1 **Trisomy 21 induces pericentrosomal crowding disrupting early stages of primary**

2 **ciliogenesis and mouse cerebellar development**

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4 Cayla E Jewett[1,2], Bailey L McCurdy[1], Eileen T O'Toole[3], Katherine S Given[1], Carrie H Lin[1],

5 Valerie Olsen[1], Whitney Martin[4], Laura G Reinholdt[4], Joaquin M Espinosa[2,5], Kelly D Sullivan[2,6],

6 Wendy B Macklin[1], Rytis Prekeris[1*], Chad G Pearson[1,2*]

7

8 [1]Department of Cell and Developmental Biology, University of Colorado-Anschutz Medical

9 Campus, Aurora, CO 80045, USA.

10 [2]Linda Crnic Institute for Down Syndrome, University of Colorado-Anschutz Medical Campus,

11 Aurora, CO 80045, USA.

12 [3]Molecular, Cellular, and Developmental Biology, University of Colorado at Boulder, Boulder,

13 CO 80302, USA.

14 [4]The Jackson Laboratory, Bar Harbor, ME 04609, USA.

15 [5]Department of Pharmacology, University of Colorado-Anschutz Medical Campus, Aurora, CO

16 80045, USA.

17 [6]Department of Pediatrics, Section of Developmental Biology, University of Colorado-Anschutz

18 Medical Campus, Aurora, CO 80045, USA.

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20 *Correspondence: rytis.prekeris@cuanschutz.edu and chad.pearson@cuanschutz.edu

21 **Abbreviations:** HSA21, human chromosome 21; MMU, mouse chromosome; PCNT,

22 pericentrin; DS, Down syndrome; Shh, Sonic hedgehog; MEF, mouse embryonic fibroblast;

23 RPE1, retinal pigmented epithelia

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25 **Keywords:** Pericentrin, primary cilia, Trisomy 21, Down syndrome, MyosinVA, CP110, Sonic

26 hedgehog, cerebellar neuronal precursor

Summary

Primary cilia are signaling organelles essential for development and homeostasis. Loss of primary cilia is lethal, and decreased or defective cilia cause multisystemic conditions called ciliopathies. Down syndrome shares clinical overlap with ciliopathies. We previously showed that trisomy 21 diminishes primary cilia formation and function due to elevated Pericentrin, a centrosome protein encoded on chromosome 21. Pericentrin is mislocalized, creating aggregates that disrupt pericentrosomal trafficking and microtubule organization. Here, we examine the cilia-related molecules and pathways disrupted in trisomy 21 and their *in vivo* phenotypic relevance. Utilizing ciliogenesis time course experiments, we reveal how Pericentrin, microtubule networks, and components of ciliary vesicles are reorganized for ciliogenesis in euploid cells. Early in ciliogenesis, chromosome 21 polyploidy results in elevated Pericentrin and microtubule networks away from the centrosome that ensnare MyosinVA and EHD1, blocking mother centriole uncapping that is essential for ciliogenesis. Ciliated trisomy 21 cells have persistent trafficking defects that reduce transition zone protein localization, which is critical for Sonic hedgehog signaling. Sonic hedgehog signaling is decreased and anticorrelates with Pericentrin levels in trisomy 21 primary mouse embryonic fibroblasts. Finally, we observe decreased ciliation *in vivo.* A mouse model of Down syndrome with elevated Pericentrin has fewer primary cilia in cerebellar granule neuron progenitors and thinner external granular layers. Our work reveals that elevated Pericentrin in trisomy 21 disrupts multiple early steps of ciliogenesis and creates persistent trafficking defects in ciliated cells. This pericentrosomal crowding results in signaling defects consistent with the neurological deficits found in individuals with Down syndrome.

Introduction

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51   Trisomy 21 or Down syndrome (DS) is a common chromosomal disorder characterized by

52   phenotypes including craniofacial abnormalities, intellectual disability, heart defects, and

53   cerebellar hypoplasia.[1-3] These pathologies overlap with those of ciliopathies—genetic disorders

54   affecting primary cilia.[4] Primary cilia are concentrated signaling hubs, particularly for the Sonic

55   hedgehog pathway (Shh), and are essential for vertebrate development. The primary cilium

56   nucleates from the centrosome and projects into the extracellular space. The centrosome is

57   comprised of a mother and daughter centriole and surrounding pericentriolar material. Cilia

58   formation requires the spatial and temporal coordination of many molecules. It begins with

59   remodeling the mother centriole, including addition of appendages that serve as docking

60   platforms for cargo delivery.[5-7] This triggers removal and proteasomal degradation of the mother

61   centriole capping proteins, allowing extension of axoneme microtubules and membrane

62   remodeling to ensheath the axoneme with a ciliary membrane.[5,8,9] A barrier complex forms at

63   the base of the cilium called the transition zone, that restricts access to and from the cilium. This

64   creates a unique ciliary compartment that is biochemically distinct from the rest of the cell,

65   allowing the cilium to function as a specialized signaling organelle.[10]

66   Building and maintaining signaling cilia requires trafficking of molecules to and from the

67   centrosome.[11,12] Molecules are trafficked in both membrane-derived vesicles and granular

68   moieties, called centriolar satellites, that move along microtubules nucleated and organized by

69   the centrosome.[13-15] Pericentrin (PCNT) is an essential centrosome scaffolding protein that,

70   together with CDK5RAP2/CEP215, organizes γ-tubulin and microtubules,[16,17] and in *Drosophila*

71   the PCNT ortholog is required for cilia function in sensory cells and sperm.[18] *PCNT* is encoded

72   on human chromosome 21 (HSA21), and we previously showed that elevated PCNT due to

73   increased copy number in trisomy 21 is necessary and sufficient to initiate cilia defects in human

74   DS-derived fibroblasts compared to age- and sex-matched controls.[19] Elevated PCNT forms

75   aggregates that colocalize with satellite proteins such as PCM1 and disrupts the flux of

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76   intracellular components to and from the centrosome, analogous to a traffic jam.[20] While we

77   showed reduced centrosome localization of the ciliary IFT20 protein in trisomy 21 cells,[19] IFT20

78   recruitment to the cilium is a later step in the process of ciliogenesis[9,21]. Thus, it is unknown

79   which molecules or pathways are disrupted in trisomy 21 that reduce cilia formation and

80   signaling, and whether these cilia defects observed in cultured cells contribute to DS-associated

81   phenotypes *in vivo*.

82   Here, we use isogenic human cell lines to eliminate genetic variability and mouse

83   models of DS to show that elevated PCNT induces trafficking defects around the centrosome

84   such that cargo trafficking events required for early steps in primary ciliogenesis, including

85   ciliary vesicle formation and mother centriole uncapping are held up in a 'pericentrosomal

86   crowd'. Of the trisomy 21 cells that do ciliate, intracellular trafficking defects persist as transition

87   zone proteins are unable to reach the centrosome efficiently, thereby decreasing their

88   localization at the transition zone. Consistent with transition zone defects, Shh signaling is

89   reduced and anticorrelates with PCNT levels. A mouse model of DS with increased *Pcnt* copy

90   number and elevated PCNT levels has reduced primary cilia in both primary mouse embryonic

91   fibroblasts (MEFs) as well as cerebellar neuronal precursor cells *in vivo*. Consistent with ciliary

92   assembly and signaling defects, these mice have a thinner external granular layer and fewer

93   neuronal protrusions. Our findings reveal how early events in ciliogenesis are disrupted by a

94   PCNT-overexpression-induced crowding phenotype and that these ciliation and signaling

95   defects have consequences for *in vivo* brain development in DS.

96

97   **Results (3500 words)**

98   *Rapid PCNT and microtubule reorganization in response to ciliation cues increases with HSA21*

99   *dosage*

100   We previously showed that elevated PCNT is necessary and sufficient for decreased ciliation in

101   trisomy 21 cells.[19,20] Whereas PCNT normally nucleates and organizes microtubules emanating

102   from centrosomes, elevated PCNT has three major consequences in trisomy 21 cells (Figure

103   1A): 1) PCNT nucleates excess microtubules, thereby increasing microtubule density around

104   the centrosome; 2) PCNT forms large protein aggregates along these microtubules; and 3)

105   PCNT nucleates cytoplasmic microtubules that are disconnected from the centrosome.[19,20]

106   Together, elevated PCNT perturbs trafficking to and from centrosomes by altering microtubule

107   networks and molecular composition of the pericentrosomal region, thereby hindering the

108   exchange of molecules required for ciliogenesis – pericentrosomal crowding. This model was

109   derived from an analysis of either cycling cells or cells 24 hours after serum depletion (0.5%

110   serum in DMEM) to induce G1 cell cycle arrest and ciliogenesis.[19,20] We questioned whether

111   elevated PCNT results in an immediate trafficking delay upon induction of ciliogenesis or if

112   trafficking defects build up over time such that only later steps of ciliogenesis are affected.

113   To differentiate between these alternatives, we used human Retinal Pigmented Epithelial

114   (RPE1) cells to perform a time course during ciliogenesis and analyzed how PCNT and

115   microtubule organization change at the centrosome. In RPE1 cells with two copies of HSA21

116   (D21), PCNT levels increased rapidly by approximately 50% near the centrosome (within a 5 μm

117   radius) 2 hours after serum depletion, and continued to gradually increase throughout the

118   remaining 48-hour time course (Figures 1B, 1C). Similarly, microtubule density around the

119   centrosome fluctuated between 0 and 8 hours post serum depletion, then remained constant

120   through the rest of the time course (Figures 1B, 1D). Isogenic human RPE1 cells genetically

121   engineered to have three or four copies of HSA21 (Trisomy 21/T21 or Tetrasomy 21/Q21), have

122   elevated PCNT prior to serum depletion both at and around the centrosome (within a 5 μm

123   radius) (Figures 1B, 1C). Microtubule intensity was more similar in all three cell lines prior to

124   serum depletion (Figures 1B, 1D). By 2 hours, both PCNT and microtubule intensities elevated

125   with increasing HSA21 dosage (Figures 1B-D). Elevated PCNT near the centrosome in T21 and

126   Q21 cells persisted through the time course and was not due to changes in whole cell protein

127   levels (Figures 1B-D, S1A-C). Instead, PCNT and microtubules in T21 and Q21 cells

128   reorganized at the centrosome (0.0-1.2 μm region from the centroid of the centrosome) and

129   pericentrosomal region (1.2-5.0 μm region from the centroid of the centrosome), such that more

130   PCNT foci and microtubules were distributed around the centrosome (Figures 1B-D, S1D-G).

131   These data support a model whereby PCNT accumulates at and around the centrosome upon

132   induction of ciliogenesis. Interestingly, by 48 hours, PCNT and microtubule intensities were least

133   changed between the three cell populations (Figures 1B-D, S1D-G), suggesting that T21 and

134   Q21 cells might adapt to an elevated PCNT state upon prolonged G1 arrest.

135   To understand the interplay between PCNT levels, microtubules, and ciliation, we

136   quantified primary cilia frequency through the time course. D21 cells demonstrated two rates of

137   ciliation: a fast phase from 0-8 hours and a slow phase from 8-48 hours (Figures 1E, 1F). In

138   contrast, T21 and Q21 cells showed a decreased initial fast phase; however, the slow phase

139   from 8-48 hours was similar to D21 cells (Figures 1E, 1F). The fast ciliation phase correlates

140   with the early increases to PCNT and microtubule intensities which are more robust in T21 and

141   Q21 cells (Figures 1B-F). The delay in ciliogenesis observed in T21 and Q21 cells is consistent

142   with a model whereby increasing HSA21 dosage disrupts pericentrosomal trafficking flux to and

143   from the centrosome early in the process of ciliogenesis.

144

145   *HSA21 ploidy does not affect centriole appendages but decreases vesicles at the mother*

146   *centriole*

147   Primary ciliogenesis requires coordination between a series of trafficking and complex assembly

148   events over several hours.[22] Centriole appendage assembly at the distal end of the mother

149   centriole is an initiating event in ciliogenesis. These appendages serve as a scaffold for

150   receiving ciliary components trafficked to the centrosome.[5-7] CEP164 and CEP83 are two distal

151   appendage proteins, with CEP83 adjacent to the centriole microtubule walls and CEP164 at the

152   tip of the appendage structure.[23] In all cell lines (D21, T21, and Q21), CEP164 and CEP83

153   localized normally at the mother centriole (Figures 2A, S2A). Moreover, the subdistal

154   appendage proteins ODF2[24,25] and Ninein[26] correctly localized to the mother centriole (Figures

155   2B, S2B). Thus, HSA21 ploidy does not affect mother centriole appendages.

156   To gain further insight into the ultrastructure of centrioles with increasing HSA21 dosage,

157   we performed 3D electron tomography. Consistent with the immunofluorescence data, D21,

158   T21, and Q21 cells showed ninefold symmetry of distal and subdistal appendages. Moreover,

159   the triplet microtubules that comprise the centriole wall were unchanged across all cell lines,

160   further confirming that HSA21 ploidy does not affect centriole structure. However, 3D modeling

161   of the tomograms revealed changes in the number of vesicles at the mother centriole. D21 cells

162   showed many small vesicles (35-65 nm diameter) near the mother centriole (91 vesicles),

163   whereas vesicle number was decreased in T21 and Q21 cells (26 and 31 vesicles, respectively)

164   (Figure 2D, red spheres). Moreover, in D21 cells, vesicles were distributed along microtubules

165   and at their ends while vesicles in the T21 and Q21 cells were not always found on microtubules

166   (Figure 2D, Video S1-3). Despite the increased microtubule density observed in Figure 1, the

167   comparable number of microtubule minus ends near the centrioles (86, 69, 90 microtubule ends

168   in D21, T21, Q21, respectively) suggests that the additional microtubules are not arising from

169   the mother centriole. Because the tomograms only capture the centrosomal region (0-1.2 μm

170   from centroid of centrosome), this is consistent with our studies showing that more microtubules

171   are found in the pericentrosomal region that is distal to the centrioles (Figure 1D).[20] Together,

172   these data suggest that HSA21 dosage does not affect centriole structure nor centriole

173   appendage formation but may alter membrane structures at and around the centrosome.

174

175   *Preciliary vesicle components contribute to PCNT-induced pericentrosomal crowding*

176   Centriolar appendages are docking sites for delivery of vesicles and molecules required for

177   ciliogenesis.[5] Our EM tomograms suggested that vesicle accumulation at the mother centriole is

178   defective with HSA21 ploidy. Because increased HSA21 ploidy resulted in changes to the

179   pericentrosomal region in early ciliogenesis, one potential explanation for decreased mother

180   centriole vesicles is disrupted trafficking in the pericentrosomal region. We thus examined

181   whether molecules required for initiating ciliogenesis are disrupted in this region. The first

182   molecules that initiate the downstream steps of ciliogenesis are termed the preciliary or distal

183   appendage vesicle and include the motor protein Myosin VA (MYOVA)[8] and the membrane

184   shaping protein EHD1.[9] At 2 hours post serum depletion, about half of D21 cells have formed a

185   MYOVA vesicle at the mother centriole (Figure 3A). In contrast, T21 and Q21 cells show

186   decreased MYOVA vesicle formation and a striking buildup of MYOVA protein in the

187   pericentrosomal region surrounding the centrosome (Figures 3A-B). These changes in

188   pericentrosomal MYOVA intensity were not due to changes in whole cell MYOVA protein levels

189   (Figure S3A). Radial analysis of MYOVA intensity surrounding the centrosome in D21 cells

190   showed high MYOVA levels at the centrosome that then decreased in intensity moving away

191   from the centrosome (Figure 3B inset). We then compared MYOVA intensity distribution at and

192   around the centrosome with increasing ploidy through the time course. Two hours after serum

193   depletion, MYOVA intensities in T21 and Q21 cells were increased in the pericentrosomal 1.2-

194   5.0 μm region from the centroid of the centrosome (Figure 3B). Moreover, centrosomal MYOVA

195   levels were decreased with increasing ploidy (Figure S3B). By 4 hours, pericentrosomal

196   MYOVA intensities in T21 and Q21 cells became more prominent, while MYOVA centrosomal

197   levels remained decreased (Figures 3A, 3C, S3C). By 24 hours, T21 and Q21 cells still showed

198   changes in MYOVA intensity distribution, but the increased intensities were tighter around the

199   centrosome (Figures 3A, 3D, S3D), consistent with the redistribution of PCNT intensities

200   observed between 4 and 24 hours (Figure 1C). Whereas MYOVA intensities increased at the

201   centrosome in T21 and Q21 cells 24 hours post serum depletion, MYOVA appeared more

202   diffuse at the mother centriole compared to D21 cells (Figure 3A, bottom panel). This suggests

203   an additional defect in vesicle coalescence. Taken together, MYOVA intensity increases in the

204   pericentrosomal region with increasing ploidy, and we define this pericentrin-induced

205   redistribution of molecules around the centrosome with increasing HSA21 ploidy as

206   pericentrosomal crowding.

207   We then asked whether elevated PCNT found with increased HSA21 ploidy was

208   sufficient to increase MYOVA in the pericentrosomal crowding region. We have previously

209   shown that reducing PCNT levels in T21 and Q21 cells with siRNA rescues ciliation.[20] Reducing

210   PCNT levels in T21 and Q21 cells to D21 levels with siRNA at 24 hours post serum depletion

211   rescued the increased centrosomal and pericentrosomal MYOVA intensity to D21 levels

212   (Figures 3E, 3F, S3E, S3F). Moreover, the pericentrosomal increase in MYOVA consistently

213   increases with increasing PCNT levels (Figures S3G, S3H). Thus, elevated PCNT from

214   increasing HSA21 ploidy induces pericentrosomal crowding where MYOVA accumulates during

215   early ciliogenesis, thereby preventing efficient MYOVA vesicle formation at the mother centriole.

216   We next examined EHD1, an early vesicle protein that is recruited to the mother

217   centriole for coalescence and fusion of preciliary or distal appendage vesicles.[9] We observed

218   decreased EHD1 accumulation at the mother centriole with increasing ploidy and a slight

219   increase in EHD1 that is caught up in the pericentrosomal region (Figure S3I). Together, our

220   data support a model whereby elevated PCNT in trisomy 21 accumulates at and around the

221   centrosome immediately after induction of ciliogenesis and induces pericentrosomal crowding,

222   disrupting multiple trafficking pathways required for early preciliary vesicle formation.

223

224 *Increased HSA21 ploidy disrupts mother centriole uncapping in a PCNT-dosage dependent*

225 *manner*

226 Delivery of preciliary vesicles occurs just prior to or coincident with mother centriole uncapping,[8]

227 which involves removal and degradation of the centriole capping proteins CP110 and

228 CEP97.[27,28] Both CP110 and CEP97 removal from mother centrioles is defective in T21 and

229 Q21 cells compared to D21 cells (Figures 4A, S4A-C). To determine if these uncapping defects

230 were due to elevated PCNT levels, we decreased PCNT levels in T21 and Q21 cells back to

231 D21 levels and examined uncapping. Strikingly, reduced PCNT levels rescued CP110 removal

232 from the mother centriole (Figure 4B). Together, this indicates that elevated PCNT from

233 increased HSA21 ploidy is sufficient to block mother centriole uncapping. Because uncapping

234 requires the trafficking of the preciliary vesicle, we propose that elevated PCNT-induced

235 crowding ensnares components required for efficient uncapping (Figure 4E).

236   The small GTPase RAB8 functions in ciliary membrane extension during later steps in

237 ciliogenesis.[9] We previously showed that IFT20 is reduced at the centrosome in patient-derived

238 trisomy 21 fibroblasts.[19] IFT20 is recruited to the mother centriole upstream of RAB8 and is

239 required for RAB8 targeting to the ciliary membrane.[9,21] We thus examined RAB8 localization to

240 the mother centriole during the ciliogenesis time course to determine if later trafficking pathways

241 are also disrupted. By 8 hours post serum starvation, T21 and Q21 cells showed decreased

242 RAB8 accumulation at the mother centriole compared to D21, and this decrease persisted

243 throughout the time course (Figures 4C-D, S4D). Given that RAB8 associates with Golgi and

244 post-Golgi membranes in the context of ciliogenesis,[29,30] one potential explanation for

245 decreased RAB8 at the centrosome is defective trafficking from the Golgi. However, we did not

246 observe changes in RAB8 intensity at the Golgi with increasing ploidy, suggesting RAB8 leaves

247 the Golgi without incident (Figures S4E-F). Thus, RAB8 trafficking defects are specific to the

248 centrosomal region and are not generally disrupted intracellularly in T21 and Q21 cells.

249   Given the observed defects in trafficked proteins required for ciliogenesis reaching the

250   centrosome and the importance of microtubules in trafficking pathways, we wondered if general

251   membrane trafficking pathways were disrupted with increasing HSA21 ploidy. *Cis-* and *trans-*

252   Golgi networks, early endosomes, and lysosomes did not exhibit changes in morphology in T21

253   and Q21 cells when compared to D21 cells (Figures S4G-Q). Moreover, whereas mild intensity

254   differences were observed in some of these organelles, they did not follow a consistent trend

255   with increasing ploidy (Figures S4G-Q). Thus, gross disruptions of the endolysosomal trafficking

256   pathways are not apparent and trafficking defects associated with trisomy 21 are focused at and

257   around the centrosome with increasing HSA21 ploidy. Collectively, this suggests that

258   ciliogenesis defects in trisomy 21 result from early PCNT-induced crowding around the

259   centrosome that captures preciliary vesicle components thereby preventing mother centriole

260   uncapping and RAB8-axoneme extension.

261

262   *Decreased transition zone protein localization in ciliated trisomy 21 cells*

263   Despite early defects in ciliogenesis, elevated PCNT from increasing HSA21 ploidy does not

264   abolish cilia. Approximately 40% of T21 and 20% of Q21 cells formed a primary cilium by 24

265   hours post serum depletion (Figure 1E). We thus wondered whether trisomy 21 induced a

266   purely kinetic block in cilia formation or if defects persisted after ciliation. To function as a

267   signaling compartment, primary cilia are exposed to the external cellular environment to send

268   and receive signals. In RPE1 cells, cilia assemble inside the cell and then fuse with the cell

269   membrane to become extracellular signaling organelles.[31] This requires remodeling of the

270   plasma membrane and cytoskeletal networks. Given the observed centrosomal trafficking

271   defects, we tested whether cilia in trisomy 21 cells were exposed to the external environment

272   using the IN/OUT assay.[32] No difference was observed in the number of cilia outside versus

273   inside the cell in D21 and T21 cells (Figure S5A-B), suggesting that trisomy 21 does not cause

274   cilia to be retained inside cells.

11

275   A second requirement for cilia function in signaling is the creation of a diffusion barrier

276 called the transition zone. The transition zone, at the base of the cilium, is composed of proteins

277 that regulate entry and exit of molecules to and from the cilium. Regulation of diffusion through

278 the transition zone is essential for proper ciliary signaling, and many genes mutated in

279 ciliopathies encode transition zone proteins.[33,34] The levels of transition zone proteins were

280 analyzed in D21 and T21 cells. Q21 cells were eliminated because of their low ciliation

281 frequency. Consistent with defects in the primary cilia transition zone, the core transition zone

282 protein CEP290[35,36] was decreased in T21 cells compared to D21 cells (Figures 5A, yellow

283 arrows, and 5B). In addition to the transition zone, CEP290 also localizes to centriolar

284 satellites[13], and we observed increased CEP290 at the pericentrosomal but not centrosomal

285 region in T21 cells relative to D21s (Figures 5A, 5C, S5C). Moreover, CEP290 colocalizes with

286 PCNT in this pericentrosomal region (Figure 5A, cyan arrows), suggesting that decreased

287 CEP290 at the transition zone may result from pericentrosomal crowding. In addition to

288 CEP290, RPGRIP1L, and two outer transition zone proteins, NPHP4 and TMEM67,[37] were also

289 decreased at the transition zone in T21 compared to D21 cells (Figures 5D-I). Collectively,

290 trisomy 21 cells show transition zone defects suggesting that trafficking problems to the

291 centrosome are not specific to building a cilium but persist even in ciliated cells. This may

292 explain the signaling defects found previously in cells with elevated PCNT.[19]

293

294 *Shh signaling is defective in primary mouse fibroblasts with elevated PCNT*

295 The barrier function of the transition zone is critical for proper ciliary signaling.[34,38] Because

296 transition zone defects were found in trisomy 21 cells, we next asked whether trisomy 21

297 negatively affects primary cilia dependent signaling. Shh signaling is the best understood cilia-

298 dependent signaling pathway and is important for developmental events that are impacted in

299 DS. Moreover, Shh signaling was previously found to be disrupted in a DS mouse model.[39] Shh

300 signaling is commonly studied in primary mouse embryonic fibroblasts (MEFs). HSA21 maps to

301   syntenic regions of three mouse chromosomes: MMU10, MMU16, and MMU17.[40] We analyzed

302   three mouse models harboring segmental genomic duplications of these syntenic regions on

303   MMU10, MMU16, or MMU17 called Dp10, Dp16, or Dp17, respectively.[41] The murine *Pcnt* gene

304   is located on MMU10, therefore only the Dp10 model contains an extra copy of the *Pcnt* gene,

305   although other cilia and centrosome-related genes can be found on MMU16 and MMU17

306   (Figure 6A).[19,42]

307   We first asked whether MEFs isolated from Dp10, Dp16, or Dp17 embryos showed

308   ciliation defects. Strikingly, only the Dp10 MEFs with elevated PCNT showed decreased primary

309   cilia frequency when compared to wild-type littermates (Figures 6B-E). Cilia frequency in Dp16

310   and Dp17 MEFs was identical to wild-type (Figures 6C, S6A-B). Dp10 mice contain

311   approximately 41 duplicated HSA21 gene orthologs,[41] and we previously tested the other cilia

312   and centrosome human orthologs on MMU10 and found no changes in ciliation.[19] Thus,

313   decreased ciliation is specific to elevated PCNT in primary MEFs from the Dp10 mouse model

314   of DS.

315   Upon induction of the Shh pathway, the transmembrane protein Smoothened (SMO)

316   translocates into the cilium and generates signals that induce nuclear GLI localization and

317   downstream transcriptional response of hedgehog target genes important for mitogenic activity

318   and developmental processes such as patterning and limb development.[4,43] Because Shh

319   signaling is disrupted in a DS mouse model,[39] and we previously found decreased GLI

320   expression in DS-derived human fibroblasts,[19] we next examined Shh signaling in MEFs where

321   we can distinguish the contributions of different regions of HSA21. We compared SMO intensity

322   in the cilium with and without induction of Shh signaling using the SMO agonist SAG. Without

323   SAG, ciliary SMO levels were undetectable in both wild-type and Dp10 MEFs (Figures 6F and

324   6H). However, upon SAG treatment, SMO robustly accumulated in the cilium in wild-type cells,

325   whereas ciliary SMO remained low in Dp10 cells (Figures 6G-H). Moreover, ciliary SMO levels

326   in Dp16 and Dp17 cell lines after SAG treatment were the opposite of PCNT levels, as Dp16

327   MEFs had slightly elevated PCNT levels and slightly decreased ciliary SMO while Dp17 MEFs

328   had slightly decreased PCNT levels and slightly increased ciliary SMO (Figures 6D-E, 6H, S6C-

329   D). Together, decreased ciliation frequency and ciliary SMO in Dp10 MEFs is consistent with

330   persistent trafficking defects from pericentrosomal crowding in ciliated trisomy 21 cells.

331   Moreover, PCNT levels anticorrelate with ciliary SMO in Dp16 and Dp17 MEFs.

332   Interestingly, one Dp10 MEF line (Dp10-2) did not show decreased ciliation compared to

333   wild-type controls (Figures S6E-F). Importantly, this line also did not exhibit elevated PCNT

334   levels or defects in ciliary SMO localization upon SAG treatment (Figures S6G-H). It is unclear

335   whether this Dp10-2 line lost the chromosome duplication containing PCNT or whether cells

336   compensated at the molecular level. Regardless, results from this line reinforce the conclusion

337   that cilia and signaling defects result from elevated PCNT levels. In summary, PCNT and ciliary

338   SMO levels anticorrelate and are disrupted in trisomy 21.

339

340   *Elevated PCNT in a DS mouse model results in decreased primary cilia and cerebellar*

341   *dysmorphology*

342   Primary cilia are ubiquitous and essential signaling organelles. They are particularly important

343   during brain development and are predicted to be disrupted in DS.[4,44] Individuals with DS

344   commonly exhibit cerebellar hypoplasia,[1] and delayed cerebellar development has been

345   observed in the Ts65Dn mouse model of DS, which harbors a duplication of MMU16 genes

346   similar to that of the Dp16 model along with a duplication of genes from MMU17 that are not

347   syntenic to human HSA21.[39,40] During cerebellar development, neuronal precursor cells in the

348   external granular layer respond to Shh from Purkinje cells.[45,46] Shh induces mitogenic activity,

349   and the amplified cells ultimately migrate to the internal granule layer where they become

350   mature neurons.[46] Primary cilia are required for neuronal precursor cell amplification and

351   defects in Shh signaling reduce proliferation and disrupt cerebellar development.[47] Yet, whether

352   T21 and elevated PCNT disrupt primary cilia during brain development *in vivo* remains

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353   unknown. We therefore examined ciliation in the external granular layer of postnatal day 4 (P4)

354   pups. Consistent with our findings in Dp10 MEFs, cerebellar neuronal precursors in Dp10

355   animals had fewer primary cilia compared to wild-type littermates (Figures 7A, 7D). Moreover,

356   Dp16 and Dp17 animals showed no change in ciliation frequency (Figures 7B-D). These data

357   are consistent with our analyses in MEFs suggesting that cilia defects occur in animals with

358   elevated PCNT.

359   Reduced Shh signaling correlates with morphological changes in the cerebellum, such

360   as decreased width of the external granular layer.[48,49] We thus measured the width of the

361   external granular layer and found decreased widths in Dp10 animals compared to wild-type

362   littermates (Figure 7E-F). Decreased width could result from decreased cell proliferation, so we

363   next examined Ki67 staining as a marker for cell proliferation. While we observed no changes in

364   the number of Ki67-positive cells in the external granular layer of Dp10 and wild-type animals at

365   P4 (Figure S7A-B), we cannot rule out changes in cell proliferation at earlier developmental time

366   points. The source of Shh for neuronal precursors comes from Purkinje cells,[46] so we next

367   examined the Purkinje cell layer. No loss of Purkinje cells was noted between Dp10 and wild-

368   type siblings (Figure S7C). Finally, given that elevated PCNT alters microtubule networks in

369   cultured RPE1 cells and that PCNT mutations produce severe neuronal defects including

370   disrupted neuronal migration,[18,50,51] we visualized doublecortin (DCX), a microtubule binding

371   protein that functions in neuronal migration.[52,53] Interestingly, Dp10 animals showed decreased

372   DCX-labeled cellular protrusions compared to wild-type (Figure S7C). While not conclusive, this

373   suggests that elevated PCNT alters non-centrosomal microtubules such as those required for

374   neuronal outgrowth.[54] Taken together, elevated PCNT results in decreased ciliation in the

375   external granular layer of the cerebellum and these cerebella demonstrated morphology

376   changes consistent with decreased Shh signaling and developmental defects.

377   To determine if decreased ciliation *in vivo* was a direct result of elevated PCNT levels,

378   we cultured cerebellar slices from P4 pups and treated slices with either control or PCNT

15

379   siRNA. Strikingly, reducing PCNT levels in Dp10 slice cultures rescued ciliation back to control

380   levels (Figure 7G-I). Taken together, this suggests that elevated PCNT from trisomy 21 is

381   sufficient to induce ciliation defects during cerebellar development *in vivo.*

382   Our data from T21 and Q21 RPE1 cells suggested that pericentrosomal crowding

383   delayed ciliogenesis; however, cells arrested in G1 for a prolonged period eventually ciliated

384   albeit at slightly lower frequencies than D21 cells (Figure 1E). Moreover, our previous work in

385   human DS-derived fibroblasts showed the strongest ciliation defects in cycling cells.[19] We thus

386   tested whether ciliation defects *in vivo* were dependent on cell cycle state by analyzing cells in

387   the inner granular layer of the cerebellum which are generally post-mitotic. Primary ciliation of

388   inner granule layer cells was unchanged between wild-type and Dp10 animals (Figure S7D).

389   This provides further evidence that trisomy 21 alters but does not abolish ciliogenesis *in vivo*,

390   with the major impact on migrating precursor cells

391

392 **Discussion**

393 *Trisomy 21 alters trafficking flux of early ciliogenesis molecules delaying ciliogenesis*

394 Here, we use a time course to establish the dynamic changes to PCNT trafficking puncta and

395 microtubules upon induction of ciliogenesis through media serum depletion. In control euploid

396 cells, the largest changes to PCNT and microtubule networks around the centrosome occur

397 early, within 2 h after induction of ciliogenesis. Moreover, we observe a slow and a fast phase to

398 ciliation in a population of cells, with the fast phase occurring within the first 8 h and the slow

399 phase from 8-48 h. In T21 and Q21 cells, PCNT and microtubule levels increase more

400 dramatically at the onset of ciliogenesis. This is accompanied by a decrease in the effectiveness

401 of the fast phase of ciliogenesis, while the slow phase from 8 to 48 h is largely the same as D21

402 cells. These data reveal that in trisomy 21, PCNT-induced pericentrosomal crowding occurs

403 immediately upon induction of ciliogenesis and delays but does not abolish ciliation.

404 Several distinct trafficking pathways required for building a primary cilium are prevented

405 from efficient recruitment to the site of assembly in the altered microtubule and PCNT landscape

406 caused by increased HSA21 dosage. MYOVA, one of the earliest proteins to localize to the

407 mother centriole, does not traffic normally but rather participates in pericentrosomal crowding in

408 T21 and Q21 cells. Mother centriole uncapping is also disrupted, perhaps through defects in

409 trafficking the ubiquitin or autophagosome machinery required for CP110 and CEP97 removal

410 and degradation, although this remains to be tested. In addition to early defects in ciliogenesis,

411 pericentrosomal crowding affects later steps such as RAB8-mediated ciliary membrane growth

412 and IFT20 centrosomal localization.[9,19] Together, our results show HSA21 dosage dependent

413 disruption in trafficking pathways important to the earliest observable stages of ciliogenesis and

414 cilia function, as molecules required for building and maintaining a cilium get hung up in PCNT-

415 induced crowding around the centrosome.

416 Consistent with a crowding model, several lines of evidence support the idea that

417 trisomy 21 does not result in a complete block to ciliogenesis, but rather a delay in the process.

17

418   First, with sustained G1 arrest, T21 and Q21 cells increase their ciliation, and these cells appear

419   to compensate for crowding such that the difference between PCNT and microtubule densities

420   at and around the centrosome is reduced between D21, T21, and Q21 cell lines. Second, a

421   similar phenomenon is observed with crowding cargo, as the amount of pericentrosomal

422   MYOVA decreases over time and MYOVA eventually accumulates at mother centrioles in T21

423   and Q21 cells. Third, trisomy 21 cells demonstrate defective signaling even after a cell builds a

424   primary cilium. This is consistent with previous work showing reduced GLI expression in human

425   trisomy 21 cells and decreased ciliary SMO trafficking in cells overexpressing PCNT.[19] Fourth,

426   crowding has the most severe consequences for molecules that are dynamically trafficking to

427   and from the centrosome, whereas longer lived structures such as the core centriole and

428   appendages[55] are unaffected by elevated PCNT. Finally, *in vivo*, only cycling neuronal

429   precursors of the external granular layer have decreased ciliation, whereas ciliation in post-

430   mitotic cells of the inner granular layer remains unaffected with trisomy 21. Collectively, we find

431   that PCNT-induced pericentrosomal crowding disrupts the dynamic flux of molecules to and

432   from the centrosome thereby disturbing the coordination and timing required for proper

433   ciliogenesis and signaling.

434

435   *Cerebellar phenotypes in animals with elevated PCNT*

436   While the timing of ciliogenesis is not vital in cultured cells, the timing of ciliogenesis and cilia-

437   dependent signaling is critical during *in vivo* development where cilia send and receive input

438   from neighboring cells to coordinate proper tissue development.[44] In line with our cultured cell

439   results, we do not observe a complete loss in cilia and signaling in primary MEFs or cerebellar

440   neuronal precursor cells with elevated PCNT but do observe consistent decreases in ciliation.

441   Moreover, the organization of cerebellar layers with a thinner external granular layer is altered

442   but not eliminated. While we might expect to see decreased cell proliferation in cells in the

443   external granular layer, Ki67 staining, a marker for proliferating cells, appears normal in Dp10

444   animals compared to wild-type littermates. This result could occur for several reasons. First, our

445   analysis was conducted only in P4 pups, where developmental delays are not yet obvious in

446   Dp10 animals. An analysis of cell proliferation at earlier or later timepoints might show changes

447   to the number of cycling cells. Second, because elevated PCNT does not result in a complete

448   loss of cilia, perhaps enough of the cell population is ciliated to receive the Shh mitogenic signal

449   and proliferate at this timepoint. Cerebellar development is a highly integrated process where a

450   trisomy 21-induced delay in ciliogenesis could alter the coordination of cellular processes such

451   that some cells never fully catch up. Because inputs are not completely lost, this cell-to-cell

452   variability could account for the spectrum of phenotypes observed in individuals with DS.

453   Most trisomy 21 animal studies have been performed in the Ts65Dn mouse model.[40]

454   While these mice show some degree of DS-like phenotypes, they are only trisomic for about half

455   of the HSA21 orthologs and contain an additional amplification of genes not found on

456   HSA21.[40,56] The Dp mouse models are a more refined genetic system to determine the

457   phenotypic contributions from different regions of HSA21. Dp10 mice contain approximately 41

458   duplicated HSA21 gene orthologs including PCNT while Dp16 and Dp17 mice have 115 and 19

459   duplicated gene orthologs, respectively, and exclude PCNT.[41] Our experiments in primary MEFs

460   and cerebellar slices largely attribute cilia defects to elevated PCNT levels in Dp10 animals.

461   However, there are other known cilia and centrosome genes found on HSA21, and potentially

462   non-coding regions that may also contribute to phenotypic consequences of trisomy 21. For

463   example, MMU16 contains the splicing factor SON, which is known to splice PCNT mRNA and

464   alters PCNT levels and distributions.[57,58] Indeed, while we do not find changes in primary cilia

465   frequency in Dp16 animals, we do observe moderately decreased ciliary SMO and moderately

466   increased PCNT levels. Interestingly, Dp16 mice have defects in the motile cilia lining the

467   ependymal cells of the brain (Figure S7E-G) suggesting that elevated gene dosage can have

468   different phenotypic consequences in different tissues. In support of this, animals with individual

469   chromosomal duplications, Dp10, Dp16, or Dp17,[41] do not show as severe phenotypic

470   consequences as combined Dp10; Dp16; Dp17 animals.[59] Therefore, while elevated PCNT

471   disrupts ciliogenesis and signaling, there are likely other contributions from additional genes on

472   HSA21 that add to the spectrum of phenotypes observed in individuals with DS.

473   In contrast to single gene disruption studies, here we show that ciliary defects arise from

474   elevated protein expression, as is often the case in chromosomal aberrations. Indeed, cilia

475   defects have been observed with increased copy number of a nuclear pore protein.[60] While

476   changes in some protein levels may be tolerated, we demonstrate that even a modest increase

477   in PCNT protein (1.5 - 2-fold) is deleterious to cilia formation and function in a dose dependent

478   manner. Elevated PCNT aggregates and reorganizes microtubule networks, inducing

479   pericentrosomal crowding that disrupts cargo transport required for early steps in ciliogenesis

480   and persists in ciliated trisomy cells. Because tight control of microtubule organization is

481   essential for many cell types and processes including neuron outgrowth, cell migration, immune

482   synapse formation, and cell polarity, these functions need further study to determine whether

483   elevated PCNT from trisomy 21 and changes in microtubule topologies alter these cell types

484   and functions.

Author Contributions

CEJ, BLM, ETO, KSG, CHL, and VO performed experiments and analyzed data. CEJ and CGP conceived the project and wrote the manuscript. WM, LR, JME, KDS, WBM, and RP provided reagents, animal models, and expertise.

Competing Interests

The authors declare no competing interests.

Figure Legends

Figure 1. Rapid PCNT and microtubule reorganization in response to ciliation cues increases

with HSA21 dosage. (A) Model figure showing the three main consequences of elevated PCNT

in trisomy 21 (T21) and tetrasomy 21 (Q21) compared to disomy 21 (D21) cells: 1) PCNT

nucleates more microtubules; 2) PCNT forms large protein aggregates on microtubules; and 3)

PCNT nucleates microtubules further away from the centrosome. These changes occur

predominantly in the pericentrosomal region defined as 1.2-5 µm from the centroid of the

centrosome. (B) Representative confocal images from time course experiments of RPE1 D21,

T21, and Q21 cells grown on coverslips and serum depleted for 0, 2, 4, 8, 24, and 48 h. Cells

were stained with DM1a to label microtubules (MTs) and PCNT. Arrows point to cilium labeled

by DM1a staining. (C) Quantitation of PCNT intensities in a 5 µm radial circle around the

centrosome throughout the time course normalized to D21 average at 0 h. Graph shows mean ±

SD. (D) Quantitation of microtubule intensities in a 5 µm radial circle around the centrosome

throughout the time course normalized to D21 average at 0 h. Graph shows mean ± SD. (E)

Quantitation of ciliation frequency throughout the time course using DM1a as a marker for cilia.

Graph shows mean ± SD. N's and statistical tests are listed in Table S1.

Figure 2. HSA21 ploidy does not affect centriole appendages but decreases vesicles at the

mother centriole. (A-B) Representative SIM images of RPE1 D21, T21, and Q21 cells grown on

coverslips and serum depleted for 24 h. Cells were stained with GT335 and the distal

appendage marker CEP164 (A) or the subdistal appendage marker ODF2 (B). Percentages

represent cells with indicated marker for 3 N's. (C) EM tomograms of RPE1 D21, T21, and

Q21cells serum depleted for 24h showing microtubule triplets and distal appendages (top panel)

and subdistal appendages (bottom panel). (D) 3D models of EM tomograms at centrosome. Top

row shows mother centriole (yellow), daughter centriole (magenta), microtubule minus ends

(light blue spheres), and vesicles (red). Bottom row shows models with microtubules (green)

531   and vesicles displayed. Tomograms and models shown are of cells prior to ciliary vesicle

532   formation.

533

534   *Figure 3. Preciliary vesicle components contribute to PCNT-induced pericentrosomal crowding.*

535   (A) Representative structured illumination microscopy (SIM) images from time course

536   experiments of RPE1 D21, T21, and Q21 cells grown on coverslips and serum depleted for 2, 4,

537   and 24 h. Cells were stained with GT335 to label centrioles and MYOVA. (B-D) Distribution of

538   MYOVA intensities moving away from the centrosome for 2 (B), 4 (C), and 24 (D) h timepoints.

539   (E) Quantitation of centrosomal MYOVA intensity in 0-1.2 μm region around centrosome for

540   control and siPCNT treated D21, T21, and Q21 cells. (F) Quantitation of pericentrosomal

541   MYOVA intensity in 1.2-5 μm region around centrosome for control and siPCNT treated D21,

542   T21, and Q21 cells. All values were normalized to the D21 average, and graphs show mean ±

543   SD. N's and statistical tests are listed in Table S1.

544

545   *Figure 4. Increased HSA21 ploidy disrupts mother centriole uncapping in a PCNT-dosage*

546   *dependent manner*. (A) Representative SIM images of RPE1 D21, T21, and Q21 cells grown on

547   coverslips and serum depleted for 24 h. Cells were stained with GT335 and the centriole

548   capping protein CP110. Percentages represent cells with indicated phenotype across 3 N's. (B)

549   Quantitation of CP110 centriole capping for control and siPCNT treated D21, T21, and Q21

550   cells. (C) Representative SIM images of RPE1 D21, T21, and Q21 cells grown on coverslips

551   and serum depleted for 4, and 8 h. Cells were stained with γ-tubulin, Actub, and RAB8.

552   Percentages represent cells with indicated phenotype across 3 N's. All time points are shown in

553   Figure S3D. (D) Quantitation of cells with RAB8-positive ciliary vesicle throughout the time

554   course. (E) Cartoon model depicting altered distribution of MYOVA from the mother centriole to

555   the pericentrosomal region and decreased mother centriole uncapping with increasing ploidy.

556   Graphs shows mean ± SD. N's and statistical tests are listed in Table S1.

557

558   *Figure 5. Decreased transition zone protein localization in ciliated trisomy 21 cells*. (A)

559   Representative confocal images of RPE1 D21 and T21 cells grown on coverslips and serum

560   depleted for 24 h. Cells were stained with GT335, PCNT, and the transition zone protein

561   CEP290. Yellow arrows point to the CEP290 transition zone population and cyan arrows point

562   to CEP290 satellites that colocalize with PCNT. (B) Quantitation of CEP290 transition zone

563   intensity. (C) Quantitation of pericentrosomal CEP290 intensity in 1.2-5 μm region around

564   centrosome. (D, F, H) Representative SIM images of RPE1 D21 and T21 cells grown on

565   coverslips and serum depleted for 24 h. Cells were stained with GT335 and the transition zone

566   proteins RPGRIP1L (D), NPHP4 (F), or TMEM67 (H). (E, G, I) Quantitation of indicated

567   transition zone protein intensities from confocal images. All values were normalized to the D21

568   average, and graphs show mean ± SD. Mean intensity values are indicated on graphs. N's and

569   statistical tests are listed in Table S1.

570

571   *Figure 6. Shh signaling is defective in primary mouse fibroblasts with elevated PCNT.* (A)

572   Cartoon model depicting mouse syntenic regions with HSA21 and corresponding Dp10, Dp16,

573   and Dp17 mouse models. PCNT is located on MMU10. Other cilia and centrosome proteins are

574   also listed. For all following experiments, WT and Dp primary MEFs were isolated from E12.5

575   dams, grown on coverslips, and serum depleted for 24 h. (B) Representative confocal images of

576   WT and Dp10 MEFs. Cells were stained with Hoechst, the ciliary marker ARL13B, and Actub.

577   (C) Quantitation of the number of cells with a primary cilium in WT and Dp MEFs. (D)

578   Representative confocal images of WT and Dp10 MEFs. Cells were stained with PCNT and

579   ARL13B. (C) Quantitation of PCNT intensities in a 5 μm radial circle around the centrosome in

580   WT and Dp MEFs. Values were normalized to the WT average. (F, G) Representative confocal

581   images of WT and Dp10 MEFs untreated (F) or treated with 100 nM SAG for the last 4 h of

582   serum depletion (G). Cells were stained with SMO and ARL13B. (H) Quantitation of ciliary SMO

583   levels in SAG-treated cells for WT and Dp MEFs. Graphs show mean ± SD. N's and statistical

584   tests are listed in Table S1.

585

586   *Figure 7. Elevated PCNT in a DS mouse model results in decreased primary cilia and cerebellar*

587   *dysmorphology.* (A-C) Representative tiled confocal images of the cerebellum from P4 wild-type

588   (WT) and Dp10 (A), Dp16 (B), and Dp17 (C) animals. Brain sections were stained with Hoechst,

589   ARL13B, and γ-tubulin. Insets show progressively zoomed in regions corresponding to the same

590   folia in each animal. (D) Quantitation of primary cilia frequency in WT and Dp animals

591   normalized to WT. (E) Representative tiled confocal images of WT and Dp10 P4 animals

592   corresponding to the same cerebellar folia in each animal. Brain sections were stained with

593   Hoechst and a Pan-neuronal marker. Yellow bracket denotes external granular layer. (F)

594   Quantitation of the external granular layer width in WT and Dp10 animals. (G) Quantitation of

595   primary cilia frequency in WT and Dp10 cerebellar slice cultures treated with control or PCNT

596   siRNA. Values were normalized to WT siControl averages. (H) Representative confocal images

597   of WT and Dp10 cerebellar slice cultures isolated from P4 pups and treated with control or

598   PCNT siRNA for 48 h in serum free media. Slices were stained with Hoechst, ARL13B, PCNT.

599   Yellow box denotes insets. (I) Quantitation of PCNT intensity in WT and Dp10 cerebellar slice

600   cultures treated with control or PCNT siRNA. Values were normalized to WT siControl

601   averages.

602 **Materials and Methods**

603 *Cell lines*

604 Disomy 21, Trisomy 21, and Tetrasomy 21 (D21, T21, Q21) hTERT-immortalized retinal

605 pigment epithelial (RPE1) cells were generated by and Drs. Andrew Lane and David Pellman at

606 the Dana-Farber Cancer Institute.[61] Cells were grown in DMEM:F12 (SH30023; Cytiva)

607 supplemented with 10% fetal bovine serum (FBS, Peak Serum; PS-FB2) and 1%

608 Penicillin/Streptomycin at 37°C and 5% CO_2. Cells were passaged 1:5 at ~80-90% confluency

609 with 0.25% Trypsin (15090-046; Gibco). Cells were routinely screened for mycoplasma. Primary

610 MEFs were generated as described in Mariani et al.[62] In brief, embryonic day 12.5 embryos

611 were dissected from pregnant females. The head and internal organs were removed, and the

612 remaining tissue was dissociated by passage through a needle. The head was used for

613 genotyping. Dissociated cells were cultured in DMEM:F12 with 10% fetal bovine serum and 1%

614 Penicillin/Streptomycin for no more than 3 passages.

615

616 *Immunofluorescence*

617 Cells were plated on collage-coated coverslips and grown in full media until 80-90% confluent.

618 To induce ciliogenesis, cells were washed once with 1x PBS and then grown in serum depleted

619 media (DMEM:F12 with 0.5% FBS) for indicated hours. For time course experiments, cells were

620 fixed according to Waterman-Storer and Salmon[63] following 0, 2, 4, 8, 24, or 48 hours post

621 serum depletion. Briefly, cells were pre-permeabilized for 5 minutes in 0.5% TritonX-100 in

622 PHEM (60mM Pipes, 25mM Hepes, 10mM EGTA, 2mM $MgCl_2$, 6.9 pH). Cells were then fixed

623 with 4% paraformaldehyde/0.5% glutaraldehyde for 20 minutes and quenched with 0.1%

624 sodium borohydride. Following quenching, cells were washed four times in 0.1% TritonX-100

625 and stored in PHEM at 4ºC until immunostaining.

626 For all other staining experiments, cells were fixed with 4% paraformaldehyde for 20

627 minutes at room temperature or with 100% ice cold Methanol for 10 minutes at -20°C. Cells

628   were blocked for 1-2 hours in block buffer (PBS, 0.1% Triton X-100, 10% normal donkey

629   serum). Primary antibodies were diluted in block buffer and incubated overnight at room

630   temperature. Cells were washed with PBSTx before adding secondary antibodies for 1-2 hours

631   at room temperature. Cells were washed again before mounting in VectaShield and sealing with

632   nail polish or mounting in Prolong Gold for SIM experiments. Coverslips for all experiments were

633   #1.5. Primary and secondary antibodies are listed in the Key Resources Table.

634   For SAG treatment, MEFs were grown on coverslips to 80-90% confluency, moved to

635   serum depleted media for 20 hours, treated with 100 nM SAG in serum depleted media for 4

636   hours, fixed with 4% PFA and stained as described above.

637   To costain PCNT with other rabbit primary antibodies, PCNT was conjugated to Alexa

638   Fluor 488 (Antibody labeling kit: Invitrogen A20181).

639

640   *Fluorescence microscopy*

641   Confocal images were acquired using either a Nikon Eclipse Ti 2 inverted A1 Confocal and

642   Nikon Elements software or Nikon Eclipse Ti inverted microscope stand equipped with a 100x

643   Plan Apochromat objective (NA 1.45), Andor iXon X3 camera, CSU-X1 (Yokogawa) spinning

644   disk, and Slidebook 6 software. Super resolution imaging was performed on a Nikon 3D SIM

645   system (Ti 2 Eclipse) with a 100x TIRF objective (NA 1.45). Images were captured with a

646   complementary metal-oxide semiconductor camera (Orca-Flash 4.0; Hamamatsu). Raw images

647   were reconstructed using the Nikon Elements image stack reconstruction algorithm. Images

648   were processed in FIJI [64]. Figures were made in Adobe Illustrator. A minimum of three biological

649   replicates were performed for each experiment unless otherwise noted. All images presented in

650   figures are max projections.

651

652   *IN/OUT cilia assay*

653   RPE1 D21, T21, and Q21 cells stably expressing pHluorin-Smoothened (pLVX-pHluorin-

654   Smoothened vector was a gift from Dr. Derek Toomre) were plated on collagen-coated

655   coverslips, fixed and stained as described in[32]. Briefly, culture media was removed, cells were

656   gently washed in PBS, and then fixed in 4% PFA in PBS for 10 minutes. Cells were then

657   blocked in 5% normal donkey serum in PBS for 30 minutes followed by incubation with anti-GFP

658   primary antibody solution for 1 hour. Cells were fixed again for 10 minutes, permeabilized with

659   0.1% Triton-X, and incubated in another primary antibody solution containing anti-Actub for 1

660   hour. After gentle washing, cells were incubated with secondary antibodies and Hoechst,

661   followed by washing and mounting on slides.

662

663   *RNAi*

664   Human PCNT siRNA (Smart Pool) (M-012172-01-0005; Dharmacon) was transfected into RPE1

665   cells with Lipofectamine RNAi MAX (13778100; ThermoFisher Scientific) according to the

666   manufacturer's protocol. Mission siRNA universal negative control #1 was used for all negative

667   controls (SIC001-1NMOL; Sigma). All siRNAs were used at a final concentration of 25 nM. Cells

668   were treated with siRNA in serum depleted media for 24 hours before fixation and subsequent

669   immunostaining steps.

670

671   *Generation of lentiviral stable cell lines*

672   RPE1 D21, T21, and Q21 lentiviral stable cell lines were generated by transfecting HEK293T

673   cells with lentiviral GFP-EHD1 or pH-Smoothened constructs and lentivirus packaging plasmids

674   using Lipofectamine 2000 (11668-027; Invitrogen). HEK293T media containing virus was

675   collected and added to target cells in the presence of 2mg/mL polybrene. Transduced cells were

676   selected using 10mg/mL puromycin for three days. To induce GFP-EHD1 expression, 0.125

677   mg/mL doxycycline was added to serum depleted media for 24 hours before fixation.

678

679   *Animal Ethics Statement*

28

680   All procedures involving mice were approved by the Institutional Animal Care and Use

681   Committee (IACUC) at the University of Colorado Anschutz Medical Campus and were

682   performed in accordance with National Institute Guidelines for the care and use of animals in

683   research.

684

685   *Mouse models of Down syndrome*

686   Mouse models were obtained from the NICHD funded Cytogenetic and Down Syndrome Models

687   Resource Jackson Laboratory. These include B6.129S7-Dp(16Lipi-Zbtb21)1Yey/J (Dp16;

688   stock# 013530), B6;129-Dp(10Prmt2-Pdxk)2Yey/J (Dp10; stock# 013529), and B6;129-

689   Dp(17Abcg1-Rrp1b)3Yey/J (Dp17; stock# 013531). Strains were maintained on a standard

690   chow diet and a 14-hour light/10-hour dark cycle. Littermate controls were used.

691

692   *Cerebellar Slice Cultures*

693   Cerebellums were dissected out of postnatal day 4 pups in ice cold Hank's media and sliced

694   300 μm thick on a tissue chopper.[65] Slices were transferred to MilliCell filter inserts (Millipore

695   PICM03050) only taking intact slices with at least 5 folia. Excess Hank's was removed from

696   filters and replaced with serum free Neurobasal media supplemented with 1% N2, 0.5%

697   Penicillin/Streptomycin, 0.25% L-glutamine, and 10 mM HEPES. Cerebellar slices were cultured

698   at 37°C and 5% CO_2 and media was changed 8 hours after plating. Slices were grown for 48

699   hours before addition of 500 μm control or PCNT siRNA (D-001910-10-05 Accell Non-targeting

700   Pool; Accell Mouse Pcnt 18541 siRNA—SMARTpool) and then cultured for an additional 48

701   hours before fixation with 4% PFA and staining as described above.

702

703   *Mouse brain immunohistochemistry*

704   Experiments were blinded to gender. P4 pups were anesthetized with Isoflurane before

705   decapitation. Tail clips were used for genotyping. Brains were dissected out and fixed in 4%

706   PFA in PBS overnight at 4°C. Brains were then moved to 30% sucrose in PBS for 1-2 days at

707   4°C. Fixed brains were sliced through the midline; each half was embedded in OCT, frozen on

708   dry ice for ~15 minutes, then stored at -80°C until ready to section. To section, blocks were

709   mounted in OCT and 20 μm sagittal sections were cut using a Leica CM 1950 cryostat

710   microtome. Sections were placed on FisherBrand charged slides (Cat # 12-550-15) and stained

711   on slides as described above.

712

713   *Genotyping*

714   Tissue from Dp10 animals or MEFs were lysed by placing tail clips or heads in Gitschiers Buffer

715   (67 mM Tris pH 8.8, 0.166 mM Ammonium sulfate, 6.7 mM magnesium chloride, 0.005%

716   TritonX, 0.1 mg/ml Proteinase K in water) for 2 hours at 55°C then 95°C for 10 minutes. Tissue

717   from Dp16 or Dp17 animals or MEFs were lysed by placing tail clips or heads in 50 mM sodium

718   hydroxide and heating at 98°C for 2 hours before neutralizing with 1M Tris pH 8 (1:10). Lysed

719   tissue was used for PCR and the resulting banding pattern from PCR was used to determine

720   genotype. Genotyping primers are as follows: Dp10For: GGCGAACGTGGCGAGAAA;

721   Dp10Rev: CCTGCTGCCAAGCCATCAG; Dp16For: CTGCCAGCC ACTCTAGCTCT; Dp16Rev:

722   AATTTCTGTGGGGCAAAATG; Dp17For: GGAGCCAGGGCTGATGGT; Dp17Rev:

723   CAACGCGGCCTTTTTACG. Primers for Cux2 were used as controls. Cux2For:

724   GGGACATCACCCACCGGTAATCTC; Cux2Rev: GACCACTGAGTCTGGCAACACG.

725

726   *Image Analysis*

727   All intensity analysis was performed on max projected images unless otherwise noted. Radial

728   fluorescence intensity was measured using the Radial Profile Extended ImageJ plugin. Briefly,

729   this plugin plots average fluorescence intensity as a function of distance from a user defined

730   centroid. 10 μm diameter ROIs were centered over the brightest pixel of a Gaussian blurred

731   maximum intensity projection of the centrosome defined by PCNT fluorescence. In cells with

732   GT335-labeled centrioles, the centroid was manually centered between the two centrioles.

733   Background was determined per field of view by the mean intensity of an extracellular ROI.

734   Background subtracted radial intensities were summed and normalized to the D21 average.

735   Centrosomal fluorescence intensity was defined as the sum of radial intensities falling within

736   0.0-1.2 μm from the centroid of analysis. Pericentrosomal fluorescence intensity was defined as

737   the sum of radial intensities falling within 1.2-5.0 μm from the centroid of analysis.

738   Whole cell intensities were calculated using the integrated density within cells outlined in

739   ImageJ. Ciliation frequency was measured by counting either DM1a (Figure 1) or ARL13B

740   (Figures 6) and dividing by the number of nuclei per field of view. For ciliation frequency in

741   cultured cerebellar slices, the number of ARL13B cilia was divided by the number of

742   centrosomes per field of view. For ciliation frequency in fixed cerebellar slices, the number of

743   ARL13B cilia was divided by the mean Hoechst intensity per field of view because individual

744   nuclei could not be resolved in the thick slices.

745   For PCNT siRNA experiments, radial MYOVA and PCNT fluorescence intensity was

746   calculated using the same methods as above. For MYOVA radial distribution, all values were

747   divided by the maximum intensity value per cell and normalized to D21 values. Correlation

748   analyses were performed by plotting 10 μm diameter PCNT intensities normalized within a cell

749   line to either centrosomal or pericentrosomal normalized MYOVA intensities. R values and

750   significance were calculated by running a correlation analysis in GraphPad. PCNT levels were

751   analyzed with a 10 μm diameter circle centered on the centrosome to measure the integrated

752   density. Background was determined with an extracellular ROI. Background subtracted values

753   were normalized to the D21 average.

754   GM130 and Golgin97 intensities were measured by drawing an ROI around the GM130

755   or Golgin97 signal and measuring the integrated density within this ROI. Background was

756   determined by an extracellular ROI. Background subtracted values were normalized to the D21

757   average. To quantify early endosome and lysosome levels, integrated density was measured

758   across a field of view and divided by the number of nuclei within the same field of view. Values

759   were normalized to the D21 average.

760   All transition zone antibodies were co-stained with GT335. The transition zone was

761   identified by the gap in GT335 signal between the mother centriole and the base of the cilium.

762   Transition zone protein intensity was determined by centering a 1 µm x 1 µm box over the

763   transition zone and measuring the integrated density. Background was determined by a 1 µm x

764   1 µm box randomly placed near the centrosome. Background subtracted values were

765   normalized to the D21 average. For TMEM67, the ROI was a 0.5 µm x 0.5 µm box to avoid

766   centrosomal TMEM67.

767

768   *Statistical Analysis*

769   All data sets were tested for Normality using D'Agostino and Pearson Omnibus Normality Test

770   in GraphPad Prism 9. Normal data sets were then tested for significance with a two-tailed

771   unpaired t-test and non-normal data sets were tested for significance using the Mann-Whitney

772   test. All graphs show mean ± SD. All experiments utilized at least three biological replicates,

773   unless otherwise noted.

774

775   *Electron Tomography*

776   RPE1 cells were grown on sapphire discs and prepared for electron microscopy using high

777   pressure freezing and freeze substitution as described in McDonald et al.[66] Briefly, 3mm

778   sapphire discs (Technotrade International) were coated with gold and a large F was then

779   scratched into the surface to help orient the cell side. The disks were coated with collagen,

780   sterilized under UV light and cells plated for culturing. Monolayers grown on sapphire discs were

781   then frozen using a Wohlwend Compact02 high pressure freezer (Technotrade International).

782   The frozen cells were then freeze substituted in 1% OsO4 and 0.1% uranyl acetate in acetone

783   at -80°C for 3 days then gradually warmed to room temperature. The discs were then flat

32

784 embedded in a thin layer of Epon resin and polymerized at 60°C. Regions containing cells were

785 identified in the light microscope, and a small square of resin containing the cells was excised

786 and remounted onto a blank resin block. The cells were then sectioned en face and serial, thick

787 sections (250-300nm) were collected onto formvar-coated slot grids. Grids were post stained

788 with 2% uranyl acetate and Reynold's lead citrate and 15 nm colloidal gold (BBI International)

789 was affixed to the section surface to serve as alignment markers.

790   Tomography was performed using a Tecnai F30 microscope operating at 300 kV

791 (Thermo Fisher Scientific, Waltham, MA). Dual axis tilt series were collected over a +/- 60°

792 range using the SerialEM image acquisition software,[67] and a Gatan OneView camera (Gatan,

793 Inc., Pleasanton, CA). For most data sets, tilt series were collected from 2-4 serial sections to

794 reconstruct a larger volume of cell data. Tomograms were computed, serial tomograms joined

795 and cellular features were modeled using the IMOD 4.9 software package

796 (https://bio3d.colorado.edu/imod/).[68,69]

797   Organelles at the centrosomes of the cells (centrioles, vesicles, positions of MT and their

798 ends) were manually traced in these reconstructions using the 3dmod program in the IMOD

799 software package.[68] Models were projected in 3D to show the arrangement of the centrioles,

800 vesicles, microtubules and the position their ends within the volume. In total, 4 centrosomes

801 from D21, 3 centrosomes from T21, and 4 centrosomes from Q21 were reconstructed and

802 modeled. The modeled centrosomes came from different stages, including at or before ciliary

803 vesicle formation, and one D21 and T21 set with a primary cilium.

804

805   **Supplemental Figure Legends**

806   *Figure S1*. (A-C) Quantitation of whole cell PCNT intensities for D21 (A), T21 (B), and Q21 (C)

807   cells throughout the time course normalized to average at 0 h. Graph shows mean ± SD. (D)

808   Quantitation of centrosomal PCNT intensities in a 0-1.2 µm radial circle around the centrosome

809   throughout the time course normalized to D21 average at 0 h. Graph shows mean ± SD. (E)

810   Quantitation of pericentrosomal PCNT intensities in a 1.2-5 µm radial circle around the

811   centrosome throughout the time course normalized to D21 average at 0 h. Graph shows mean ±

812   SD. (F) Quantitation of centrosomal microtubule intensities in a 0-1.2 µm radial circle around the

813   centrosome throughout the time course normalized to D21 average at 0 h. Graph shows mean ±

814   SD. (G) Quantitation of pericentrosomal microtubule intensities in a 1.2-5 µm radial circle

815   around the centrosome throughout the time course normalized to D21 average at 0 h. Graph

816   shows mean ± SD. N's and statistical tests are listed in Table S1.

817

818   *Figure S2.* (A, B) Representative SIM images of RPE1 D21, T21, and Q21 cells grown on

819   coverslips and serum depleted for 24 h. Cells were stained with GT335 and the distal

820   appendage marker CEP83 (A) or centrin, Acetylated tubulin (Actub), and the subdistal

821   appendage marker Ninein (NIN) (B). Percentages represent cells with indicated marker for 3

822   N's.

823

824   *Figure S3.* (A) Quantitation of whole cell MYOVA intensity for D21, T21, and Q21 cells. (B-D)

825   Distribution of centrosomal MYOVA intensities moving away from the centrosome for 2 (B), 4

826   (C), and 24 (D) h timepoints. Values were normalized to the D21 average. Graph shows mean ±

827   SD. (E) Representative SIM images of RPE1 D21, T21, and Q21 cells grown on coverslips and

828   serum depleted for 24 h. Cells were either treated with control or PCNT siRNA during the 24 h

829   serum depletion. Cells were stained with GT335 and MYOVA. (F) Quantitation of PCNT

830   intensities in a 5 µm radial circle around the centrosome in control and PCNT siRNA treated

34

831   cells normalized to the D21 average. Graph shows mean ± SD. (G, H) Correlation analysis

832   between PCNT and MYOVA levels for D21, T21, and Q21 cells 24 h after serum depletion. (I)

833   Representative confocal images of RPE1 D21, T21, and Q21 cells stably expressing GFP-

834   EHD1 grown on coverslips and serum depleted for 24 h. Cells were stained for PCNT and

835   Actub. N's and statistical tests are listed in Table S1.

836

837   *Figure S4.* (A) Quantitation of CP110 centriole capping for D21, T21, and Q21 cells 24 h after

838   serum depletion. (B) Representative SIM images of RPE1 D21, T21, and Q21 cells grown on

839   coverslips and serum depleted for 24 h. Cells were stained with GT335 and the centriole

840   capping protein CEP97. Percentages represent cells with indicated phenotype across 3 N's. (C)

841   Quantitation of CEP97 centriole capping for D21, T21, and Q21 cells. (D) Representative SIM

842   images of RPE1 D21, T21, and Q21 cells grown on coverslips and serum depleted for 2, 4, 8,

843   24, and 48 h. Cells were stained with γ-tubulin, Actub, and RAB8. Percentages represent cells

844   with indicated phenotype across 3 N's. (E) Representative confocal images of RPE1 D21, T21,

845   and Q21 cells grown on coverslips and serum depleted for 24 h. Cells were stained with

846   Hoechst, PCNT, Golgin97, and RAB8. (F) Quantitation of RAB8 Golgi intensity normalized to

847   the D21 average. (G) Cartoon model depicting intracellular trafficking pathways. (H)

848   Representative confocal images of RPE1 D21, T21, and Q21 cells grown on coverslips and

849   serum depleted for 24 h. Cells were stained with Hoechst, γ-tubulin, and the cis-Golgi marker

850   GM130. (I-J) Quantitation of GM130 intensity (I) and GM130-labeled Golgi area (J) normalized

851   to the D21 average. (K) Representative confocal images of RPE1 D21, T21, and Q21 cells

852   grown on coverslips and serum depleted for 24 h. Cells were stained with Hoechst, PCNT, and

853   the trans-Golgi marker Golgin97. (L-M) Quantitation of Golgin97 intensity (L) and Golgin97-

854   labeled Golgi area (M) normalized to the D21 average. (N) Representative confocal images of

855   RPE1 D21, T21, and Q21 cells grown on coverslips and serum depleted for 24 h. Cells were

35

856   stained with Hoechst, γ-tubulin, and the early endosome marker EEA1. (O) Quantitation of

857   EEA1 intensity normalized to the D21 average. (P) Representative confocal images of RPE1

858   D21, T21, and Q21 cells grown on coverslips and serum depleted for 24 h. Cells were stained

859   with Hoechst, PCNT, and the lysosome marker CD63. Q) Quantitation of CD63 intensity

860   normalized to the D21 average. Graphs shows mean ± SD. N's and statistical tests are listed in

861   Table S1.

862

863   *Figure S5.* (A) Representative confocal images of RPE1 D21 and T21 cells stably expressing

864   pH-Smoothened (pH-SMO) grown on coverslips and serum depleted for 24 h. The IN/OUT

865   assay was performed, and cells were stained with an anti-GFP antibody pre-permeabilization

866   and Actub after. Anti-GFP labeled cilia are extracellular. (B) Quantitation of extracellular cilia in

867   D21 and T21 cells. (C) Quantitation of centrosomal CEP290 intensity in 0-1.2 µm region around

868   centrosome.

869

870   *Figure S6.* For all following experiments, WT and Dp primary MEFs were isolated from E12.5

871   dams, grown on coverslips, and serum depleted for 24 h. (A, B) Representative confocal images

872   of WT and Dp16 (A) or Dp17 (B) MEFs. Cells were stained with Hoechst, the ciliary marker

873   ARL13B, and γ-tubulin. (C, D) Representative confocal images of WT and Dp16 (C) or Dp17 (D)

874   MEFs. Cells were stained with PCNT and ARL13B. (E) Representative confocal images of WT

875   and the Dp10-2 MEF line that does not have decreased cilia. Cells were stained with Hoechst,

876   the ciliary marker ARL13B, and Actub. (F) Quantitation of the number of cells with a primary

877   cilium in WT and Dp10-2 MEFs. (G) Quantitation of PCNT intensities in a 5 µm radial circle

878   around the centrosome in WT and Dp10-2 MEFs. Values were normalized to the WT average.

879   (H) Quantitation of ciliary SMO levels in SAG-treated cells for WT and Dp10-2 MEFs. Values

880   were normalized to the WT average. Graphs show mean ± SD. N's and statistical tests are

881   listed in Table S1.

882

883   *Figure S7.* (A) Representative tiled confocal images of WT and Dp10 P4 animals corresponding

884   to the same cerebellar folia in each animal. Brain sections were stained with Hoechst and the

885   cell proliferation marker Ki67. (B) Quantitation of the number of Ki67-positive cells in the

886   external granular layer of WT and Dp10 animals. (C) Representative tiled confocal images of

887   WT and Dp10 P4 animals corresponding to the same cerebellar folia in each animal. Brain

888   sections were stained with Hoechst and the Purkinje cell marker calbindin (CALB1) and the

889   microtubule binding protein doublecortin (DCX). The far-right panel shows insets of DCX

890   staining. (D-F) Representative tiled confocal images of multiciliated ependymal cells lining the

891   ventricle next to the cerebellum from P4 wild-type (WT) and Dp10 (E), Dp16 (F), and Dp17 (G)

892   animals. Brain sections were stained with Hoechst, ARL13B, and γ-tubulin.

893

894   *Video S1-3.* Movies of D21 (S1), T21 (S2), and Q21 (S3) cells from Figure 2 showing the EM

895   tomogram volume, then models projecting from the images, then the model turning without

896   images. Mother centriole (yellow), daughter centriole (magenta), microtubule minus ends (light

897   blue spheres), microtubules (green), and vesicles (red). Note the proximity of vesicles to

898   microtubules.

899

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Figure 1
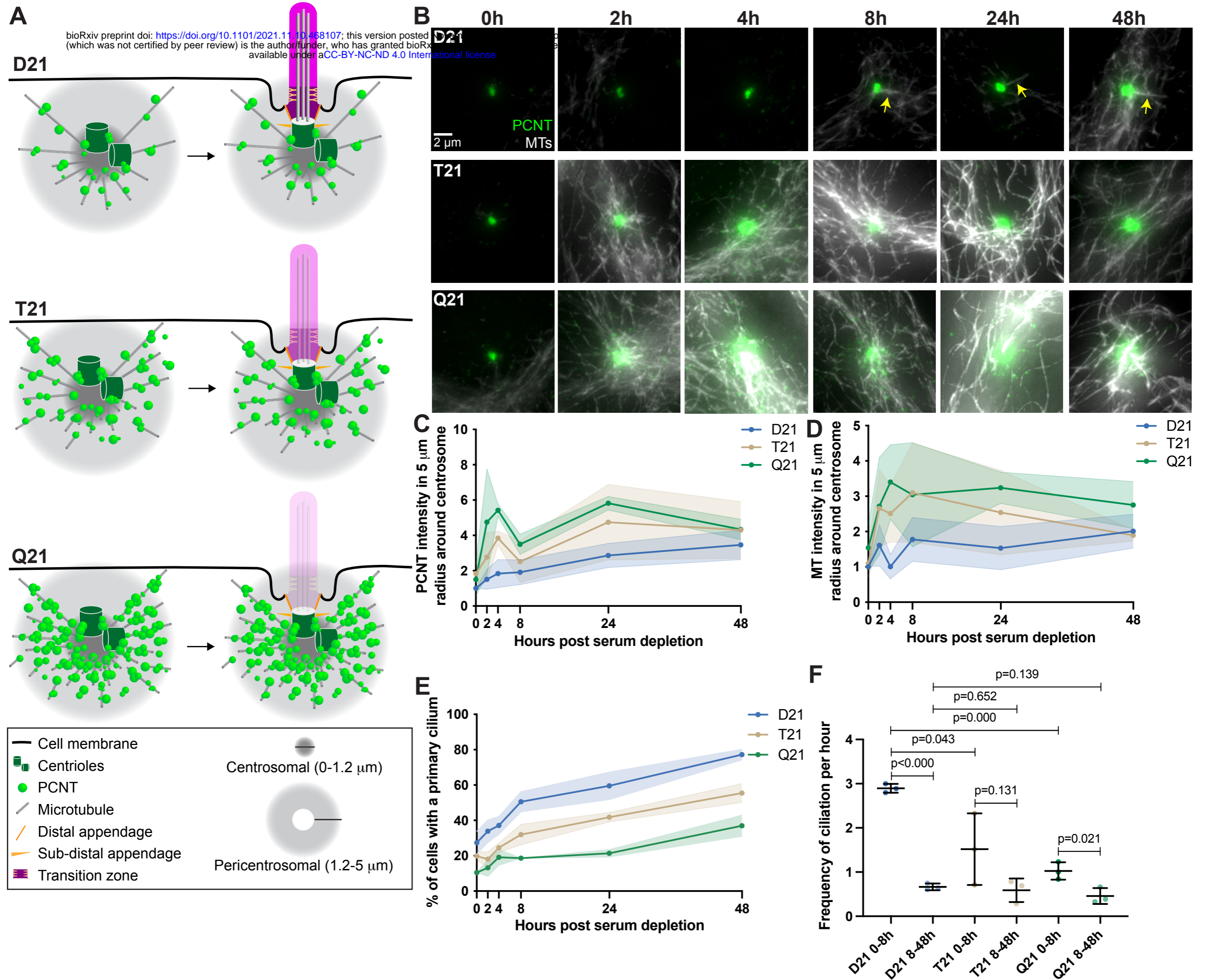
Figure 2
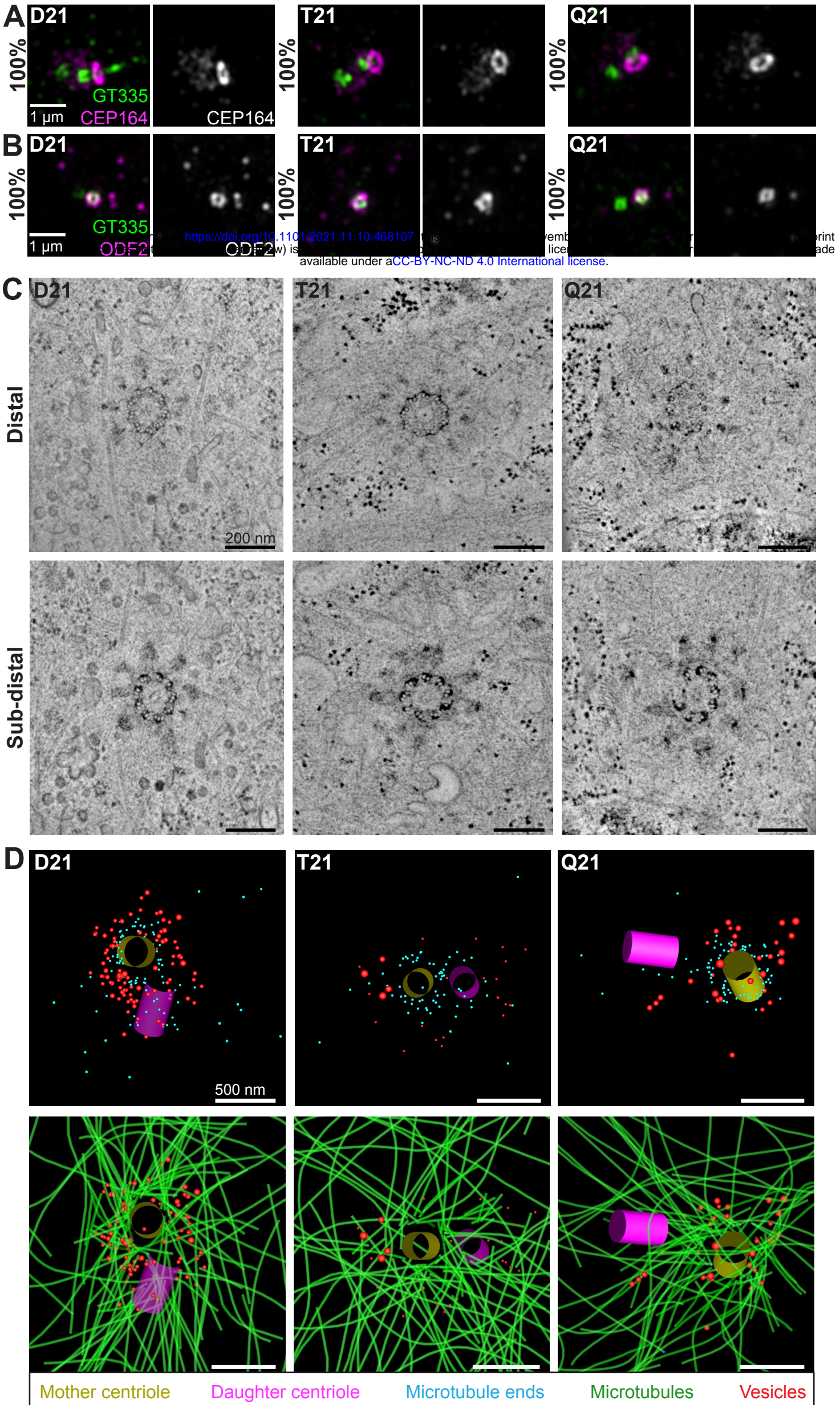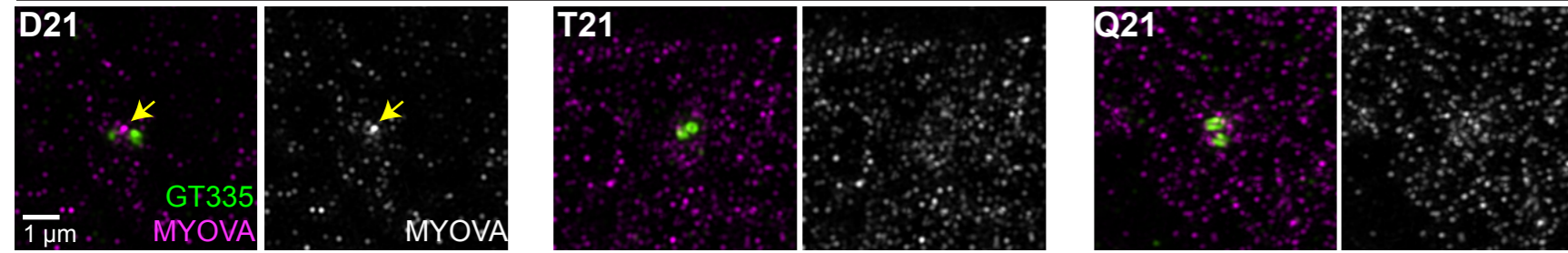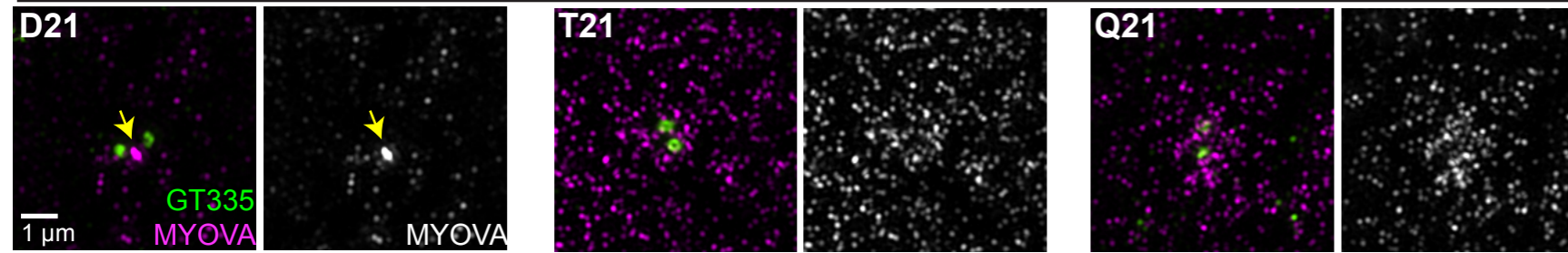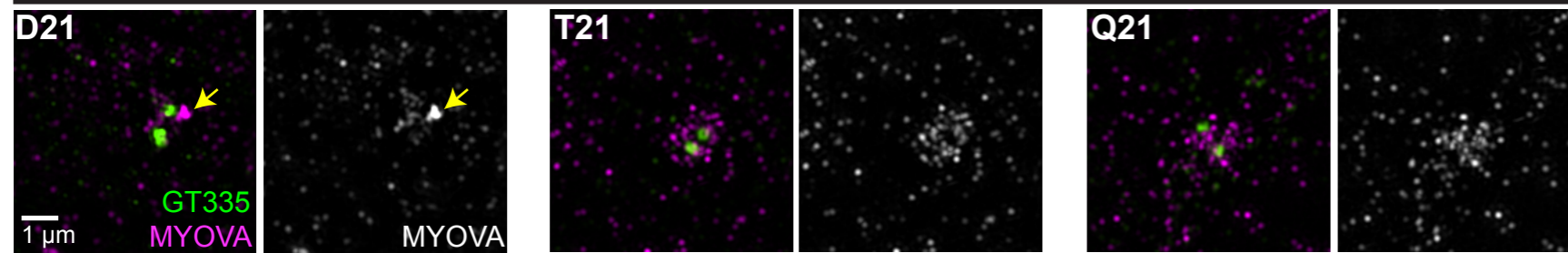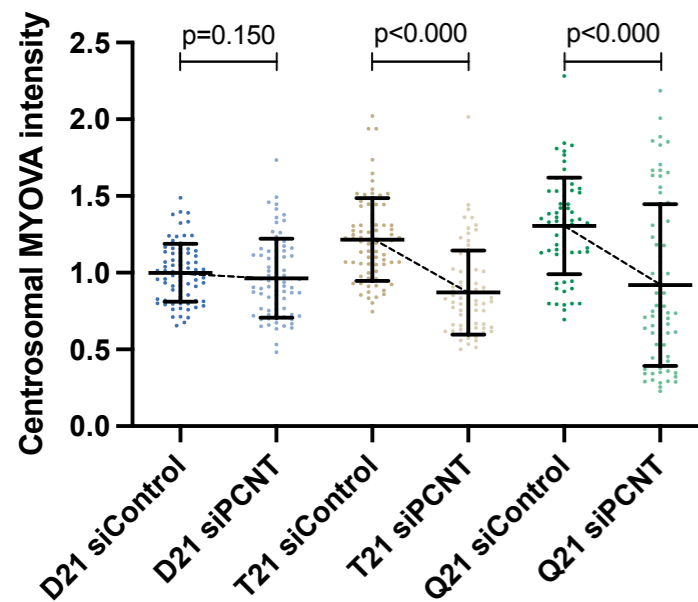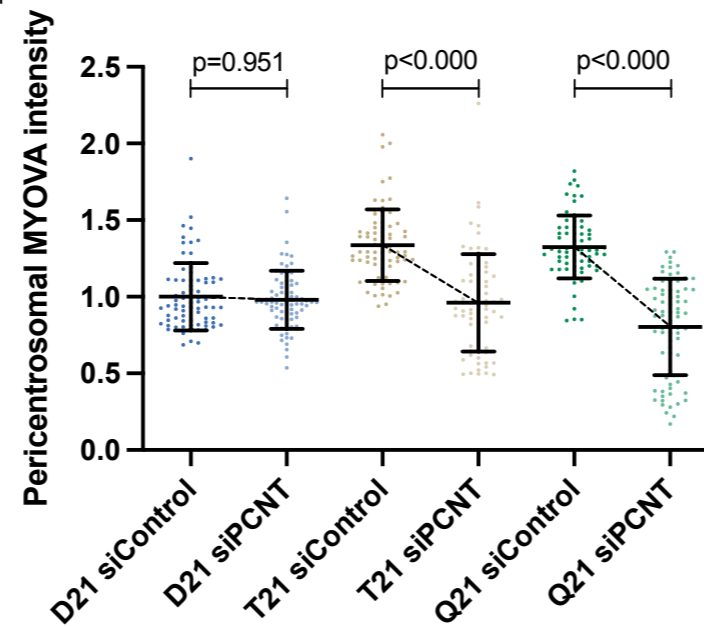
Mother centriole   Daughter centriole   Microtubule ends   Microtubules   Vesicles

Figure 3

Figure 4

A — 24h serum depletion

D21 | T21 | Q21

Capped 26% | 47% | 77%

GT335 / CP110

Uncapped 74% | 53% | 23%

1 μm

B

% of cells with both centrioles capped

p=0.580   p=0.005   p=0.001

D21 siControl, D21 siPCNT, T21 siControl, T21 siPCNT, Q21 siControl, Q21 siPCNT

C — 4h serum depletion

D21 | T21 | Q21

No CV 84% | 90% | 100%

γ-tub / Actub / RAB8 ; RAB8

RAB8+ CV 16% | 10%

1 μm

8h serum depletion

D21 | T21 | Q21

No CV 66% | 87% | 100%

γ-tub / Actub / RAB8 ; RAB8

RAB8+ cilium 34% | 13%

1 μm

D

% of cells with RAB8 CV

D21, T21, Q21

0 2 4 8 24 48
Hours post serum depletion

Legend:
— Cell membrane
■ Centrioles
● PCNT
╱ Microtubule
╱ Distal appendage
— Sub-distal appendage
▥ Transition zone
● Pre-ciliary vesicles
● Ciliary vesicle
⬬ Mother centriole cap

Centrosomal (0-1.2 μm) | Pericentrosomal (1.2-5 μm)

E
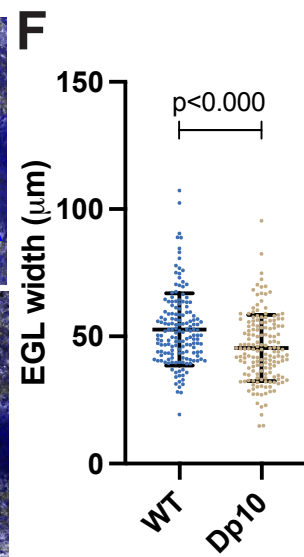
D21 | T21 | Q21
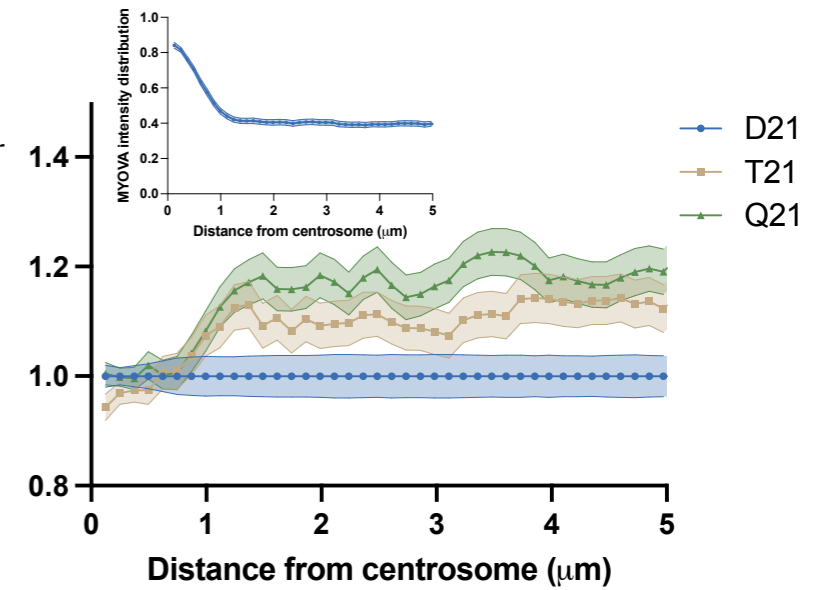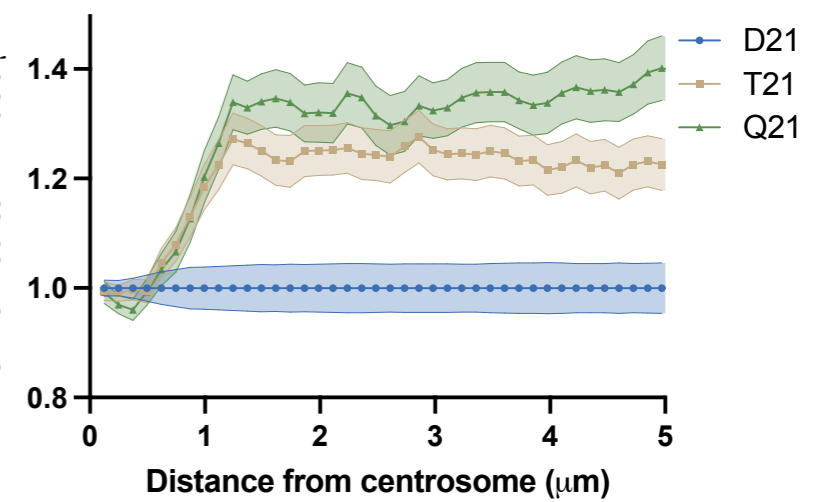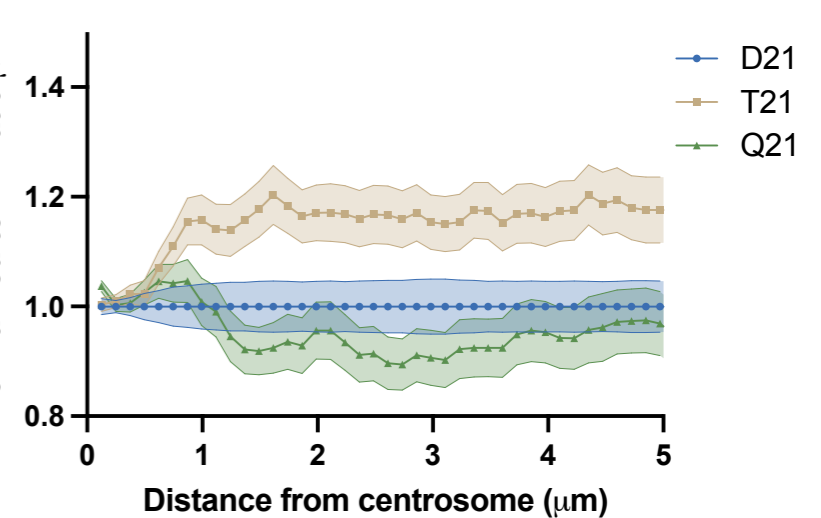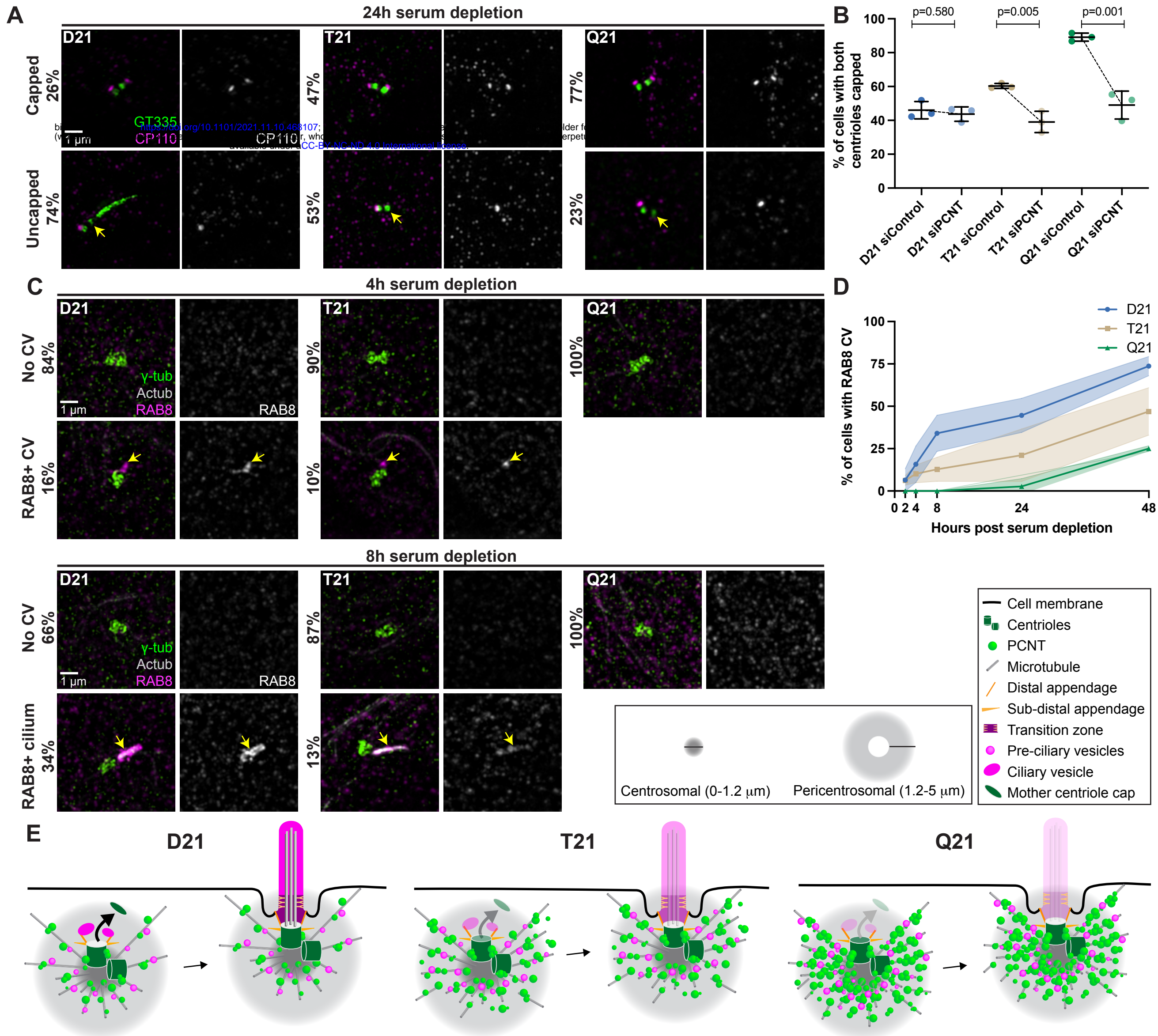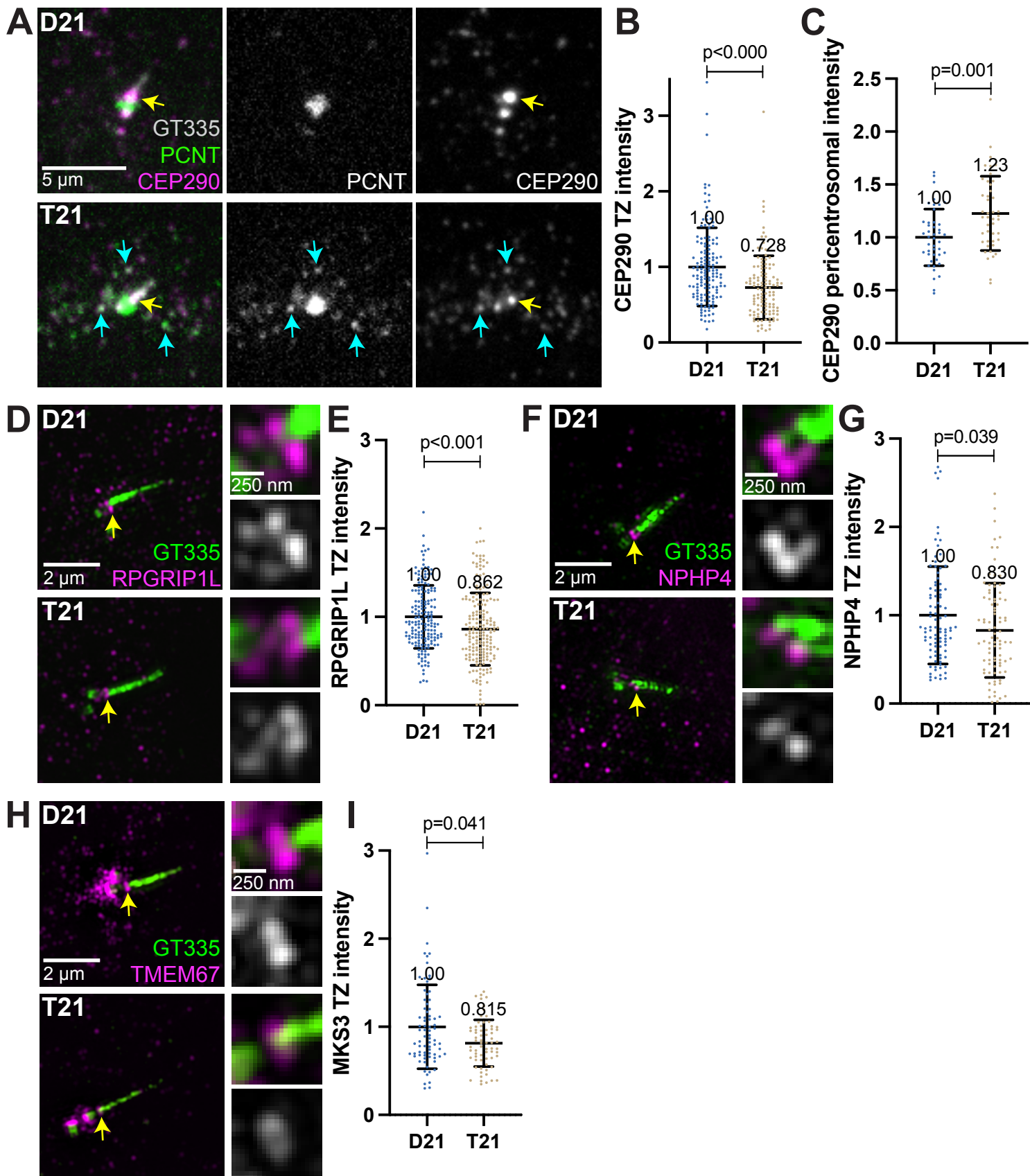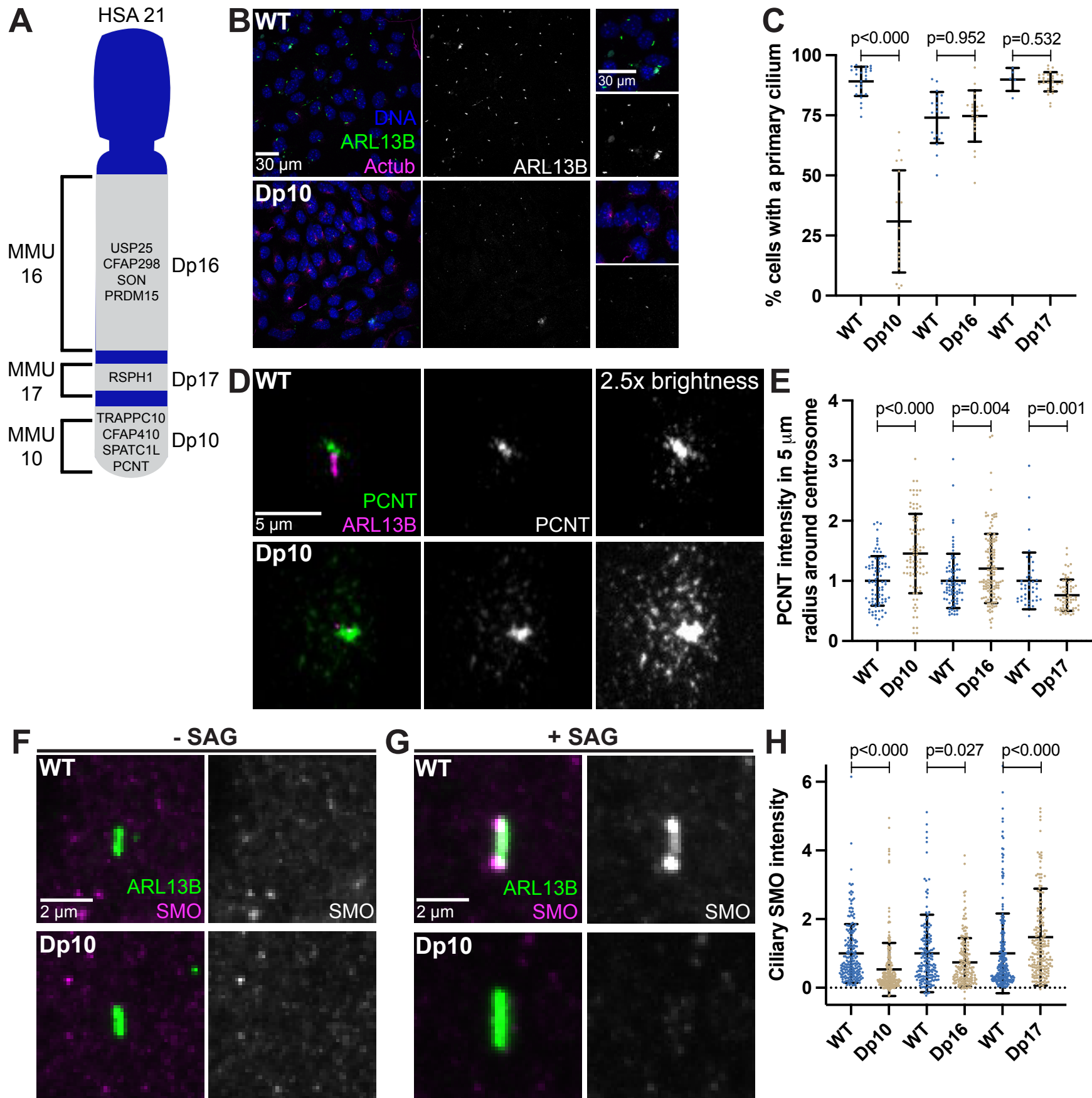
Figure 5

Figure 6

Figure 7

P4 Fixed Sagittal Sections

Cerebellar Slice Cultures