The oral-facial-digital syndrome gene C2CD3 encodes a positive regulator of centriole elongation

Christel Thauvin-Robinet1,2,17, Jaclyn S Lee3,17, Estelle Lopez1, Vicente Herranz-Pérez4,5, Toshinobu Shida3, Brunella Franco6,7, Laurence Jegó1, Fan Ye3, Laurent Pasquier8, Philippe Loget9, Nadège Gigot1,10, Bernard Aral1,10, Carla A M Lopes11, Judith St-Onge1,10, Ange-Line Bruel1, Julien Thevenon1,2, Susana González-Granero4,5, Caroline Alby12,13, Arnold Munnich12–14, Michel Vekemans12–14, Frédéric Huet1,2, Andrew M Fry11, Sophie Saunier13,15, Jean-Baptiste Rivière1,10, Tania Attié-Bitach12–14, Jose Manuel Garcia-Verdugo4,5, Laurence Faivre1,2, André Mégarbané16 & Maxence V Nachury13

Centrioles are microtubule-based, barrel-shaped structures that initiate the assembly of centrosomes and cilia1,2. How centriole length is precisely set remains elusive. The microcephaly protein CPAP (also known as MCHP6) promotes procentriole growth3–5, whereas the oral-facial-digital (OFD) syndrome protein OFD1 represses centriole elongation6,7. Here we uncover a new subtype of OFD with severe microcephaly and cerebral malformations and identify distinct mutations in two affected families in the evolutionarily conserved C2CD3 gene. Concordant with the clinical overlap, C2CD3 colocalizes with OFD1 at the distal end of centrioles, and C2CD3 physically associates with OFD1. However, whereas OFD1 deletion leads to centriole hyperelongation, loss of C2CD3 results in short centrioles without subdistal and distal appendages. Because C2CD3 overexpression triggers centriole hyperelongation and OFD1 antagonizes this activity, we propose that C2CD3 directly promotes centriole elongation and that OFD1 acts as a negative regulator of C2CD3. Our results identify regulation of centriole length as an emerging pathogenic mechanism in ciliopathies.

OFD syndromes are ciliopathies characterized by malformations of the face, oral cavity and digits8. A 4-year-old male born from consanguineous parents presented with canonical OFD syndrome as well as severe microcephaly, micropenis and severe intellectual disability (Fig. 1a,b and Supplementary Table 1), indicative of an unclassified OFD syndrome. Brain magnetic resonance imaging (MRI) showed the presence of molar tooth sign (MTS; a cerebellar anomaly characteristic of Joubert syndrome and related disorders9) and several other cerebral malformations (Fig. 1c–e and Supplementary Table 1). The proband’s younger sister presented with similar anomalies and cardiac malformation, leading to neonatal death. Homozygosity mapping identified a candidate region of 4 Mb at 11q13.4-q14.1 (Supplementary Fig. 1a), and coupled exome sequencing identified a homozygous nonsense mutation in the C2CD3 gene (NM_015531.5: c.184C>T in exon 2; p.Arg62*), which was confirmed by Sanger sequencing and was found to be heterozygous in his parents (Supplementary Fig. 1b).

We next screened the coding exons of C2CD3 for mutations in 34 individuals with OFD who were negative for mutations in known OFD-associated genes. We identified a male fetus with compound heterozygous C2CD3 mutations consisting of one missense variant (NM_015531.5: c.3085T>G; p.Cys1029Gly) and a substitution in the splice acceptor consensus sequence of exon 22 (NM_015531.5: c.3911–2A>T; p.Arg62*). Analysis of the affected fetus showed that the c.3911–2A>T substitution caused splicing of the 5′ end of exon 22 to a downstream cryptic splice site, leading to a 4-nt frameshift deletion (NM_015531.5: c.3911_3914delCAAG; p.Ala1304Valfs*3) (Supplementary Fig. 1c–e). In close similarity to the index case, the second individual with OFD exhibited severe microcephaly combined with canonical OFD symptoms (Fig. 1f–j and Supplementary Table 1). Both mutations were absent from the Exome Variant Server (see Supplementary Table 1).
Figure 1 Clinical presentation of individuals with OFD with mutations in C2CD3. (a–e) Characterization of the 4-year-old (case 1) showing microcephaly with trigonocephaly, facial dysmorphism including telecanthus, up-slanting palpebral fissures and microretrognathia (a), bilateral broad duplicated and deviated hallux (b) and brain MRI with corpus callosum hypoplasia (c), MTS (d), subarachnoid cysts in the right occipital lobe (d) and the posterior fossa (e), and incomplete myelination of the white matter (c–e). Informed consent to publish these images was obtained from this individual’s family. (f–j) Pictures of the fetus at 22 weeks of gestation (case 2) showing bilateral hand postaxial polydactyly (f) and broad duplicated hallux (g) and X-rays showing normal thorax (h) and postaxial polydactyly of the left (i) and right (j) hands. (k) Domain organization of C2CD3 (C2 calcium-dependent domain containing 3). The six orange ovals correspond to canonical C2 domains, and the yellow oval represents the signature C2CD3N C2 domain. Alterations identified in cases are indicated in red on top of the diagram, mouse mutants are shown under the diagram, and peptides identified in LAP-BBS4 preparations are mapped onto the diagram by blue bars. The splice-site mutation c.3911–2A>T leads to a 4-nt frameshift deletion (c.3911–2A>T) and peptides identified in LAP-BBS4 preparations are mapped onto the diagram by blue bars. The splice-site mutation c.3911–2A>T leads to a 4-nt frameshift deletion (c.3911–2A>T) predicted to result in a premature stop codon at position 1307.

Figure 2 C2CD3 colocalizes with OFD1 at the distal end of centrioles and procentrioles and physically interacts with OFD1. (a–h) IMCD3 cells expressing GFP fused mouse C2CD3 were stained for the centriolar marker branched glutamylated tubulin (GT335; pink) (a), the distal centriole and procentriole marker centrin (red) (b), the procentriole marker SASS6 (red) (c), ninein (red; marks the subdistal appendages and the proximal end of the centrioles and procentrioles) and glutamylated tubulin (GT335; pink; marks the ciliary axoneme) (d), CEP164 (red; marks the distal appendages) and glutamylated tubulin (GT335; pink) (e), CEP290 (red; marks the transition zone) and glutamylated tubulin (GT335; pink) (f), OFD1 (red) and γ-tubulin (pink; marks the pericentriolar material near the proximal part of the centriole) (g), and the distal cap marker CP110 (red) and γ-tubulin (pink) (h). All cells were treated with nocodazole before processing for immunofluorescence. All scale bars indicate 1 µm. (i) Immunoprecipitation of OFD1 from RPE cell extract recovers C2CD3. For the C2CD3 immunoblot, 160 equivalents of the eluates and 1 equivalent of lysate were run on the gel. For the OFD1 immunoblot, 4 equivalents of the eluates and 1 equivalent of lysate were run on the gel. The eluate and lysate panels are cropped from the same film exposure. MW, molecular weight; IP, immunoprecipitation; IB, immunoblot; GST, glutathione-S-transferase; MBP, maltose-binding protein; GST-OFD1, GST fusion with OFD1; MBP-GFP, maltose-binding protein-GFP; GST-C2CD3, GST fusion with C2CD3. MBP-GFP-FKBP (negative control) expressed in HEK293FT cells were captured by bacterially expressed GST fusions with OFD1. Captured proteins were detected by immunoblotting for GFP; 80 equivalents of the eluates and 1 equivalent of lysate were run on the gel.
in C5orf42 in Joubert syndrome (JBTS17; MIM 614615) and OFD VI (refs. 14, 15), and the mutations in TCTN3 identified in OFD IV (OFD4; MIM 258860), Meckel-Gruber syndrome and Joubert syndrome16, our identification of a new OFD subtype with severe cerebral malformations including MTS further reinforces the clinical continuum of the ciliopathy spectrum and the inclusion of OFD in the ciliopathies.

Besides its requirement for cilium assembly11, the function of C2CD3 remains largely elusive. A first clue was provided by the mass spectrometry-based identification of C2CD3 as a BBSome-interacting protein (Fig. 1k and Supplementary Fig. 2a). The BBSome is a complex of proteins that is altered in the ciliopathy Bardet-Biedl syndrome (BBS; MIM 209900) and present in cilia and cytoplasmic granules termed centriolar satellites17–21. To determine the localization of C2CD3, we expressed GFP-fused mouse C2CD3 at low levels in a stable clone of mouse IMCD3 Flp-In kidney cells. Whereas C2CD3 was never detected inside cilia, we found robust colocalization of C2CD3 with the core centriolar satellite component PCM-1 (Supplementary Fig. 2b). However, knockdown of C2CD3 in human retinal pigmented epithelial RPE1-hiHERT cells and C2cd3 mutations in immortalized mouse embryonic fibroblasts (MEFs) did not perturb centriolar satellite function (Supplementary Fig. 2c).

In addition to finding C2CD3 localized at centriolar satellites, we consistently found C2CD3 at two to four bright juxtaplume foci that did not overlap with PCM-1 foci (Supplementary Fig. 2b, insets). Costaining with a monoclonal antibody against glutamylated tubulin (GT335)—a modified tubulin found at centrioles and ciliary axonemes—showed that these PCM-1–negative foci of C2CD3 were centrioles (Supplementary Fig. 3a). Although centriolar satellites deliver proteins such as pericentrin to the centrosome17,21, inhibiting the movement of centriolar satellites using the microtubule poison nocodazole did not affect C2CD3 abundance at centrioles (Supplementary Fig. 3b).

We then mapped the precise location of C2CD3 within centrioles. We frequently observed two juxtaposed foci of C2CD3 associated with each other (Fig. 1k). Because the two juxtaposed foci of C2CD3 were each positive for centrin, which marks the distal lumen of mature centrioles and procentrioles (Fig. 2b and Supplementary Fig. 4a), and because procentriole microtubules are not polyglutamylated22, the C2CD3 focus negative for glutamylated tubulin (Supplementary Fig. 4a) is present at procentrioles (Fig. 2b). We identified by a single focus of glutamylated tubulin (Supplementary Fig. 4a). We identified by a single focus of glutamylated tubulin (Supplementary Fig. 4a), and this focus had a stringent colocalization with the core centriolar satellite component PCM-1 (Supplementary Fig. 4a). In addition, we found that the localization of C2CD3 was not perturbed by the centrosome inhibitor ninein, which suggests that C2CD3 is not required for PCM-1 localization.
or OD2 (Fig. 2d and Supplementary Fig. 4b). C2CD3 was precisely located between the CEP164-marked distal appendages (Fig. 2e). Finally, the transition zone25 marker CEP290 was clearly distal to C2CD3 (Fig. 2f), leading us to conclude that C2CD3 is localized near the distal tip of centrioles. CP110 and OD1 have also been found to localize near the distal end of centrioles, with CP110 marking a slightly more distal location than OD1 (ref. 7), namely, the centriole cap whose removal allows for elongation of the ciliary axoneme36. C2CD3 perfectly colocalized with OD1 (Fig. 2g) and was slightly proximal to CP110 (Fig. 2h).

Because C2CD3 colocalizes with OD1 and both of these proteins are altered in OFD syndromes, we tested whether these proteins physically interact. Using antibodies raised against the endogenous proteins, C2CD3 and OD1 were found to communoprecipitate out of human RPE cells (Fig. 2i). To further confirm this interaction, glutathione S-transferase (GST) fusions of human OD1 fragments were expressed in bacteria, immobilized on beads and used to capture GFP-fused mouse C2CD3 expressed in HEK293FT cells. Remarkably, the central fragment of OD1, which is also responsible for homodimerization27, specifically interacted with GFP-C2CD3 (Fig. 2j and Supplementary Fig. 4c). We conclude that C2CD3 forms a complex with OD1 at the distal end of centrioles, where it colocalizes with other known regulators of centriole elongation, such as centrin28 or POC5 (ref. 29).

Because OD1 and C2CD3 associate physically and cytologically and because the genes encoding them are mutated in the same syndrome, we hypothesized that C2CD3 deletion would mimic loss of OD1 and result in centrin-positive hyperelongation of centrioles25. Surprisingly, centrin was undetectable in the vast majority of C2cd3Hty/Hty and C2cd3Gt/Gt centrioles (Fig. 3a,b) even though total centrin levels stayed constant (Supplementary Fig. 5a), in agreement with a recent study30. Similarly, staining for glutamylated tubulin did not show centriole hyperelongation in C2cd3-mutant MEFS (Fig. 3c). Thus, unlike loss of OD1, loss of C2CD3 does not result in elongation of the distal centriole compartment.

Remarkably, distal appendages (stained by CEP164; Fig. 3c,d) as well as subdistal appendages (stained by OD2; Fig. 3e,f) were absent from C2cd3-mutant centrioles. Ninein staining confirmed the absence of subdistal appendages and the intactness of the proximal end of centrioles (Supplementary Fig. 5b). Similarly, CPAP, a marker of the proximal segment of centrioles, was retained in C2cd3 mutants (Fig. 3g,h). OD1 levels were greatly reduced at C2cd3-mutant centrioles (Fig. 3i,j), suggesting that C2CD3 recruits OD1 to centrioles through the physical interaction of these proteins. Whereas C2cd3-mutant centrioles lost most markers of the distal centrioles, the centriole cap protein CP110 was still present at the distal end of centrioles (Fig. 3k), concordant with the inability of C2cd3-mutant cells to assemble a primary cilium11.

An appealing explanation for the loss of distal centriole structures in C2cd3-mutant cells is incomplete centriole elongation. Alternatively, it is conceivable that maturation of the distal centriole is affected independently of alterations in centriole length. In support of the former hypothesis, the distance between the centriole cap and centriole center—measured by fluorescence microscopy—was significantly ($P < 1 \times 10^{-4}$) reduced in C2cd3Gt/Gt cells compared to control cells (Fig. 4a,b). To precisely assess the structural defects in C2cd3-mutant centrioles, we performed thin-section electron microscopy on C2cd3-mutant MEFS. Confirming our immunofluorescence findings, distal and subdistal appendages were absent from C2cd3-mutant centrioles (Fig. 4c–e and Supplementary Fig. 6a,b). Total centriole length was precisely assessed by thin-section electron microscopy, and we found C2cd3-mutant centrioles to be shorter than controls by ~150 nm (Fig. 4f). We conclude that C2CD3 is required for the extension of procentrioles into full-length centrioles.

To gain insight into the mechanisms by which C2CD3 controls centriole length, we examined the effects of overexpressing GFP-fused mouse C2CD3 (Fig. 5). Strikingly, in RPE cells, we observed one or two juxtanuclear straight rods of >2 µm in length marked by GFP-C2CD3 and acetylated microtubules along their length (Fig. 5a, insets) and resistant to nocodazole treatment (Supplementary Fig. 7a). By synchronizing cells in S phase and releasing them in G2 phase31, we found that 15–25% of U2OS human osteosarcoma cells transfected with plasmid encoding GFP-C2CD3 possessed GFP-positive rods, in line with previously described centriole-lengthening activities31,32. The morphological resemblance of centrioles from
these cells to the hyperelongated centrioles in cells overexpressing CEP120 or CPAP3–5,31,32 hinted that the C2CD3-positive rods might represent hyperelongated centrioles (Fig. 5b–e). These juxtanuclear C2CD3-positive rods were also positive for CPAP and CEP164 at their bases (Fig. 5b,c) and for glutamylated tubulin and centrin along their lengths (Fig. 5d,e) and showed an enrichment of CP110 at their distal ends (Fig. 5e). The absence of the cilium maker IFT88 at the C2CD3-positive rods (Fig. 5d) and the presence of CP110 at the end of these structures (Fig. 5e) strongly suggested that these structures represent hyperelongated centrioles and not cilia. To confirm that C2CD3 promotes centriole hyperelongation, we imaged the GFP-C2CD3–positive rods by correlative light electron microscopy33 (CLEM) and identified continuous extensions of the centriolar microtubules up to 1.3 µm in length in three separate cells (Fig. 5f, Supplementary Fig. 7b and Supplementary Video 1). In addition to localization at hyperelongated centrioles, overexpressed GFP-C2CD3 was found alongside bright microtubules rods (Supplementary Fig. 7c) enriched in acetylated tubulin in various regions of the cytoplasm (Fig. 5a). As in previous observations with CPAP overexpression3, CLEM showed that these cytoplasmic microtubule structures were reminiscent of centrioles, albeit much less organized. The mixed C2CD3-positive microtubule rods contained microtubules spaced 250 nm apart by an electron-dense matrix (Fig. 6a), and doublet microtubules could sometimes be distinguished (Fig. 6a, bottom). Congruently, the C2CD3-positive microtubule rods were also positive for centrin (Fig. 6b). Although the biogenesis...
of these centriole-like structures is presently unknown, they may have originated and then broken off from hyperelongated centrioles (Supplementary Fig. 7b and Supplementary Video 1).

The ability of C2CD3 to induce centriole hyperelongation enabled a test of the functional antagonism between C2CD3 and OFD1. Cotransfection of plasmid encoding mouse OFD1 (but not of a control plasmid) with plasmid for mouse C2CD3 in U2OS cells led to a drastic reduction in the frequency of hyperelongated centrioles (Fig. 5g).

Our finding that the OFD-relevant gene products OFD1 and C2CD3 antagonize one another in the regulation of centriole length suggests a model in which C2CD3 promotes centriole lengthening and OFD1 inhibits the activity of C2CD3 through direct physical contact. In this model, the inhibition exerted by OFD1 on C2CD3 would need to be minimal in the early stages of procentriole elongation and increase as centrioles reach their mature length. Further insights may be gained by studying how the centriole-lengthening activity of C2CD3 is related to its suggested ability to bind and stabilize microtubules and how C2CD3 function is coordinated with the procentriole-elongating activities of CPAP and CEP120, in particular, with respect to their microtubule-stabilizing activities9,10. Finally, an unexplored aspect of C2CD3 lies in its six canonical C2 domains, which are predicted to bind Ca2+ and phospholipid headgroups10. Centrin, which colocalizes precisely with CPAP and CEP120, in particular, with respect to their microtubule-stabilizing activities31,32. C2CD3, long thought to be a centriolar satellite protein, is also found in the perinuclear cytoplasm. OFD1 brings new understanding.

Author Contributions
M.V.N. conceived and supervised the functional characterization of C2CD3. C.T.-R. designed and conducted the gene identification strategy with assistance from T.A.-B., B.F., L.E., L.L., E.I., J.-B.R., C.A., N.G., B.A., A. Mégarbané, J.T.-S., J.-O. and A.-L.B. I.-B.R. and S.S. performed mapping analysis, genetic screening and mutation analysis. A. Mégarbané, B.F., L.P., P.L. and C.T.-R. identified and recruited subjects. S.G.-G., A. Munnich, F.H. and M.G. gave technical support and conceptual advice. A.M.F. and C.A.M.L. provided OFD1 reagents. M.V.N., J.S.L. and T.S. designed, executed and analyzed the immunohistochemistry, oncopression and protein interaction assays. F.Y. designed and executed the centriole length measurement by light microscopy. V.H.-P. and J.M.-G.V. designed and executed electron microscopy imaging and analysis. C.T.-R. and M.V.N. wrote the manuscript. All authors reviewed the manuscript.

Competing financial interests
The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Paintrand, M., Moudjou, M., Delacroix, H. & Bornens, M. Centrosome organization and centriole architecture: their sensitivity to divalent cations. J. Struct. Biol. 108, 107–128 (1992).
2. Blument-court-Dias, M. & Glover, D.M. Centrosome biogenesis and function: centrosomics brings new understanding. Nat. Rev. Mol. Cell Biol. 8, 451–463 (2007).
3. Kohlmaier, G. et al. Overly long centrioles and defective cell division upon excess of the SAS-4–related protein CPAP. Curr. Biol. 19, 1012–1018 (2009).
4. Tang, C.-J.C., Fu, R.-H., Wu, K.-S., Hsu, W.-B. & Tang, T.K. CPAP is a cell-cycle regulated protein that controls centriole length. Nat. Cell Biol. 11, 825–831 (2009).
5. Schmidt, T.I. et al. Control of centriole length by CPAP and CPAP1. Curr. Biol. 19, 1005–1011 (2009).
6. Ferrante, M.I. et al. Identification of the gene for oral-facial-digital type I syndrome. Am. J. Hum. Genet. 68, 569–576 (2001).
7. Sirigla, V., Romague-Ros, M. & García-Verdugo, J.M. Ofd1, a human disease gene, regulates the length and distal structure of centrioles. Dev. Cell 18, 410–424 (2010).
8. Gurrieri, F., Franco, B., Torriolo, H. & Neri, G. Oral-facial-digital syndromes: review and diagnostic guidelines. Am. J. Med. Genet. A. 143A, 3314–3323 (2007).
9. Patel, S. & Barkovich, A.J. Analysis and classification of cerebellar malformations. AJNR Am. J. Neuroradiol. 23, 1074–1087 (2002).
10. Zhang, D. & Aravind, L. Novel transglutaminase-like peptidase and C2 domains elucidate the structure, biogenesis and evolution of the ciliary compartment. Cell Cycle 11, 3861–3875 (2012).
11. Hoover, A.N. et al. C2cd3 is required for cilia formation and Hedgehog signaling in mouse. Development 135, 4049–4058 (2008).
12. Zohn, I.E., Anderson, K.V. & Niswander, L. Using genomewide mutagenesis screens to identify the genes required for neural tube closure in the mouse. Birth Defects Res. A Clin. Mol. Teratol. 73, 583–590 (2005).
13. Poretti, A. et al. Delineation and diagnostic criteria of oral-facial-digital syndrome type VI. Orphanet J. Rare Dis. 7, 4 (2012).
14. Slaczka, J. et al. Mutations in C5ORF42 cause Joubert syndrome in the French Canadian population. Am. J. Hum. Genet. 90, 693–700 (2012).
15. Lopas, E. et al. C5or42 is the major gene responsible for OFD syndrome type VI. Hum. Genet. 133, 367–377 (2014).
16. Thomas, S. et al. TCTN3 mutations cause Mohr-Majewski syndrome. Am. J. Hum. Genet. 91, 372–378 (2012).
17. Dammernann, A. & Merdes, A. Assembly of centrosomal proteins and microtubule organization depends on PCM-1. J. Cell Biol. 159, 255–266 (2002).
18. Kim, J.C. et al. The Bardet-Biedl protein BBS4 targets cargo to the pericentriolar region and is required for microtubule anchoring and cell cycle progression. Nat. Genet. 36, 462–470 (2004).
19. Nachury, M.V. et al. A core complex of BBS proteins cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis. Cell 129, 1201–1213 (2007).
20. Jin, H. et al. The conserved Bardet-Biedl syndrome proteins assemble a coat that traffics membranes proteins to cilia. Cell 141, 1208–1219 (2010).
21. Lopas, C.M. et al. Centriolar satellites are assembly points for proteins implicated in human ciliopathies, including oral-facial-digital syndrome 1. J. Cell Sci. 124, 600–612 (2011).
22. Klyeylin-Sohn, J. et al. Pirk-induced centriole biogenesis in human cells. Dev. Cell 13, 190–202 (2007).
23. Strnad, P. et al. Regulated HsSAS-6 levels ensure formation of a single procentriole per centriole during the centrosome duplication cycle. Dev. Cell 13, 203–213 (2007).
24. Morgenstern, M.M., Bornens, M., Malik, A., Piel, M. & Bouckson-Castaing, V. Microtubule minus-end anchorage at centrosomal and non-centrosomal sites: the role of ninein. J. Cell Sci. 113, 3013–3023 (2000).
25. Garcia-Gonzalo, F.R. & Reiter, J.F. Scoring a backstage pass: mechanisms of ciliogenesis and ciliary access. J. Cell Biol. 197, 693–709 (2012).
26. Spokrot, A., Tson, W.Y., Knoch, D. & Dynlacht, B.D. Cep97 and CP110 suppress a cilia assembly program. Cell 130, 678–690 (2007).
27. Giorgio, G. et al. Functional characterization of the OFD1 protein reveals a nuclear localization and physical interaction with subunits of a chromatin remodeling complex. Mol. Biol. Cell 18, 4397–4404 (2007).
28. Salisbury, J.L., Suino, K.M., Busby, R. & Springett, M. Centrin-2 is required for centriole duplication in mammalian cells. Curr. Biol. 12, 1287–1292 (2002).
29. Azimzadeh, J. et al. HPOC5 is a centrin-binding protein required for assembly of full-length centrioles. J. Cell Biol. 185, 101–114 (2009).
30. Balestra, F.R., Strnad, P., Flückiger, I. & Gönczy, P. Discovering regulators of centriole biogenesis through siRNA-based functional genomics in human cells. Dev. Cell 25, 555–571 (2013).
31. Comartin, D. et al. CEP120 and SPICE1 cooperate with CPAP in centriole elongation. Curr. Biol. 23, 1360–1366 (2013).
32. Lin, Y.-N. et al. CEP120 interacts with CPAP and positively regulates centriole elongation. J. Cell Biol. 202, 211–219 (2013).
33. Reddick, L.E. & Alto, N.M. Correlative Light and Electron Microscopy (CLEM) as a tool to visualize microinjected molecules and their eukaryotic sub-cellular targets. J. Vis. Exp. (63), e3650 (2012).
34. Thompson, J.R., Ryan, Z.C., Salisbury, J.L. & Kumar, R. The structure of the human centrin 2-xeroderma pigmentosum group C protein complex. J. Biol. Chem. 281, 18746–18752 (2006).
ONLINE METHODS

Research subjects. The consanguineous family and additional families were recruited worldwide on the basis of the presence of at least one individual with an OFD syndrome proven with oral features (lingual hamartoma, cleft/lobulated tongue, gingival frenulae and/or cleft lip/palate), facial dysmorphism and digital anomalies, as well as Meckel syndrome. Whenever possible, individuals underwent a full diagnostic protocol, and a standardized clinical questionnaire was administered to assess the extent of multiorgan involvement. Informed consent was obtained for all participating subjects and their families; the study was approved by the ethics boards of the Necker–Enfants Malades Hospital (Paris) and the Dijon University Hospital. For fetuses, pregnancies were terminated after genetic counseling, in accordance with local legislation. Chromosome analysis and clinicopathological examination were performed for all cases. Genomic DNA was extracted from frozen tissue or amniocyte cultured cells in fetal cases and from peripheral blood samples for patients and their parents using standard procedures.

Genome-wide SNP genotyping and homozygosity mapping. Genome-wide SNP genotyping was performed using the Human Mapping 500K Nsp array (Affymetrix) at the Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC, Illkirch). Sample processing and labeling were performed according to the manufacturer’s instructions (Affymetrix GeneChip Mapping 500K Assay Manual, 701930 Rev.3). Hybridization was performed with a GeneChip hybridization oven 640 at 49 °C and 60 r.p.m. for 16 h, and arrays were washed with a GeneChip Fluidics Station 450 and scanned with a GeneChip hybridization oven 640 at 49 °C and 60 r.p.m. for 16 h. Hybridization was performed for all cases. Genomic DNA was extracted from frozen tissue or amniocyte cultured cells in fetal cases and from peripheral blood samples for patients and their parents using standard procedures.

Exome sequencing. Exome capture was performed at the Genoscope Centre National de Génotypage (CNG; Evry, France) on 8 µg of DNA sample from the affected proband using the SureSelect Human All Exon 50Mb kit (Agilent Technologies) according to standard procedures. The resulting libraries underwent 2 × 100-bp paired-end sequencing on an Illumina HiSeq 2000 in accordance with the manufacturer’s recommendations. Reads were aligned to the human reference genome (GRCh37/hg19) with the Burrows-Wheeler Aligner26 (BWA 0.5.6), and we removed potential duplicate paired-end reads using Picard tools.1.22 (see URLs). The Genome Analysis Toolkit (GTAK) 1.0.57 was used for base quality score recalibration and indel realignment, as well as for single-nucleotide variant and indel discovery and genotyping using standard hard filtering parameters.7,28 Homozygous variants with quality scores of >30, sequencing depth of >4, quality/density ratios of >5.0 and strand bias of <−0.10 were conserved from subsequent analyses. Coverage was assessed with the GTAK Depth of Coverage tool by ignoring reads with mapping quality of <20 and bases with base quality of <30. Candidate events were then inspected using the Integrative Genomics Viewer (IGV). The resulting variants were excluded when the frequency was greater than 1 in 1,000 in the Exome Variant Server, National Heart, Lung, and Blood Institute (NHBLI) (see URLs).

Sanger sequencing validation. Mutation screening of C2CD3 (NM_015531.5) was performed by direct sequencing of the PCR products of the 31 coding exons and the adjacent intronic junctions in 34 individuals with OFD. PCR primers (sequences available upon request) were designed with Primer3 (ref. 39; see URLs) according to reference sequence NM_015531.5. PCR products were purified using the Exo-SAP cleanup kit (USB). Sequencing was performed using the ABI BigDye Terminator Cycle Sequencing kit (v3.1) (Applied Biosystems) according to the manufacturer’s instructions on an ABI 3130 sequencer 7 (Applied Biosystems). Sequence data were analyzed with SeqScape v.2.7 (Applied Biosystems). The impact of missense and splice-site mutations was assessed using PolyPhen-2 (ref. 40; see URLs) and Human Splicing Finder v.2.4.1 online software41 (see URLs), respectively.

cDNA sequencing. To assess the impact of the c.3911–2A>T substitution on splicing, we extracted total RNA from skin biopsies obtained from case 2 and two control individuals using the RNeasy Plus Universal Mini kit (Qiagen) and performed RT–PCR using the Quantitect Reverse Transcription kit (Qiagen) according to the manufacturer’s instructions. Analysis of C2CD3 cDNA was carried out by standard PCR amplification using primers annealing between exons 20 and 23 as well as between exons 21 and 23 and subsequent Sanger sequencing as described above.

Cell culture. IMCD3 cells (American Type Culture Collection) were cultured in DMEM/F12 medium (Life Technologies) containing 10% FBS (HyClone) at 37 °C in 5% CO2. Medium was supplemented with 0.25% Na2CO3 for RPE cells. DMEM with 10% FBS was used to grow HEK293FT (Life Technologies) and SV40-immortalized MEFs derived from C2cd3-mutant mice and mice heterozygous for disruption of C2cd3 (gifts from A. Liu)11. To induce ciliosis, cells were grown in serum starvation medium (0.2% FBS) for 24 h. A stable IMCD3 clonal cell line expressing GFP-fused mouse C2CD3 under the control of the EEF1A1 promoter was generated using the Flp-In system (Invitrogen), as described previously.20 Transfections of RPE, IMCD3 and HEK293FT cells were carried out with X-tremeGENE9 (Roche). U2OS cells (American Type Culture Collection) were transfected with Lipofectamine 2000. To maximize the frequency of cilioe pole elongation observed upon C2CD3 overexpression, 48 h after transfection, U2OS cells were arrested in S phase by the addition of hydroxyurea (8 mM) or aphidicolin (2 µg/ml) for 20 h and then released into G2 phase by changing the medium to roscovitine (RO-3306; 10 µM) for 16 h before fixation. All cell lines used were tested for mycoplasma.

Immunoblotting, immunoprecipitation and immunohistochemistry. All antibodies used in this study are listed in Supplementary Table 2. Immunofluorescence was conducted as described.32 Briefly, cells were fixed for 5 min with 4% paraformaldehyde and immediately plunged into methanol at −20 °C before rehydration in PBS and processing for immunofluorescence (except for CPAP immunofluorescence, for which the paraformaldehyde step was omitted). For studying the localization of C2CD3 within centrosomes (Fig. 2 and Supplementary Fig. 4), cells were treated with 5 µM nocodazole for 1 h before processing for immunofluorescence. All microscopy (except for the measurement of cilioe pole length) was performed with a 63×, 1.4 N.A. PlanApo objective mounted on a Zeiss Axio Imager.M1 microscope connected to a Lambda XL light source (Sutter Instruments), and fluorescence was gated by a Sedat filter set (Semrock). Images were captured with a CoolSNAP HQ2 camera (Photometrics), and the system was controlled by Slidebook 5 (Intelligent Imaging Innovations).

To measure the distance between CP110 and GT335 foci in MEFs, z-stack images of centrosomes were taken on a DeltaVision workstation (GE) equipped with an Olympus PlanApo 60×, 1.42 N.A. oil lens and an eCMOS camera and were deconvolved using softWoRx software. Only those centrosomes with both CP10 and GT335 staining in the same focal plane were selected for distance measurement. The center points (centroids) of GT335 and CP110 staining were mapped using the SpotTracker plugin in ImageJ software, and the distances between these two centroids were measured.

Immunoblotting, cotransfection and coimmunoprecipitation experiments, and GST capture assays were carried out as described.20,27 Tandem affinity purification of the BBsome using the RPE–(LAP-BBS4) stable cell line has been described.19

Immunoprecipitation of endogenous OFD1 was conducted as follows: two 15-cm dishes of RPE cells were lysed in buffer Co-IP 200 (50 mM Tris, pH 7.4, 200 mM NaCl, 1% Triton X-100, 1 mM DTT), lysate was cleared by centrifugation at 16,000g, and 50 µg of antibody to OFD1 or 2 µg of rabbit IgG was added to 5 mg of cell lysate. After a 45-min incubation at 4 °C, antibody-antigen complexes were captured on 10 µl of protein A–Sepharose beads through end-over-end incubation for 1 h. Beads. were washed four times with buffer Co-IP 200, and complexes were eluted in NuPage LDS sample buffer (247 mM Tris base, 2% LDS, 10% glycerol, 0.51 mM EDTA, 0.22 mM Coomassie G250, 0.175 mM phenol red, pH 8.5).

Electron microscopy. Cells were seeded on 8-well Permanox chamber slides (Nalgene Nunc International) and were subsequently fixed in 3.5% glutaraldehyde in 0.1 M phosphate buffer (PB) for 10 min at 37 °C. The buffer was replaced with fresh, prewarmed 3.5% glutaraldehyde, and cells were
incubated for 1 h at 4 °C. Cells were postfixed in 2% OsO$_4$ for 1 h at room temperature and stained in 2% uranyl acetate in the dark for 2 h at 4 °C. Finally, cells were rinsed in distilled water, dehydrated in ethanol and embedded overnight in Durcupan resin (Fluka, Sigma-Aldrich). After polymerization, serial ultra-thin sections (0.06–0.08 μm) were cut with an UC-6 Ultracut (Leica) and stained with lead citrate. Photomicrographs were obtained under an FEI Tecnai G2 Spirit transmission electron microscope (FEI Europe) using a Morada digital camera (Olympus Soft Image Solutions). Quantitative measurements of centriole length were made on longitudinal sections using Fiji software.

**Correlative light electron microscopy.** U2OS cells were seeded onto photo-etched, gridded coverslips (Bellco) and transfected with pEGFP-C2CD3 either alone or in combination with pRFP-PACT. After 48 h, cells were fixed with glutaraldehyde as described above, and regions of interest (ROIs) were identified on an inverted fluorescence microscope (Deltavision, GE Healthcare). The coordinates of each ROI were recorded at low magnification to enable the identification of the ROI after embedding. Processing of samples for electron microscopy was performed as described above, except for the insertion of a block-trimming step as described. Serial sections were aligned using the TrakEM2 plugin from Fiji.

**Human reference sequences.** C2CD3 cDNA sequence (NM_015531.5); C2CD3 protein sequence (NP_056346.3).

35. Seelow, D., Schuelke, M., Hildebrandt, F. & Nurnberg, P. HomozygosityMapper—an interactive approach to homozygosity mapping. *Nucleic Acids Res.* 37, W593–W599 (2009).
36. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25, 1754–1760 (2009).
37. McKenna, A. et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* 20, 1297–1303 (2010).
38. DePristo, M.A. et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat. Genet.* 43, 491–498 (2011).
39. Koressaar, T. & Remm, M. Enhancements and modifications of primer design program Primer3. *Bioinformatics* 23, 1289–1291 (2007).
40. Adzhubei, I.A. et al. A method and server for predicting damaging missense mutations. *Nat. Methods* 7, 248–249 (2010).
41. Desmet, F.-G. et al. Human Splicing Finder: an online bioinformatics tool to predict splicing signals. *Nucleic Acids Res.* 37, e67 (2009).
42. Breslow, D.K., Koslover, E.F., Seydel, F., Spakowitz, A.J. & Nachury, M.V. An in vitro assay for entry into cilia reveals unique properties of the soluble diffusion barrier. *J. Cell Biol.* 203, 129–147 (2013).
43. Cardona, A. et al. TrakEM2 software for neural circuit reconstruction. *PLoS ONE* 7, e38011 (2012).
44. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682 (2012).