transfer to nitrocellulose membranes; primary antibodies were detected using HRP-conjugated secondary antibodies
408 (Jackson ImmunoResearch, West Grove, PA) and enhanced chemiluminescence (ECL, GE Healthcare, Cardiff, United
409 Kingdom). Immunoblots in Fig. 6 were developed using an Odyssey Sa imager (Li-Cor, Cambridge, UK).

410 GEL FILTRATION

411 The following buffer was used for gel filtration: 30 mM HEPES (pH 7.0), 150 mM KOAc, 2 mM MgSO₄, 0.68 M glycerol, 2
412 mM DTT. A 24 ml Superose 6 column (GE Healthcare) was calibrated using 70S ribosome, apoferritin, b-amylase, bovine
413 serum albumin and carbonic anhydrase. Cells expressing either GFP or mGFP-WDR34 were lysed at 4°C using a ball
414 bearing cell homogenizer with 10 μ m clearance (Isobiotec, Heidelberg) in gel filtration buffer and cleared by
415 centrifugation for 30 minutes at 25,000 xg . Fractions (0.5ml each) were collected and fluorescence of each sample
416 measured using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara, CA) at 25°C with
417 excitation at 490 nm (5 nm excitation slit) and emission scanning 500-600 nm with 5 nm emission slits.

418 IMMUNOLABELLING AND MICROSCOPY

419 Medium was removed and cells were subsequently washed with PBS. Cells were then fixed using cold methanol for 4
420 minutes at -20°C. For images in Figure 1E and 1F, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with
421 0.1% Triton X-100 for 5 minutes and then washed in PBS. After two washes in PBS, cells were blocked using a 3% bovine
422 serum albumin (BSA) in PBS for 30 minutes at room temperature. Three washes with PBS of 5 minutes each at room
423 temperature were done after each of the primary and secondary antibody incubations. Nuclear staining was done using
424 DAPI (4,6-Diamidino-2-phenylindole (Life Technologies) diluted at 1:5000 in distilled water) for 3 minutes at room
425 temperature, cells were then rinsed twice in PBS. Cells were imaged using an Olympus IX-71 or IX-81 widefield
426 microscope with a 63x 1.42 N.A. objective, and excitation and emission filter sets (Semrock, Rochester, NY) controlled
427 by Volocity software (v. 4.3, Perkin-Elmer, Seer Green, UK).

428 PROCESSING AND QUANTIFICATION OF IMAGE DATA AND STATISTICAL ANALYSIS

429 Representative images are shown, all experiments were repeated independently at least three times each. Samples
430 were compared using Kuskall-Wallis one-way analysis of variance with Dunn's post-hoc test using GraphPad Prism v 4.
431 All images were prepared with Adobe Photoshop and Adobe Illustrator. All data and resulting statistical analyses arise
432 from 3 independent experiments.

433

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440 The authors declare no competing interests.

441

442

443 CONTRIBUTIONS

444 D.A. performed experiments and analyzed data; N.S. performed experiments and analyzed data; D.J.S. conceived and
445 directed the project, analyzed data, and wrote the manuscript. All authors reviewed and contributed to the writing of
446 the manuscript.

447 FIGURE LEGENDS

448 Figure 1: Localization of mGFP-WDR34. (A) mGFP-WDR34 (green) localizes to a cytosolic pool and to primary cilia
449 colocalizing with acetylated tubulin (red). (B) γ -tubulin labelling, (C) ODF2, and (D) OFD1 (red in each case) demonstrate
450 the accumulation of mGFP-WDR34 at the base of the cilium with some labelling evident along its length. (E) Cells
451 expressing mGFP-WDR34 were fixed with paraformaldehyde and labelled to detect acetylated tubulin and WDR34 as
452 indicated. Two examples are shown. (F) Cells expressing GFP were processed as in (E) to demonstrate an absence of
453 GFP from primary cilia. Bars (all panels) = 5 μ m.

454 Figure 2: Immunoprecipitation from serum-starved RPE1 cells stably expressing either mGFP or mGFP-WDR34. GFP-
455 traps of cells expressing mGFP alone or mGFP-WDR34 were separated by SDS-PAGE and immunoblotted to detect (A)
456 WDR34, (B) WDR60, (C) IC74 (DYNC1I2), (D) GAPDH, (E) p150^{Glued}, (F) p50^{dynamitin}, (G) LIS1, (H) Tctex-1, (I) NudCD3, or (J)
457 TCTEX1D2 as indicated. Molecular weight markers are shown (kDa). In each case the lanes show I = input, U = unbound
458 fraction, B = bound fraction as indicated.

459 Figure 3: Sucrose density gradient centrifugation of cell lysates from RPE1 cells. (A) RPE1 cells were lysed and loaded
460 onto a 5-40% sucrose density gradient. After centrifugation, 1 ml fractions were removed from the gradient and probed
461 by immunoblotting to determine the distribution across the gradient of DIC74 (DYNC1I2), WDR34, or GAPDH. (B) RPE1
462 cells stably expressing (B) GFP or (C) mGFP-WDR34 were lysed and loaded onto a 5-20% sucrose density gradient.
463 Twenty 500 μ l fractions were collected post-centrifugation and alternate fractions analysed by SDS PAGE and
464 immunoblotting for GFP, DIC74, GAPDH and WDR60. (D) Gel filtration of lysates from GFP expressing cells (gray bars)
465 and mGFP-WDR34 expressing cells (black bars). Molecular mass calibration is shown below the graph aligned to the
466 fraction numbers.

467 Figure 4: The stabilities of WDR34 and WDR60 are interdependent. (A, B) Cells were transfected with siRNA duplexes
468 targeting WDR34 or WDR60 as indicated and following serum starvation, fixed and immunolabelled to detect acetylated
469 tubulin and (A) WDR60 or (B) WDR34. (C) Cells depleted of WDR60 were effectively depleted (shown by loss of
470 immunoreactivity around the centrosome) and labelling showed either longer cilia (example shown for WDR60-1) or a
471 failure to generate cilia (example shown for WDR60-2). (D) Quantitation of the proportion of cells that produced cilia in
472 response to 24 hours serum starvation. Asterisks indicate statistical significance (* $p < 0.05$; ** $p < 0.01$). (E) Lengths of
473 remaining cilia were measured for control (GL2) or WDR60-depleted cell cultures. Bars show mean and s.d.; asterisks
474 indicate statistical significance (***) $p < 0.001$. (F) Distribution of cilium length represented as a cumulative frequency
475 chart of the percentage of total cilia found in 0.25 μ m bins. Data plotted is the same as in (E). (G) Immunoblotting
476 confirmed the efficacy of WDR34 and WDR60 siRNAs. Immunoblotting for WDR34 following suppression of WDR60 and
477 vice versa was also used to test interdependency of these subunits. Lamin A/C is included as a negative control, GAPDH
478 as a loading control. Molecular weight markers are indicated (kDa). (H, I) Results were analysed to determine whether
479 depletion of both WDR34 and WDR60 simultaneously could enhance the phenotypes seen in terms of (H) the number
480 of cells producing cilia or (I) the length of remaining cilia. In both cases no additive effect is seen. Bars show mean and
481 s.d.; asterisks indicate statistical significance (***) $p < 0.001$. (J) Distribution of cilium length represented as a cumulative
482 frequency chart of the percentage of total cilia found in 0.25 μ m bins. Data plotted is the same as in (I). Bar (all panels)
483 = 10 μ m.

484 Figure 5: Suppression of giantin results in a loss of pericentrosomal WDR60. (A) Cells depleted of giantin were labelled
485 to detect acetylated tubulin and WDR60. (B) The area of pericentrosomal WDR60 labelling was measured and plotted
486 (arbitrary units; bars indicate mean and s.d.; asterisks indicate statistical significance: ***) $p < 0.001$. (C) The loss of
487 pericentrosomal WDR60 labelling correlates with the efficacy of giantin suppression. (D) Data were quantified and the
488 correlation tested using both Pearson's and Spearman coefficients. Colour coding indicates siRNA transfection as
489 indicated. Bar (all panels) = 10 μ m.

490 Figure 6: NudCD3 is required for ciliogenesis and cilia length control. Two independent siRNA duplexes targeting
491 NudCD3 were used. (A) Immunoblots show tubulin as a loading control, lamin A/C as a siRNA control and NudCD3. (B)
492 Cells were immunolabelled to detect pericentrin and acetylated tubulin. Enlargements highlight the failure to extend
493 cilia from the basal body in NudCD3-depleted cells. (C) The proportion of ciliated cells was quantified; asterisks indicate

494 statistical significance (* $p < 0.05$). (D) Further examples of NudCD3 depleted cells show those in which cilia are evident.
495 (E) Quantitation shows that in cells depleted of NudCD3 that extended cilia, these were longer than those seen in
496 control cells. Bars show mean and s.d.; asterisks indicate statistical significance (***) $p < 0.001$. (F) Distribution of cilium
497 length represented as a cumulative frequency chart of the percentage of total cilia found in 0.25 μm bins. Data plotted
498 is the same as in (E). (G) Depletion of NudCD3 results in a loss of pericentrosomal WDR34 (red) as shown by
499 immunofluorescence. The centrosome and cilia are labelled with acetylated tubulin. (H) Data were quantified and
500 statistical significance is indicated, ** = $p < 0.05$, *** = $p < 0.01$. (I) Suppression of NudCD3 does not affect the
501 accumulation of OFD1 (red) around the centrosome (acetylated tubulin, green). Bar (all panels) = 10 μm .

502 Figure 7: TCTEX1D2 depletion increases cilia length. RPE1 cells were depleted of TCTEX1D2 using 3 different siRNA
503 duplexes as indicated and then serum starved for 24 hours prior to (A) analysis by QPCR or (B-D) fixation and
504 immunolabelling to detect acetylated tubulin and pericentrin. (A) Relative TCTEX1D2 mRNA expression in transfected
505 cells, as determined by RT-PCR. Error bars show s.e.m. (n=3). (B) Representative maximum intensity z-stack projections
506 of control and depleted cells. Enlargements highlight the increase in cilia length upon TCTEX1D2 depletion. Bar = 10 μm .
507 (C) Dot plot comparing cilia lengths in control and TCTEX1D2 depleted cells. Bars show mean and s.d. ($p < 0.001$, n=3).
508 (D) Distribution of cilium length represented as a cumulative frequency chart of the percentage of total cilia found in
509 0.25 μm bins. Data plotted is the same as in (B). (E) The percentage of ciliated cells found in control and TCTEX1D2
510 depleted cells. There is no consistent change following siRNA treatment. Error bars show s.e.m.

511 Figure 8: Schematic of cytoplasmic dynein-1 and dynein-2 complexes. Light intermediate chains are shown as hexagons,
512 intermediate chains as extended ovals, and light chains as circles. Additional interacting partners are seen for dynein-1
513 (left) that are not associated with dynein-2 (right). NudCD3 associates with both cytoplasmic dynein complexes. It
514 remains unclear whether TCTEX1D2 is present as a monomer or dimer within the complex.

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TABLE 1: PROTEOMIC ANALYSIS OF mGFP-WDR34 VERSUS mGFP IN STABLY-TRANSFECTED, SERUM-STARVED HTERT-RPE1 CELLS.

TABLE 1A: CYTOPLASMIC DYNEIN-2 SUBUNITS, NOT FOUND ASSOCIATED WITH DYNEIN-1

Accession	# AAs	MW [kDa]	Description	Score mGFP	Coverage mGFP	# PSM mGFP	# Peptides mGFP	Score mGFP-WDR34	Coverage mGFP-WDR34	# PSM mGFP-WDR34	# Peptides mGFP-WDR34	Annotation
Q96EX3	536	57.8	WD repeat-containing protein 34 GN=WDR34	137.93	44.59	31	16	3732.64	79.85	873	34	DYNEIN WDR34
Q8WVS4	1066	122.5	WD repeat-containing protein 60 GN=WDR60	n.d.	n.d.	n.d.	n.d.	329.54	37.24	89	33	DYNEIN WDR60
B011S0	4307	492.3	DYNC2H1 variant protein GN=DYNC2H1	n.d.	n.d.	n.d.	n.d.	37.31	2.48	10	10	DYNEIN HEAVY CHAIN 2
E5RJK4	72	7.9	Cytoplasmic dynein-2 light intermediate chain GN=DYNC2LI1	n.d.	n.d.	n.d.	n.d.	5.38	20.83	1	1	DYNEIN LIC3
Q8WW35	142	16.1	Tctex1 domain-containing protein 2 GN=TCTEX1D2	n.d.	n.d.	n.d.	n.d.	110.43	45.07	24	5	DYNEIN TCTEX1D2

n.d. = not detected

TABLE 1B: DYNEIN-1 LIGHT CHAINS ALSO FOUND ASSOCIATED WITH DYNEIN-2

Accession	# AAs	MW [kDa]	Description	Score mGFP	Coverage mGFP	# PSM mGFP	# Peptides mGFP	Score mGFP-WDR34	Coverage mGFP-WDR34	# PSM mGFP-WDR34	# Peptides mGFP-WDR34	Annotation
P63172	113	12.4	Dynein light chain Tctex-type 1 GN=DYNLT1	n.d.	n.d.	n.d.	n.d.	84.63	60.18	19	4	DYNEIN TCTEX1
P51808	116	13.1	Dynein light chain Tctex-type 3 GN=DYNLT3	n.d.	n.d.	n.d.	n.d.	7.97	23.28	3	2	DYNEIN TCTEX3
A4D1D1	297	32.5	Similar to dynein, cytoplasmic, light peptide; 8kD LC; dynein LC8; protein inhibitor of neuronal nitric oxide synthase GN=LOC392067	n.d.	n.d.	n.d.	n.d.	4.21	7.41	2	1	DYNEIN LC8
P63167	89	10.4	Dynein light chain 1, cytoplasmic OS=Homo sapiens GN=DYNLL1	23.71	64.04	7	5	405.41	64.04	109	6	DYNEIN LIGHT CHAIN 1
Q96FJ2	89	10.3	Dynein light chain 2, cytoplasmic OS=Homo sapiens GN=DYNLL2	19.29	25.84	5	3	255.84	65.17	81	6	DYNEIN LIGHT CHAIN 2
Q9NP97	96	10.9	Dynein light chain roadblock-type 1 OS=Homo sapiens GN=DYNLRB1	52.23	55.21	16	4	709.81	85.42	202	10	DYNEIN ROADBLOCK
Q8TF09	96	10.8	Dynein light chain roadblock-type 2 OS=Homo sapiens GN=DYNLRB2	19.08	16.67	6	2	170.55	33.33	54	3	DYNEIN ROADBLOCK 2

n.d. = not detected

TABLE 1C: DYNEIN-1 ACCESSORY FACTORS ALSO FOUND IN ASSOCIATION WITH DYNEIN-2

Accession	# AAs	MW [kDa]	Description	Score mGFP	Coverage mGFP	# PSM mGFP	# Peptides mGFP	Score mGFP-WDR34	Coverage mGFP-WDR34	# PSM mGFP-WDR34	# Peptides mGFP-WDR34	Annotation
B0FTY2	361	40.8	NudC-like protein OS=Homo sapiens GN=NUDCD3	n.d.	n.d.	n.d.	n.d.	7.77	5.54	3	2	NUDCD3
Q9Y266	331	38.2	Nuclear migration protein NudC OS=Homo sapiens GN=NUDC	7.33	3.32	2	1	27.18	10.57	7	3	NUDC

n.d. = not detected

TABLE 1D: DYNEIN-1 ACCESSORY FACTORS THAT WERE NOT FOUND IN ASSOCIATION WITH DYNEIN-2

Accession	# AAs	MW [kDa]	Description	Score mGFP	Coverage mGFP	# PSM mGFP	# Peptides mGFP	Score mGFP-WDR34	Coverage mGFP-WDR34	# PSM mGFP-WDR34	# Peptides mGFP-WDR34	Annotation
G5E9H4	1139	126.7	Dynactin 1 (P150, glued homolog, Drosophila), isoform CRA_a OS=Homo sapiens GN=DCTN1	2.70	1.14	1	1	10.04	1.05	1	1	DYNACTIN
H0YIC1	228	25.4	Bicaudal D-related protein 1 (Fragment) OS=Homo sapiens GN=CCDC64	n.d.	n.d.	n.d.	n.d.	2.43	4.39	1	1	BICAUDAL D1
B4DWL0	270	30.9	Bicaudal D-related protein 1 OS=Homo sapiens GN=CCDC64	19.73	5.19	2	1	n.d.	n.d.	n.d.	n.d.	BICAUDAL D RELATED PROTEIN 1
I3L2U8	345	38.9	Platelet-activating factor acetylhydrolase IB subunit alpha OS=Homo sapiens GN=PAFAH1B1	6.44	9.28	2	2	n.d.	n.d.	n.d.	n.d.	LIS1

n.d. = not detected

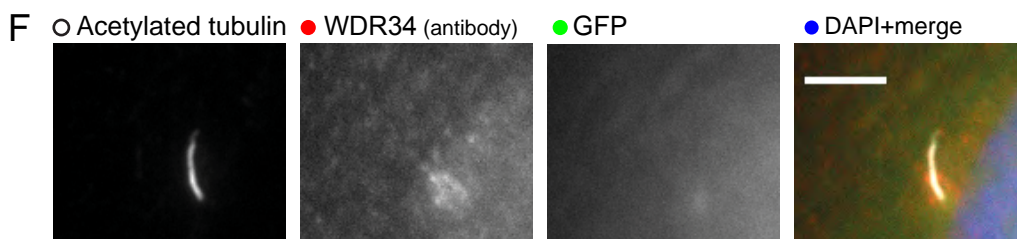
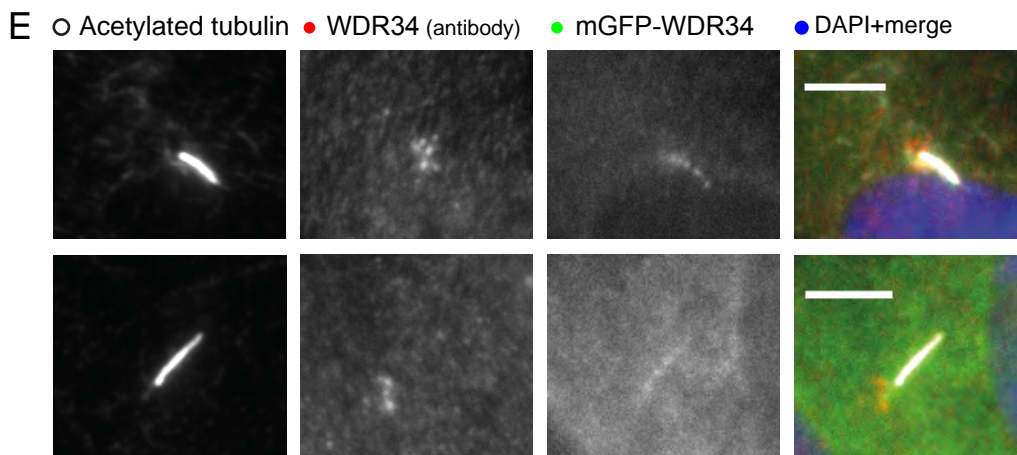
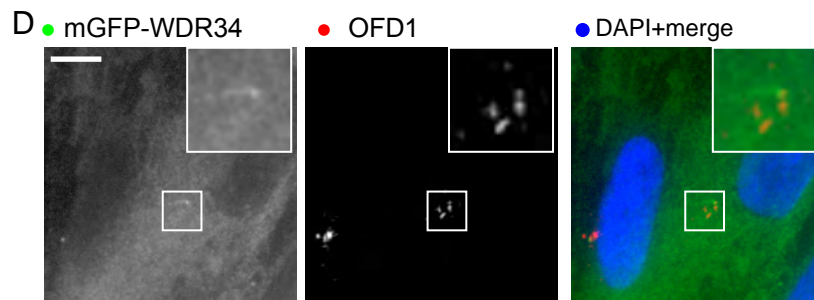
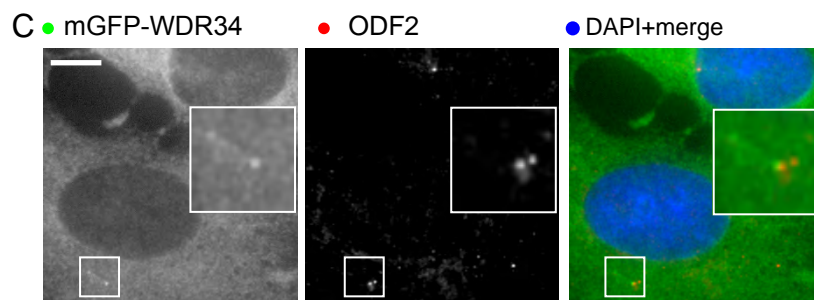
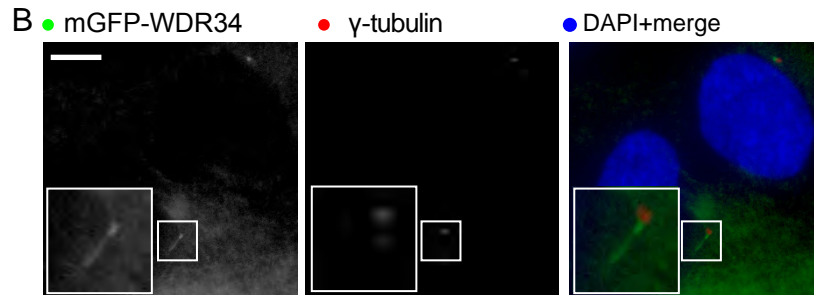
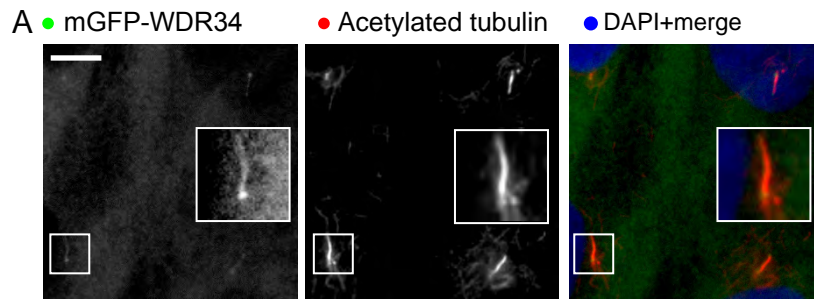


Figure 2

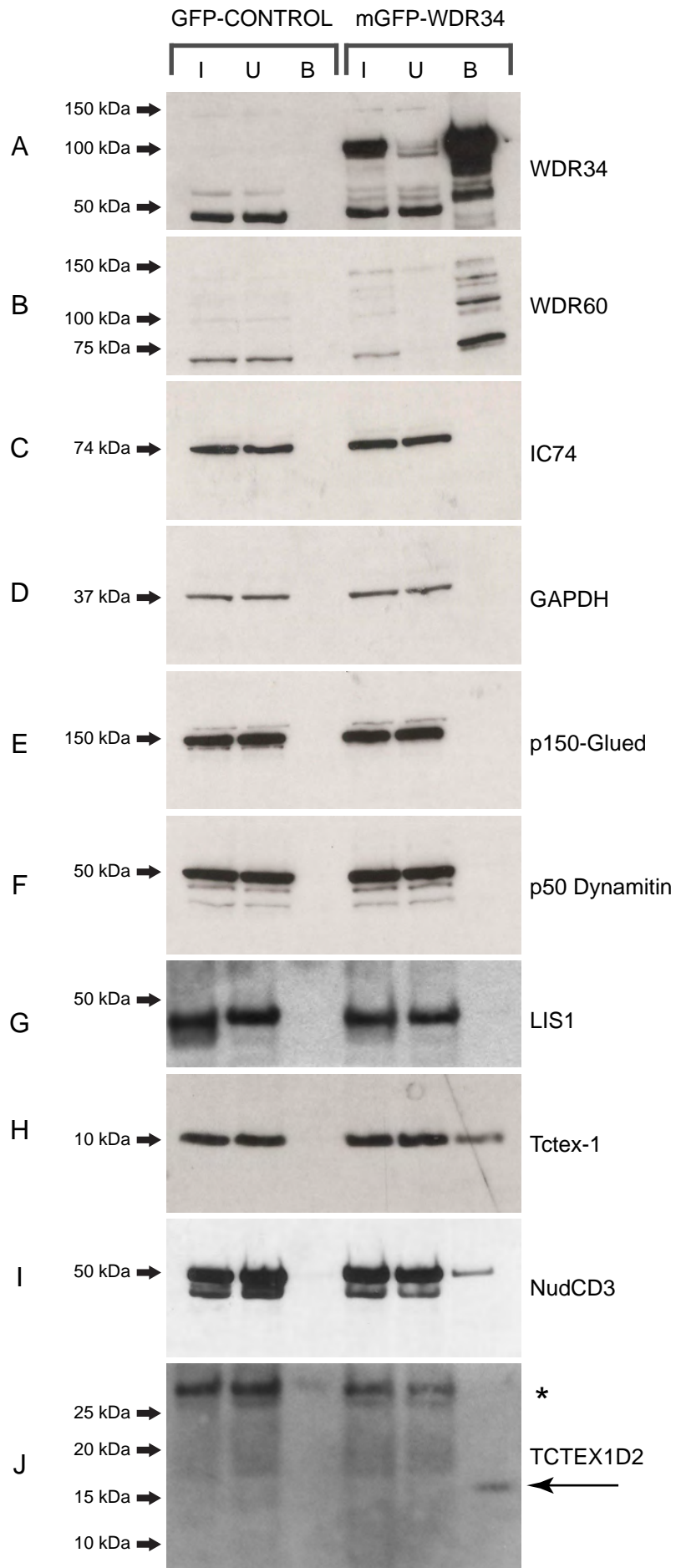


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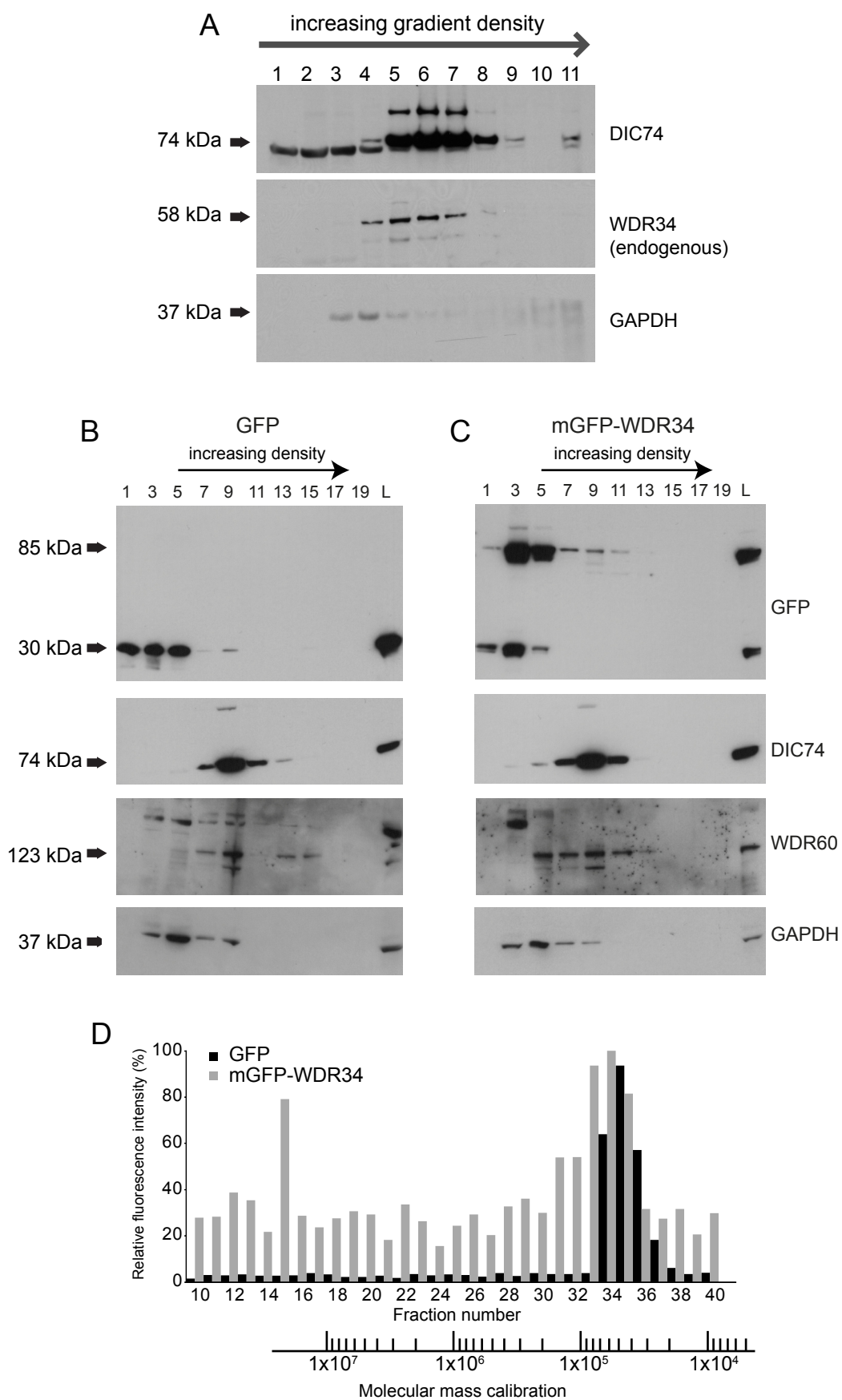


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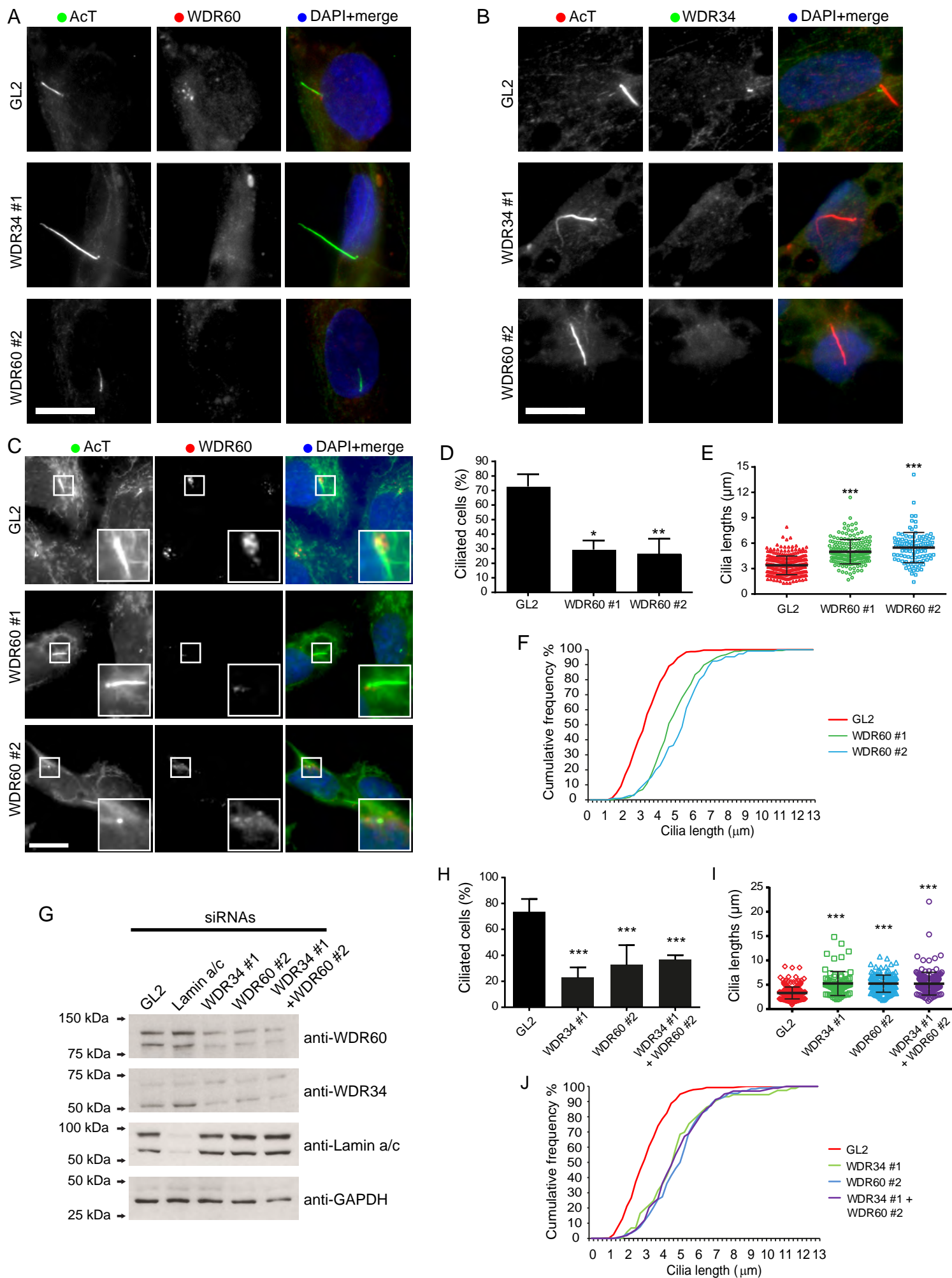


Figure 5

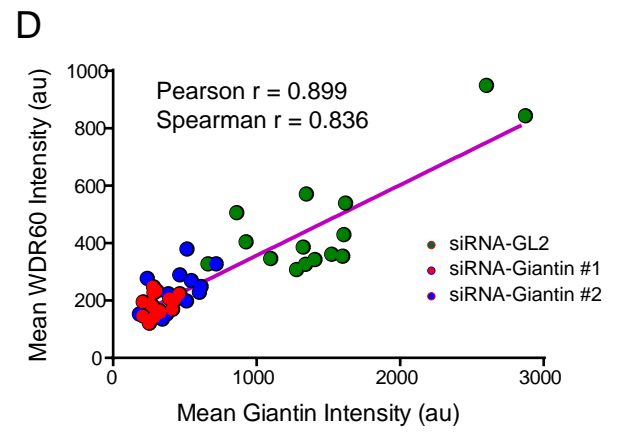
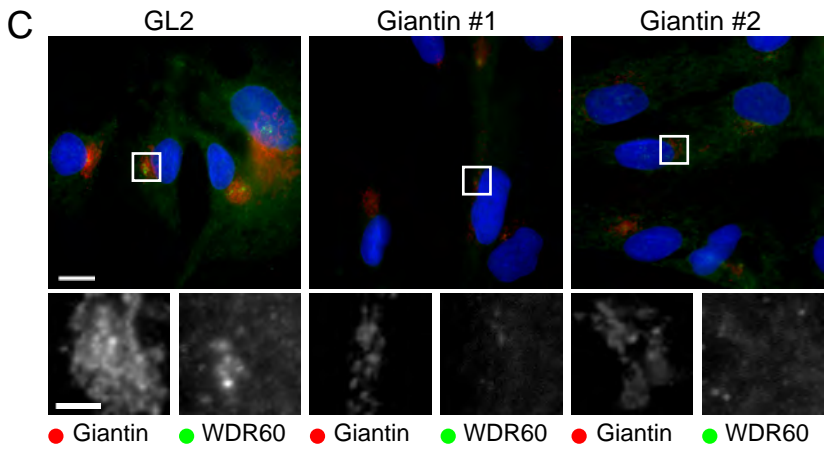
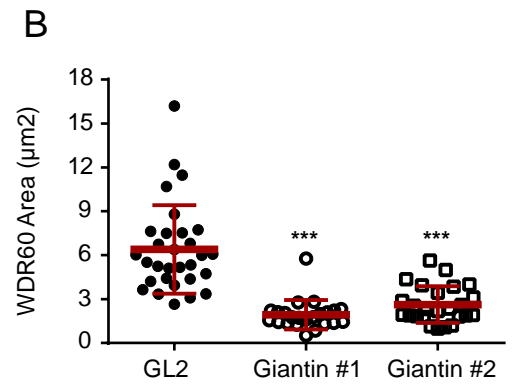
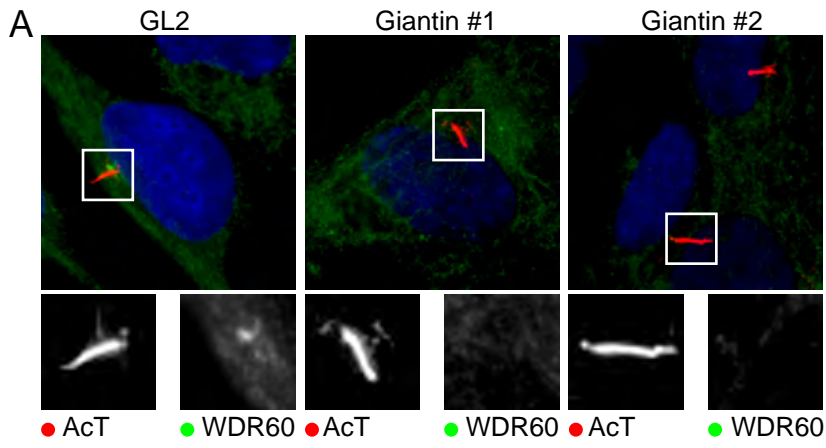


Figure 6

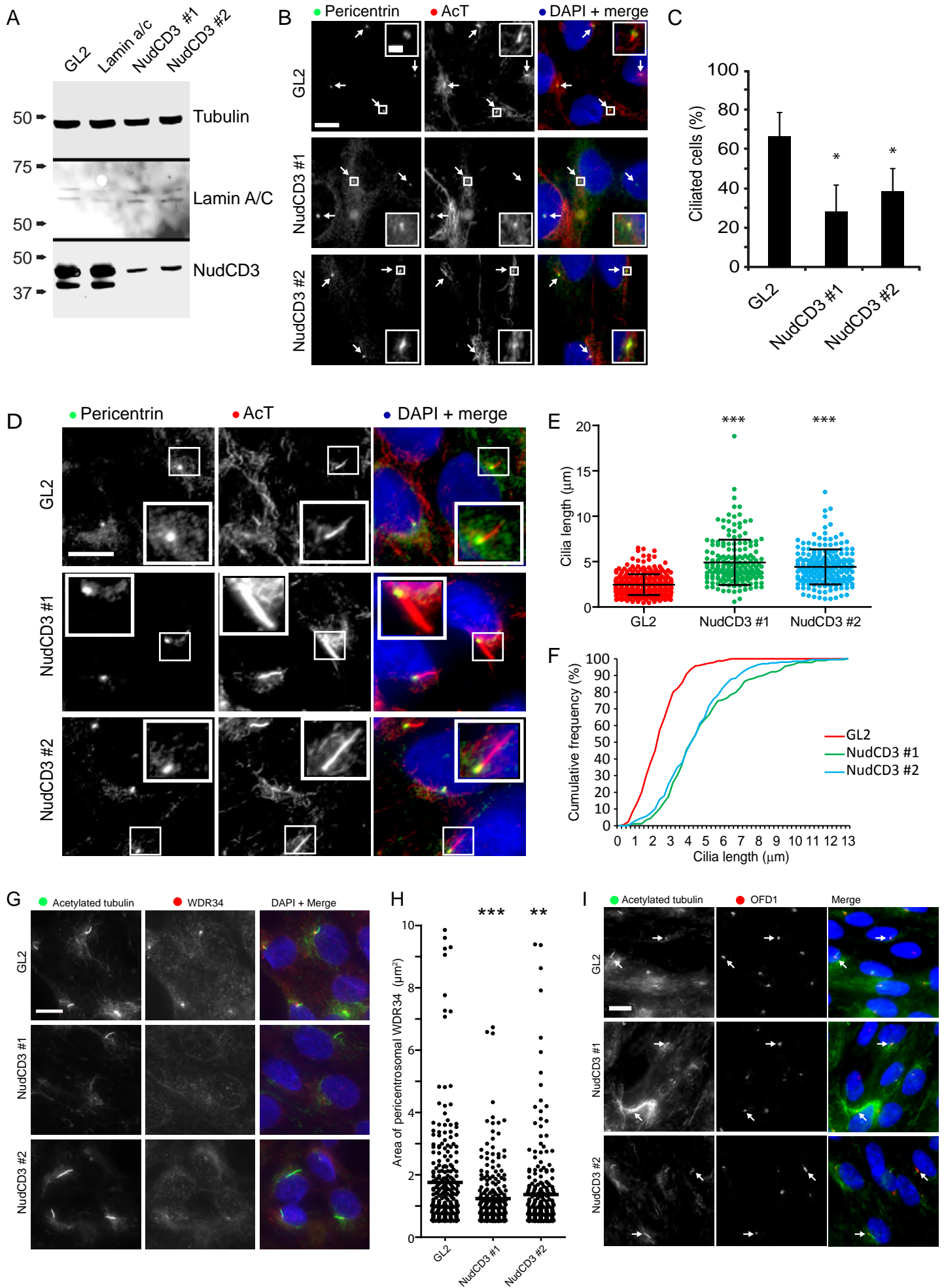


Figure 7

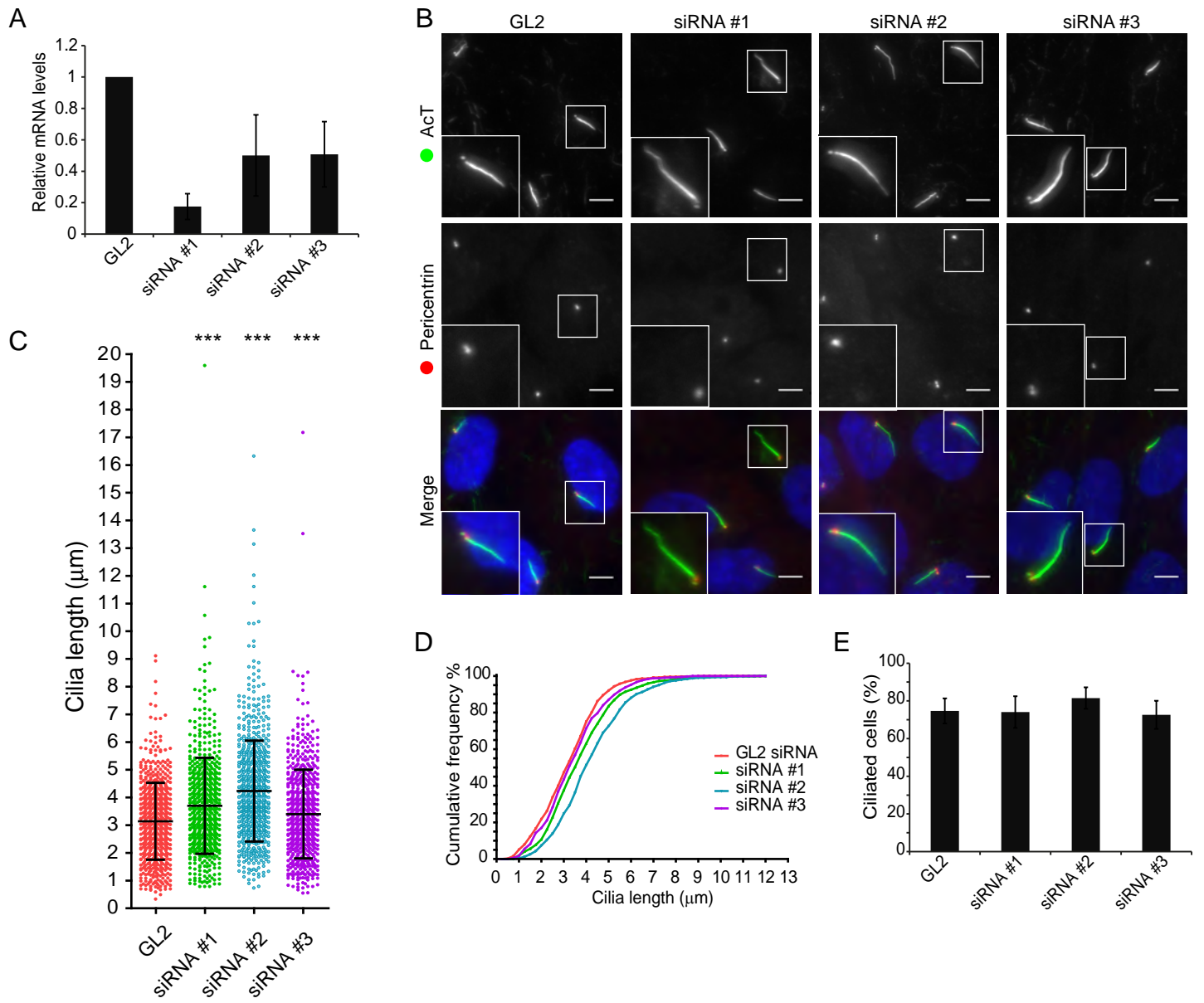


Figure 8

