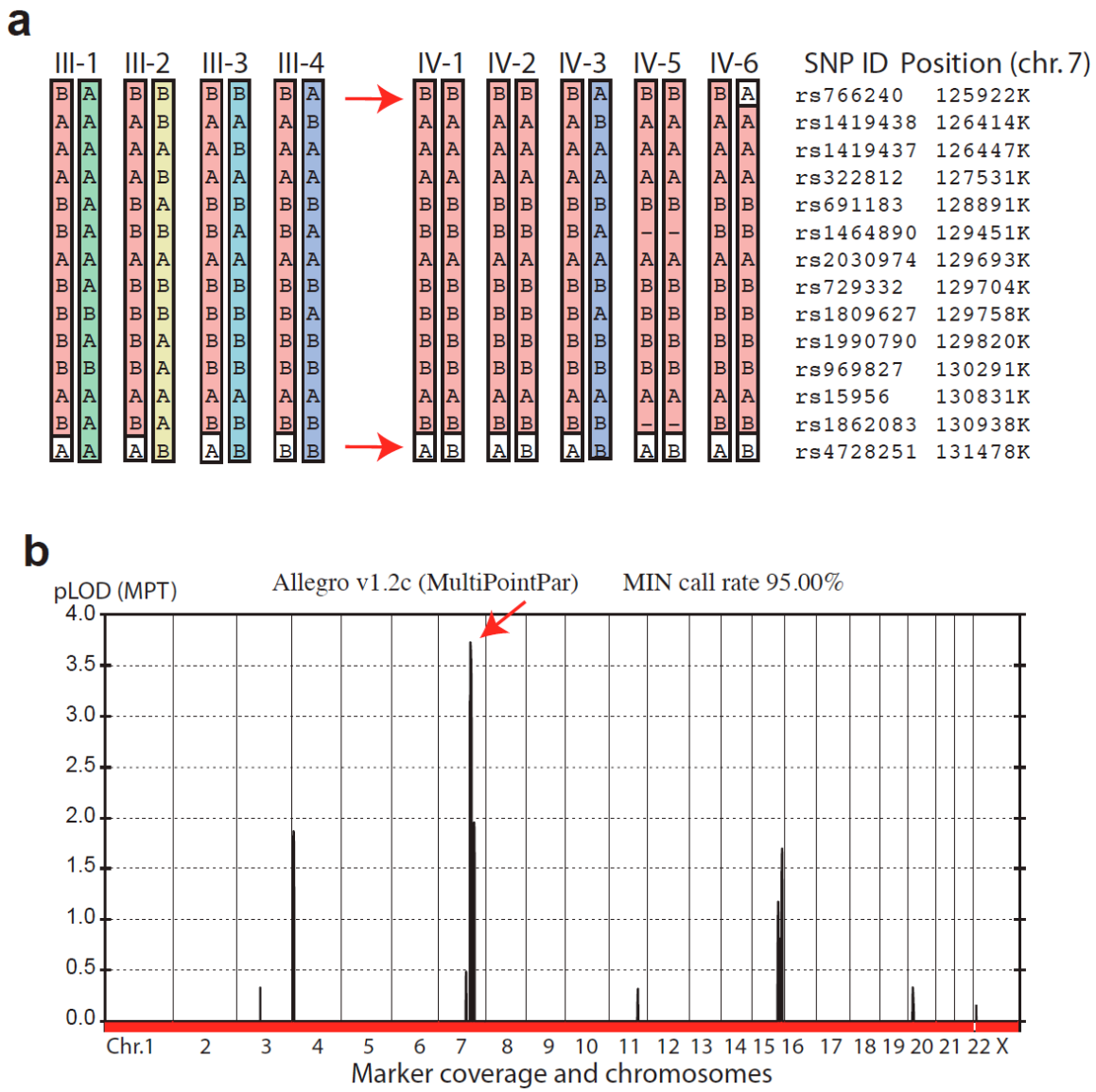
# Supplementary Information

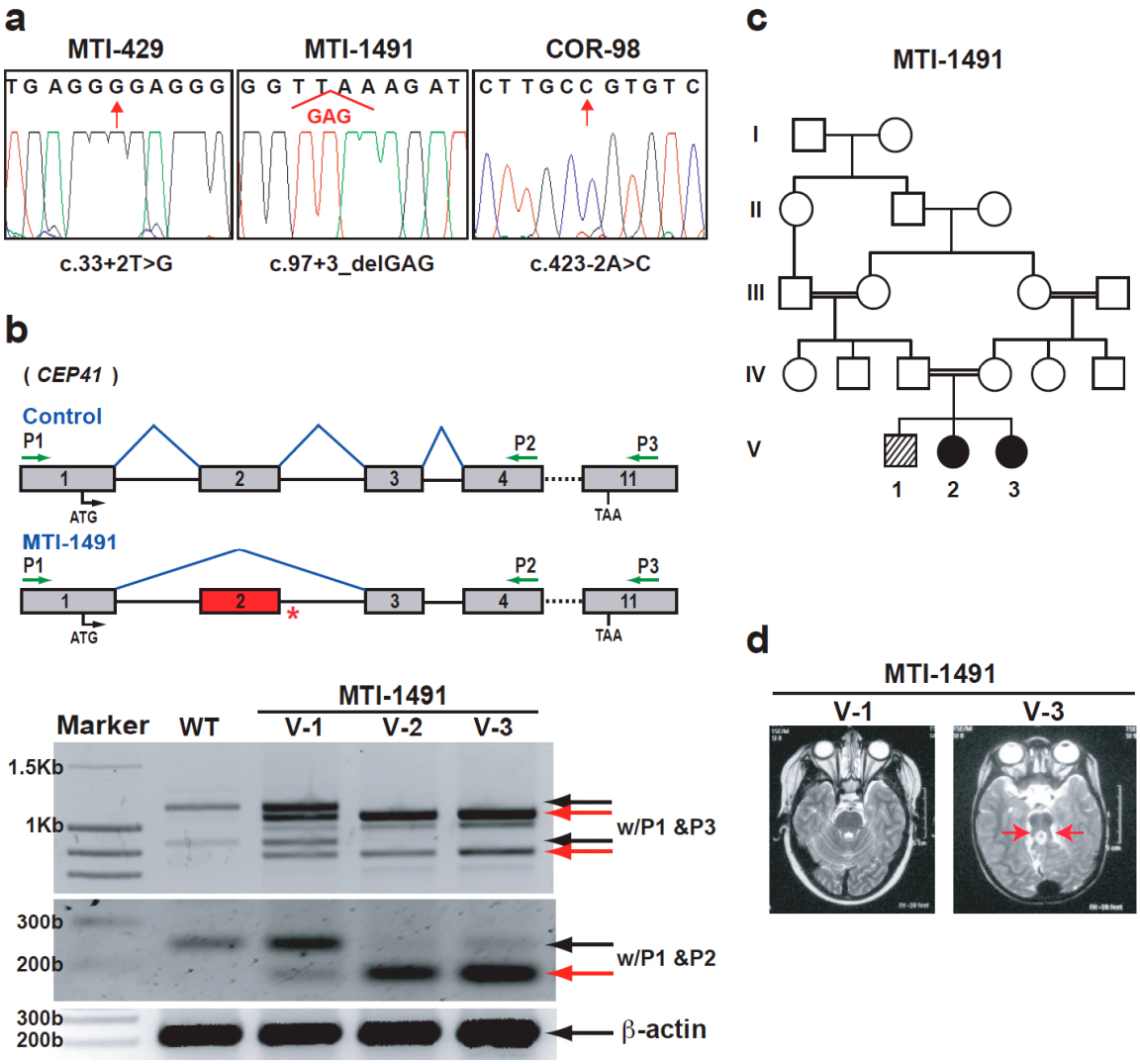
## *CEP41* is mutated in Joubert syndrome and is required for tubulin glutamylation at the cilium

Ji Eun Lee, Jennifer L. Silhavy, Maha S. Zaki, Jana Schroth, Stephanie L. Bielas, Sarah E. Marsh, Jesus Olvera, Francesco Brancati, Miriam Iannicelli, Koji Ikegami, Andrew M. Schlossman, Barry Merriman, Tania Attié-Bitach, Clare V. Logan, Ian A. Glass, Andrew Cluckey, Carrie M. Louie, Jeong Ho Lee, Hilary R. Raynes, Isabella Rapin, Ignacio P. Castroviejo, Mitsutoshi Setou, Clara Barbot, Eugen Boltshauser, Stanley F. Nelson, Friedhelm Hildebrandt, Colin A. Johnson, Daniel A. Doherty, Enza Maria Valente, Joseph G. Gleeson

Correspondence should be addressed to J.G.G. (jogleeson@ucsd.edu)



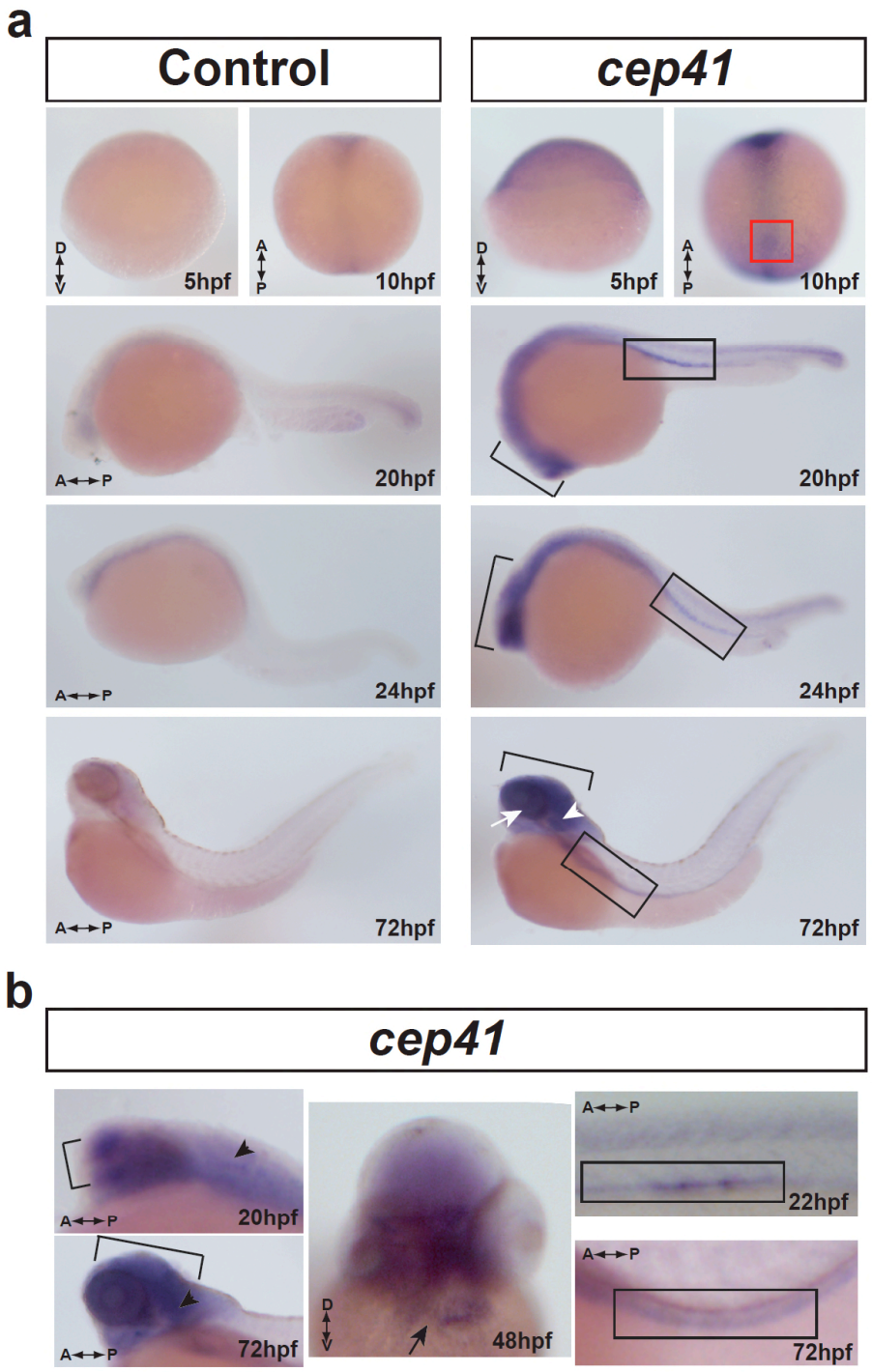

**Supplementary Figure 1.** Genotypes for affected individuals and linkage plots for family MTI-429. **(a)** Affected haplotype (red) with recombinations (red arrows) delineating the candidate interval. Base position of SNP markers according to the Mar. 2006 assembly of the human genome browser. **(b)** Genome-wide LOD score plot, showing linkage to Chr.7q (red arrow) and peak multipoint score of 3.71.



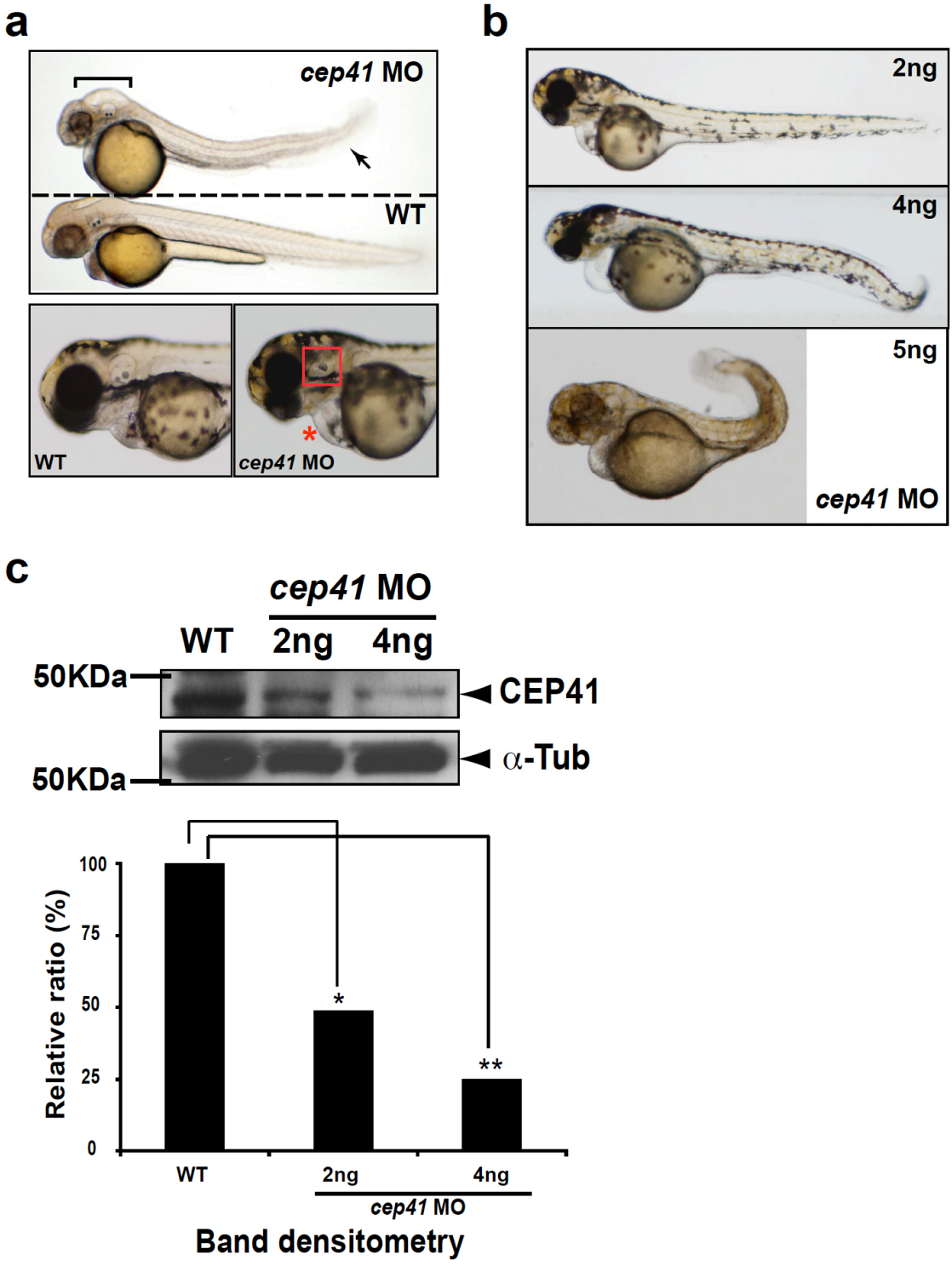

e

| | 35 | 36 | 37 | 88 | 89 | 90 | 178 | 179 | 180 | 359 | 360 | 361 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Homo sapiens | S | M | T | I | Q | R | V | R | D | P | R | S |
| Pan troglodytes | S | M | T | I | Q | R | V | R | D | P | R | S |
| Macaca mulatta | S | M | T | I | Q | R | V | R | D | P | R | S |
| Canis familiaris | S | I | T | I | Q | R | V | R | D | A | R | S |
| Rattus norvegicus | S | M | T | I | Q | R | V | R | D | S | R | T |
| Mus musculus | S | M | T | I | Q | R | V | R | D | P | R | T |
| Gallus gallus | S | L | T | I | H | K | V | R | D | S | R | M |
| Felis catus | S | M | T | I | Q | R | V | R | D | - | - | - |
| Xenopus tropicalis | S | M | T | L | Q | R | V | R | D | S | P | V |

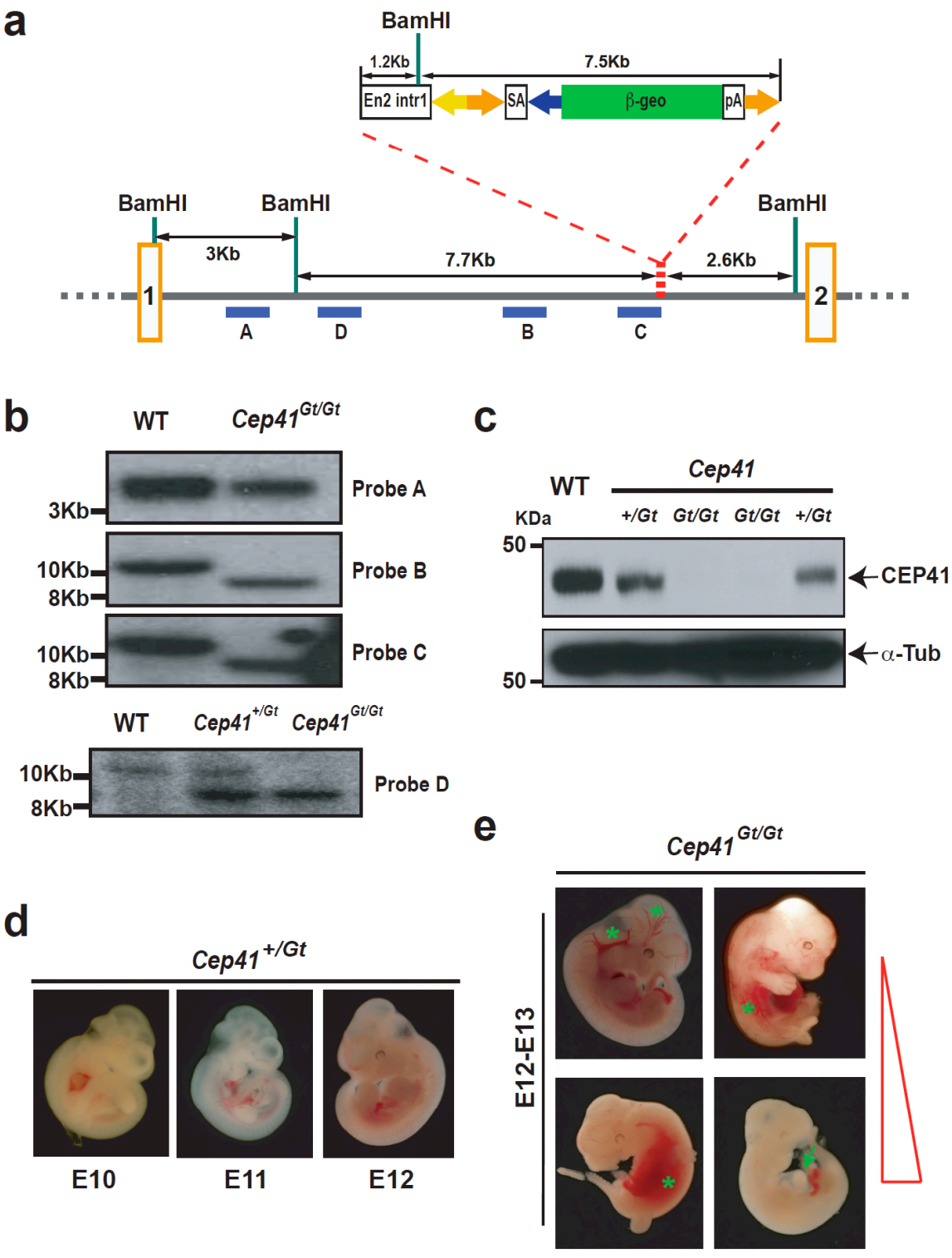
**Supplementary Figure 2.** Additional *CEP41* mutations in individuals with JBTS. (**a**) Sequence chromatograms from one affected member of each family, showing the nucleotide changes (red arrows or bracket) listed below. (**b**) RT-PCR confirmation of the splicing defect of *CEP41* in the MTI-1491. The c.97+3_5delGAG mutation induces the skipping of exon 2 (red rectangle), thereby creating a premature stop codon in exon 3. Both affected individuals (V-2 and V-3) of the family generated truncated *CEP41* mRNA products (exon 2= 64 bp, red arrows), while the control generated predicted length mRNAs (include one shorter isoform, black arrows) in two different RT-PCR analyses. The BBS patient (V-1) in family MTI-1491 carried a heterozygous mutation in *CEP41*, thus showed both normal product on one allele and truncated product on the other allele in the RT-PCR results. *β-actin* was amplified as an internal control of this PCR. In the diagram, green arrows indicate the positions of primers and an asterisk marks the region of splice mutation out in the MTI-1491. (**c**) The pedigree structure of MTI-1491 having one BBS male patient (V-1, hatched square) and two JBTS female patients (V-2 and V-3, black circles) in generation V. (**d**) Axial brain MRIs showing molar tooth sign (red arrows) in the JBTS patient, but not in the BBS patient in MTI-1491. (**e**) Protein sequence alignment showing evolutionary conservation of altered amino acids found in the *CEP41* heterozygous mutations (MTI-109 family: p.36M>T, MKS-1012 family: p.89Q>E, COR-130 family: p.179R>H, and COR-210: p.360R>C).



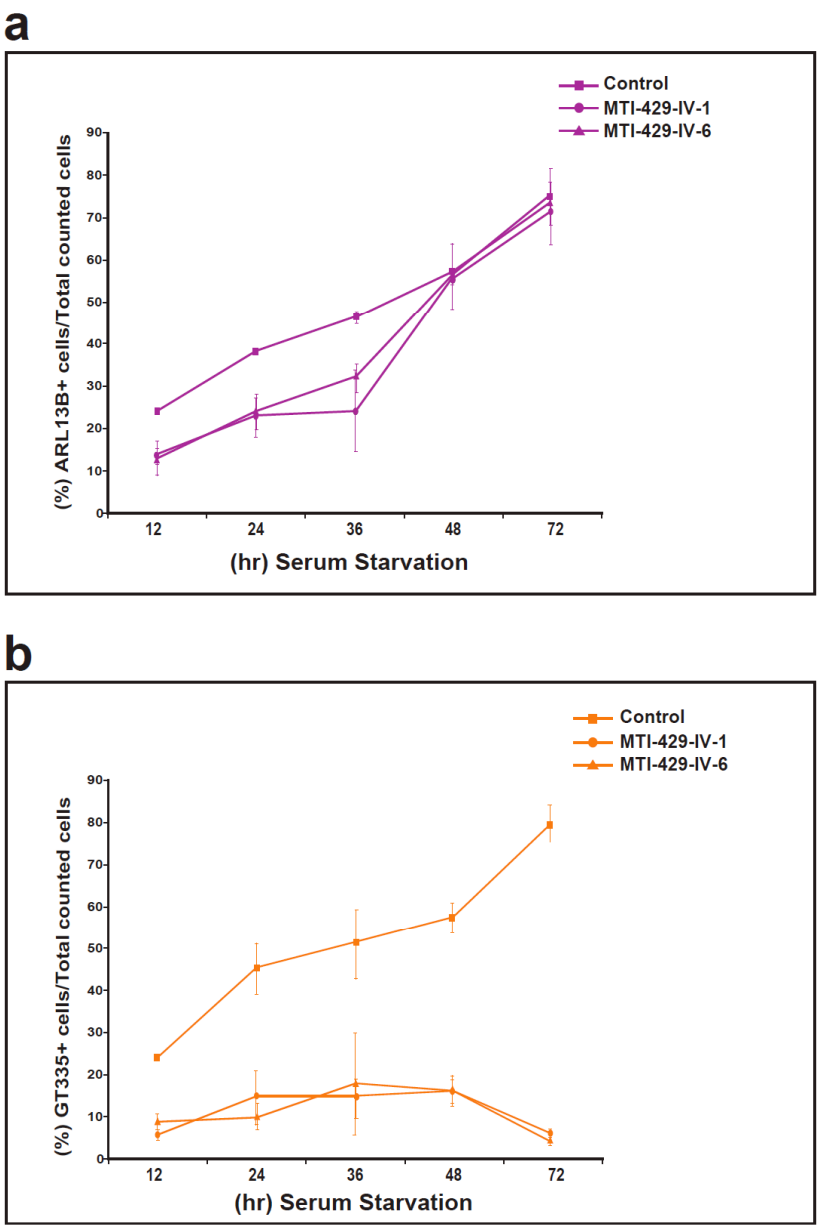

**Supplementary Figure 3.** Expression of *cep41* in zebrafish. (**a**) Zebrafish *cep41* mRNA is expressed ubiquitously at early embryonic stages (5 hpf), but is strongly expressed in specific organs such as Kupffer's vesicle (red box), ears (arrowhead), brain (brackets), eyes (arrow), pronephric duct (black boxes) at later stages. (**b**) Magnified images of *cep41*-expressed zebrafish organs. Brackets mark brain area, arrowheads indicate ears, arrow marks heart, black boxes mark pronephric ducts. A, anterior; D, dorsal; P, posterior; V, ventral.


a
cep41 MO
WT
WT
cep41 MO
b
2ng
4ng
5ng
cep41 MO
c
cep41 MO
WT
2ng
4ng
50KDa
CEP41
50KDa
α-Tub
Relative ratio (%)
100
75
50
25
0
*
**
WT
2ng
4ng
cep41 MO
Band densitometry

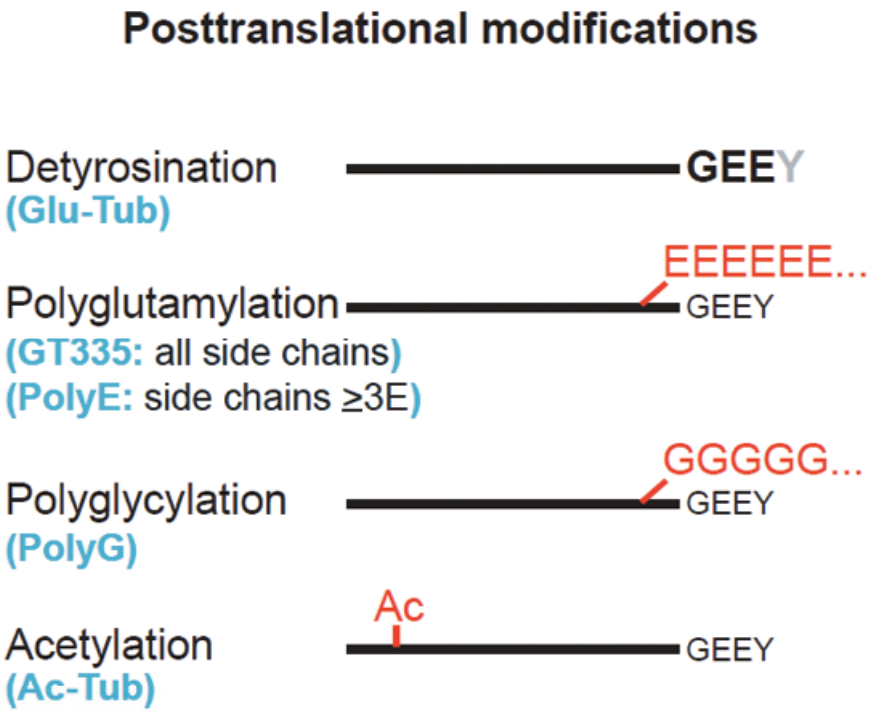
**Supplementary Figure 4.** Efficiency of *cep41* knockdown in zebrafish embryos. (**a**) Injection of translation-blocking morpholino antisense oligonucleotide (MO) targeting *cep41* into zebrafish embryos causes ciliary phenotypes such as hydrocephalus (bracket), abnormal number/orientation of ear otolith (red box), along with defects of curved tail (arrow) and peripheral cardiac edema (asterisk). The embryos were raised in either 1-phenyl 2-thiourea (PTU) media to suppress pigmentation (top panel) or regular embryo media (bottom panel) until 72 hpf and observed for morphology analysis. (**b**) Dose-dependent phenotypic severity of *cep41* morphants. Embryos were observed at 72 hpf without suppressing pigmentation. **(c)** Dosage-dependent reduction of Cep41 in *cep41* morphants was confirmed by western blot using the anti-CEP41 antibody. Graph shows normalized band densitometry to compare Cep41 products between wild type control embryos and two different dosages (2 ng: 52 % reduction, 4 ng: 75 % reduction). $*P < 0.01$, $**P < 0.001$.


a
BamHI
1.2Kb
7.5Kb
En2 intr1
SA
β-geo
pA
BamHI
BamHI
BamHI
3Kb
7.7Kb
2.6Kb
1
2
A
D
B
C
b
WT
Cep41Gt/Gt
3Kb
Probe A
10Kb
8Kb
Probe B
10Kb
8Kb
Probe C
WT
Cep41+/Gt
Cep41Gt/Gt
10Kb
8Kb
Probe D
c
Cep41
WT
KDa
+/Gt
Gt/Gt
Gt/Gt
+/Gt
50
CEP41
50
α-Tub
d
Cep41+/Gt
E10
E11
E12
e
Cep41Gt/Gt
E12-E13

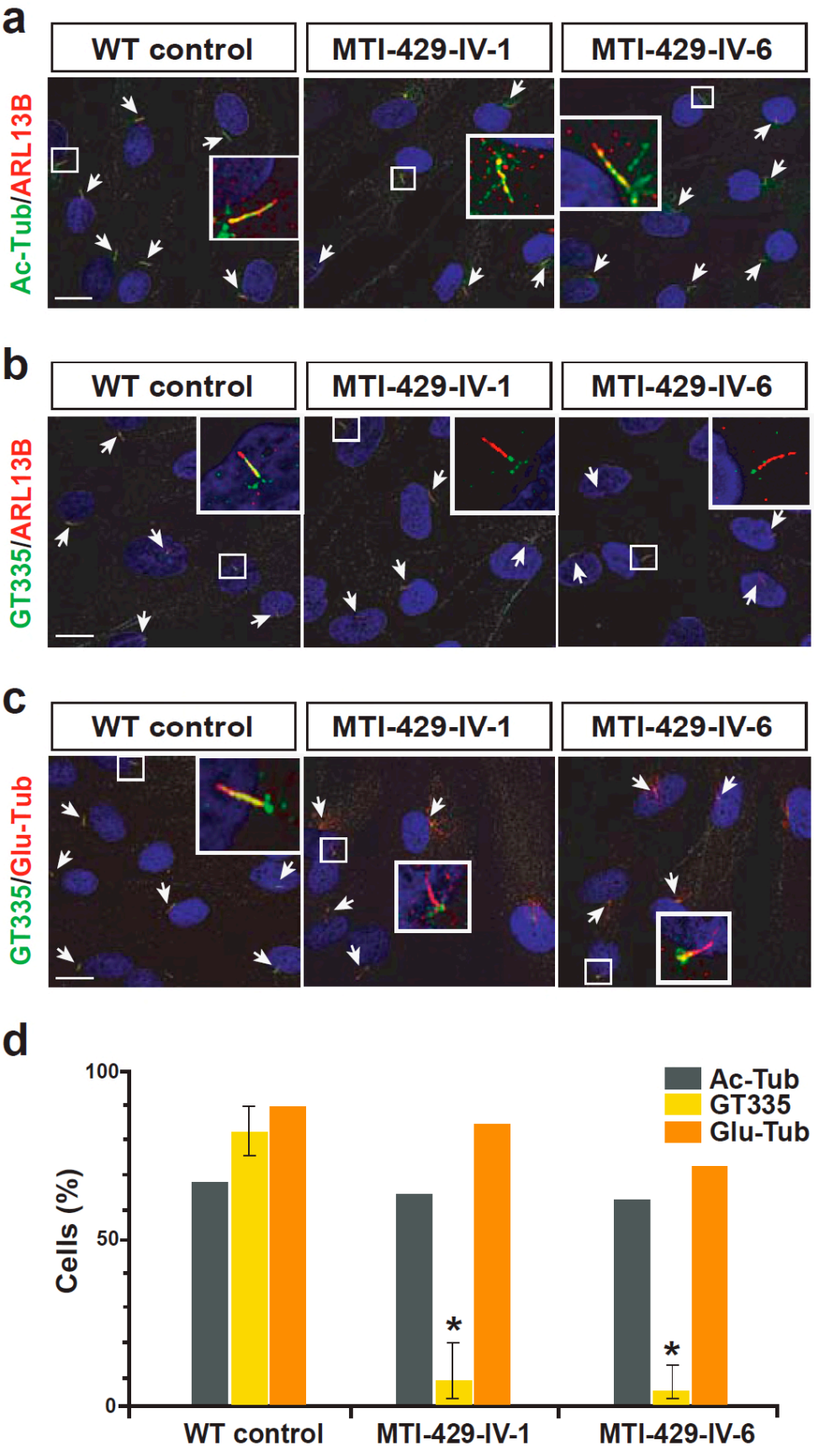
**Supplementary Figure 5.** Characterization of *Cep41* knockout mouse embryos. (**a**) Schematic of gene-trap strategy. Mutant mice were generated from gene-trap lines carrying $\beta$-*Geo* insertion within intron 1. (**b**) Southern blot confirmation of the genotypes of mouse embryos. Positions of probes used in the Southern blot are shown in the diagram (blue rectangles). (**c**) Western blot confirmation of the production of Cep41 protein from four different adult gene-trap mice. (**d**) Phenotypes of $Cep41^{+/Gt}$ embryos at E10-12. Most *Cep41* heterozygous mice embryos developed normally. (**e**) Phenotypes of $Cep41^{Gt/Gt}$ embryos at E12-13. Various phenotypes of *Cep41* homozygous mice embryos included hemorrhages in the brain, neck and body, and embryonic lethality with visible phenotypes such as hemorrhage and abnormal heart. Surviving embryos: top panel, non-surviving embryos: bottom panel. Hemorrhages: asterisks, dilated pericardial sac: arrow.



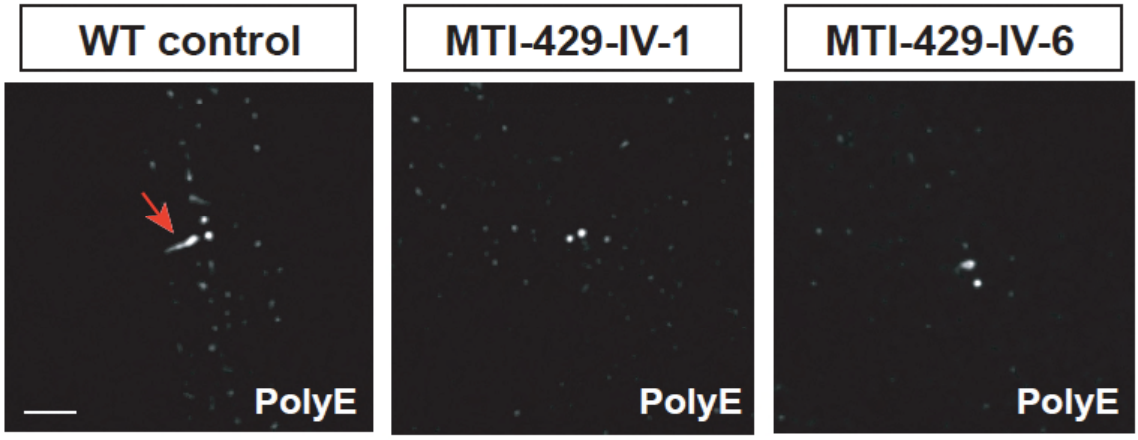

**Supplementary Figure 6.** Microtubule glutamylation of the cilium and ciliogenesis are not correlated. **(a)** Primary cultured fibroblast cells were stained with anti-ARL13B antibody after serum starvation at different time points and ARL13B-positive cells were counted as cells generating cilia. Two different *CEP41* mutant fibroblast cells (MTI-429-IV-1 and -IV-6) showed less cilia assembly than control cells for a short time after serum starvation, but 32 - 48 hr after serum starvation, the numbers of ciliated cells recovered (~70 %) to those of control cells at 72 hr serum starvation. **(b)** *CEP41* mutant fibroblast cells were stained with GT335 antibody after serum starvation at different time points and cells with GT335-positive cilia were counted. Both mutant fibroblasts had less glutamylated ciliated cells compared with those of control fibroblast at all post-serum starvation time points tested. Error bars in the graphs show s.e.m.



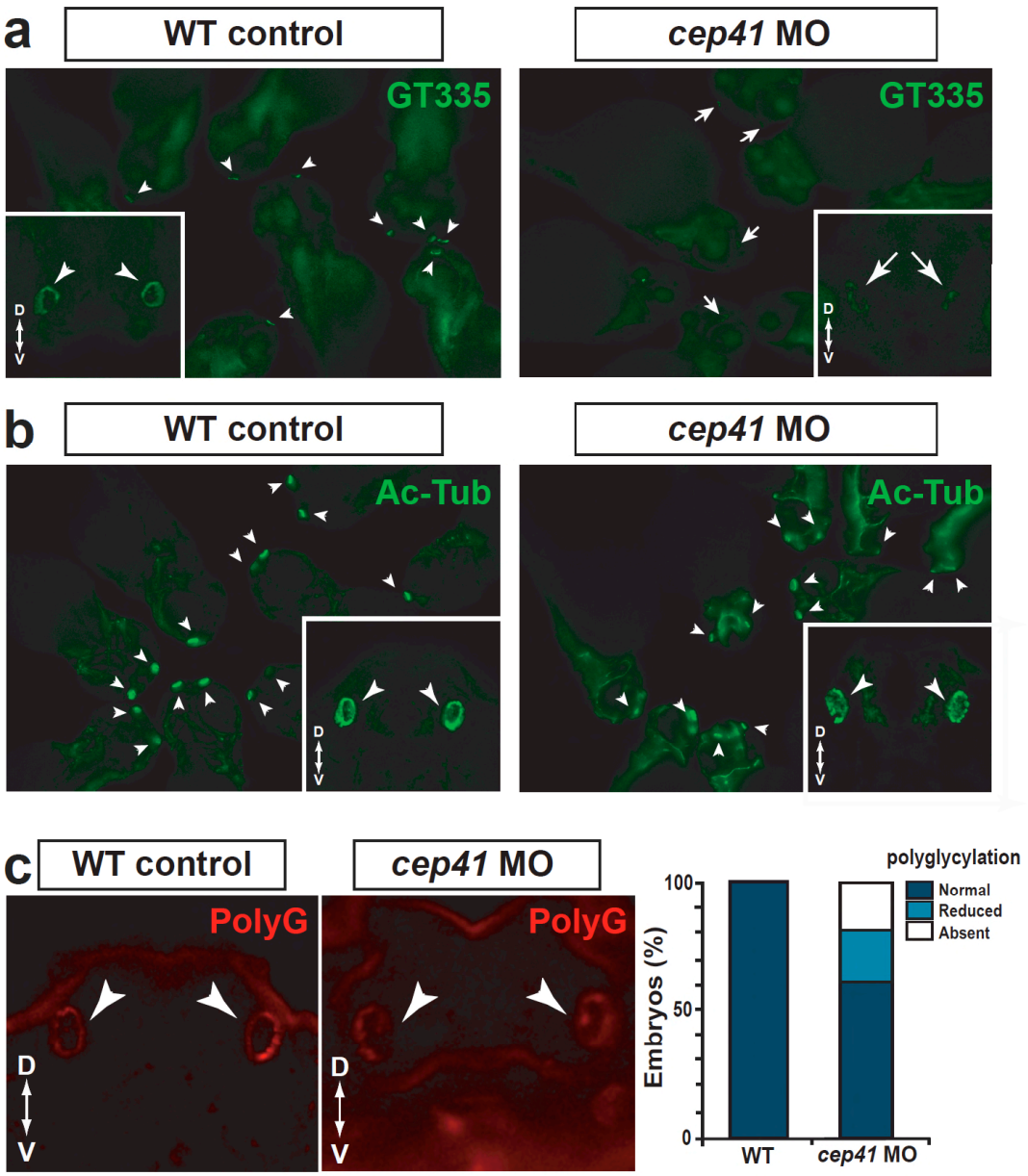

**Supplementary Figure 7.** Summary of tubulin posttranslational modifications (PTMs), which occur in ciliary axoneme. Several PTMs, known to be involved in ciliogenesis/ciliary function are listed and the PTM-specific antibodies used in this study are presented in the brackets.


a
WT control
MTI-429-IV-1
MTI-429-IV-6
Ac-Tub/ARL13B
b
WT control
MTI-429-IV-1
MTI-429-IV-6
GT335/ARL13B
c
WT control
MTI-429-IV-1
MTI-429-IV-6
GT335/Glu-Tub
d
Cells (%)
100
50
0
Ac-Tub
GT335
Glu-Tub
*
*
WT control
MTI-429-IV-1
MTI-429-IV-6

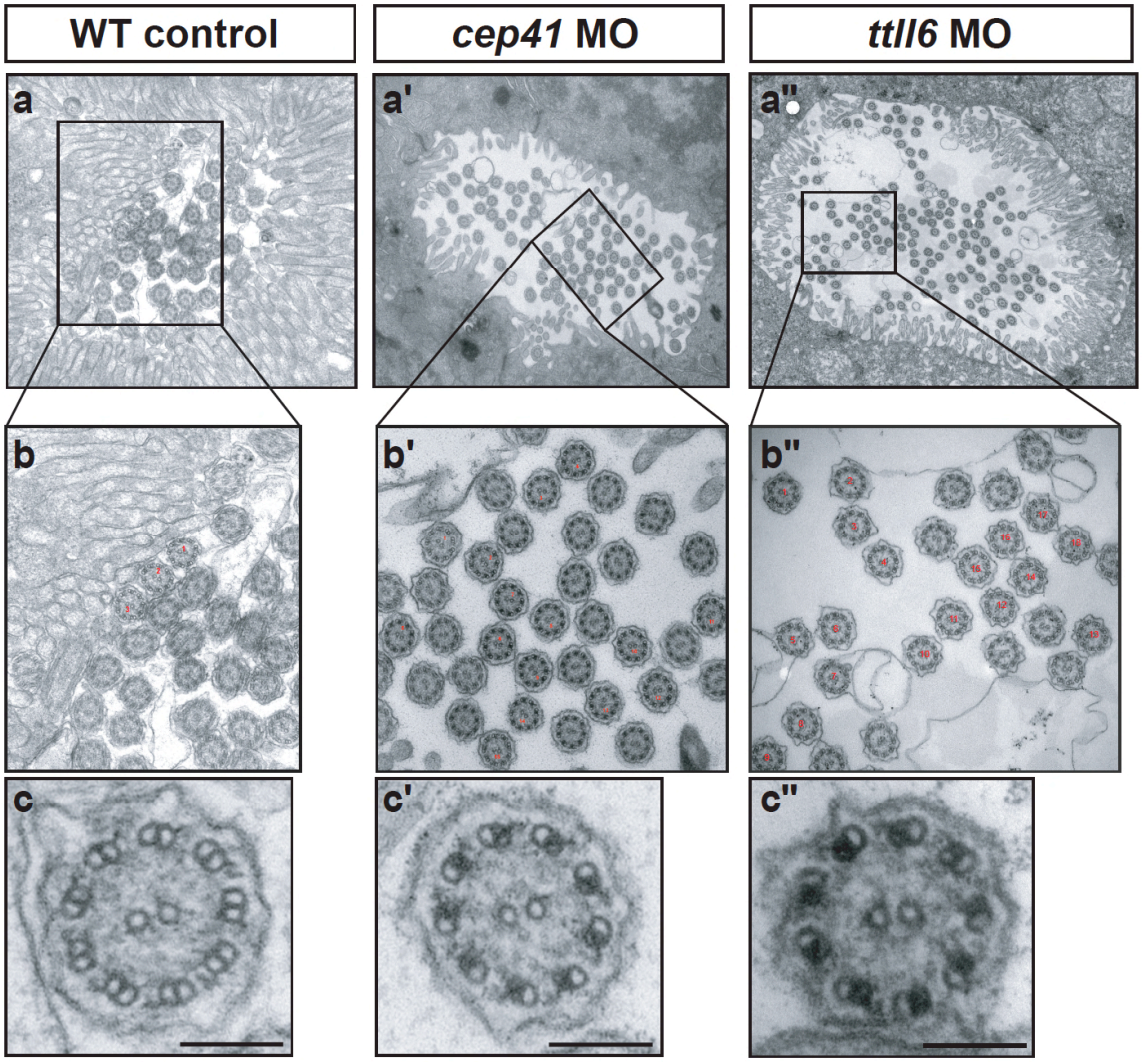
**Supplementary Figure 8.** Effects of *CEP41* loss on PTMs at the ciliary axoneme. (**a**) Both control and *CEP41* mutant fibroblasts co-stained with Ac-Tub (green), a marker for acetylated tubulin, and ARL13B (red), a marker for cilia. (**b**) Tubulin polyglutamylation is affected in *CEP41* mutant ciliary axonemes. Control cells have cilia marked by ARL13B (red), co-stained with GT335 (green), a marker for glutamylated tubulin. The majority of mutant cells display no glutamylated tubulin in the cilium. (**c**) Defects of tubulin detyrosination were not apparent in *CEP41* mutant cilia. Mutant cells showed only intact Glu-tubulin (red, marker for detyrosinated tubulin), whereas control cells labeled with both markers. Scale bar= 5 μm. (**d**) Quantification of ciliary axonemal PTM, Ac-Tub-, GT335-, and Glu-Tub- positive cilia. *$P$ < 0.001 and error bars: s.e.m.



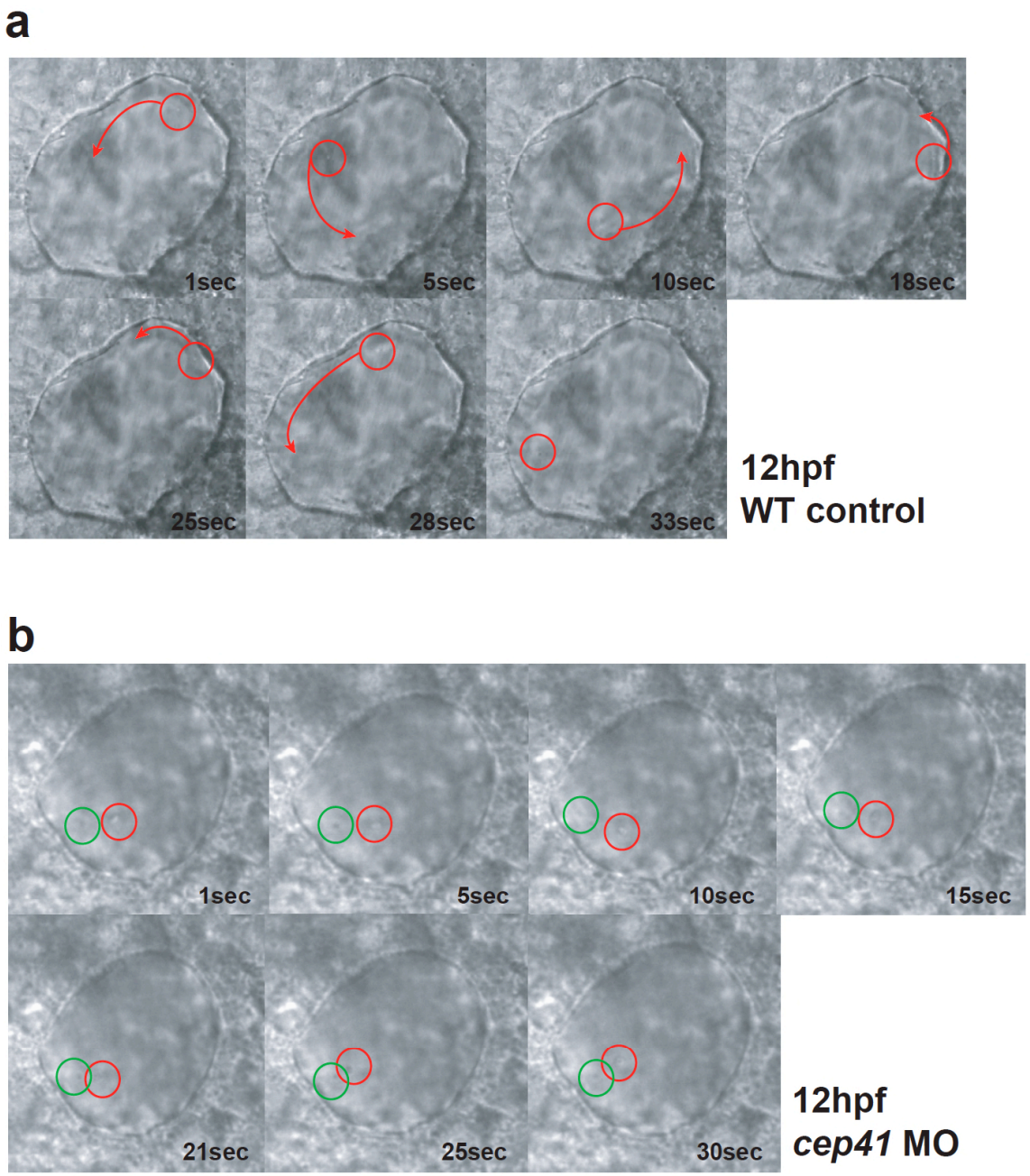

**Supplementary Figure 9.** Effect of *CEP41* loss on tubulin polyglutamylation in human fibroblasts. WT control and two individual *CEP41* mutant fibroblasts (MTI-429-IV-1 and -IV-6) were stained with PolyE antibody after three days of serum starvation. Tubulin in the ciliary axoneme and centrosome were normally polyglutamylated in WT cells, while *CEP41* mutant cells had defective polyglutamylation in the cilium, but normal modified centrosome. An arrow indicates primary cilium stained with PolyE antibody. Scale bar is 5 μm.



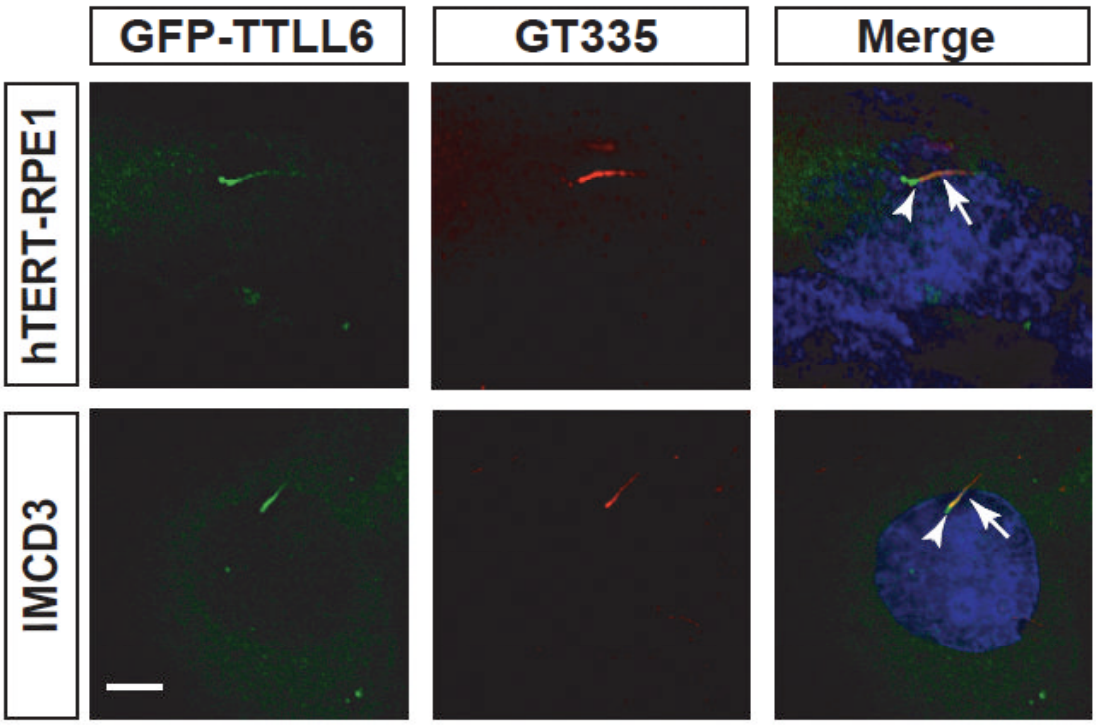

**Supplementary Figure 10.** Effect of *cep41* loss on PTM in the zebrafish olfactory placode cilia. (**a**) *cep41* MO-injected zebrafish embryos show reduced or absent glutamylation in the olfactory placode cilia (arrowheads-normal and arrows-reduced). (**b**) Acetylation occurs normally in the olfactory placode of *cep41* MO-injected zebrafish embryos (94 % in total 95 embryos). Insets magnify the stained cilia in a single embryo (**a-b**). (**c**) Majority of *cep41* morphants have normally polyglycylated cilia in the olfactory placode, but some show reduced or absent polyglycylation in the cilia (reduction: 20 %, absence: 18 % in total 49 embryos). Arrowheads mark normally modified (including glutamylation, acetylation and glycylation) cilia (**a-c**) and arrows indicate reduced glutamylated cilia (**a**). D, dorsal; V, ventral directions. Embryos were mounted in anterior view (head is up and tail is down) for imaging (**a-c**).



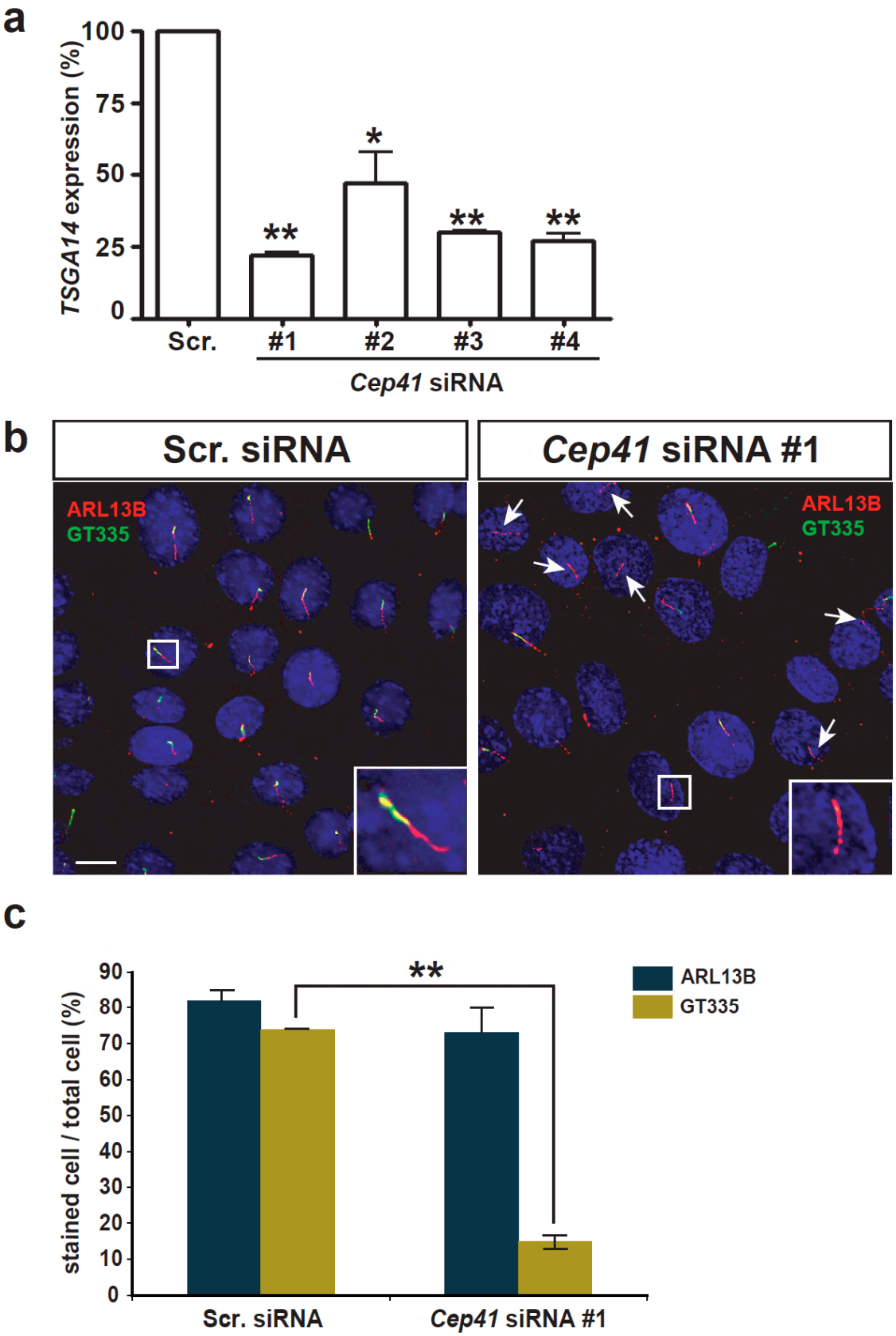

**Supplementary Figure 11.** TEM ultrastructure analysis of zebrafish pronephric cilia at 72 hpf. (**a-a"**) TEM images of overall zebrafish renal cilia. TEM was performed after cross-section of kidneys in wild type and *cep41*- or *ttll6*-MOs injected embryos at 72 hpf (n ≥3 in each genotype). Orientation of cilia was variable, thus some of cilia were not sectioned 100% horizontally, resulting in blurry or smashed surface of sectioned cilia. (**b-b"**) Higher-power images of insets in (**a-a"**). To observe the ultrastructure of renal cilia in detail, clean images selected and quantified in higher magnification. Red numbers indicate selected cilia for counting. (**c-c"**) TEM images of the single ciliary ultrastructure. Scale bars= 50 nm.




**Supplementary Figure 12.** Effects of *cep41* knockdown on KV cilia-driven fluid flow in zebrafish. Images were captured every ~5 sec. from Supplementary Movie 1 and 2. Movement of a small piece of debris in a 6 somite KV between wild type control embryo (**a**) and *cep41* morphant (**b**). (**a**) WT embryo shows debris following a circular path. (**b**) The *cep41* morphant shows the debris stalled throughout the acquisition.




**Supplementary Figure 13.** Localization of TTLL6 to the basal body and cilium in ciliated cells. Mouse IMCD3 and human hTERT-RPE1 ciliated cells, transfected with GFP-TTLL6 plasmid show predominant localization of TTLL6 at the basal body/cilium. Arrowheads mark basal body and arrows indicate cilium. GT335 antibody was used for marking basal body/cilium. Scale bar= 5 μm.


a
TSGA14 expression (%)
100
75
50
25
0
**
*
**
**
Scr.
#1
#2
#3
#4
Cep41 siRNA
b
Scr. siRNA
Cep41 siRNA #1
ARL13B
GT335
ARL13B
GT335
c
stained cell / total cell (%)
90
80
70
60
50
40
30
20
10
0
**
ARL13B
GT335
Scr. siRNA
Cep41 siRNA #1

**Supplementary Figure 14.** Efficiency of *Cep41* knockdown in mouse inner medullary collecting duct cells (IMCD3). (**a**) qPCR confirmation of knockdown efficiencies of four different *Cep41* siRNAs. *Cep41* mRNA expression was measured 48 hr after each siRNA transfection at 75 nM. *β-actin* expression was used to normalize qPCR data. *$P < 0.01$, **$P < 0.001$ and error bars show s.e.m. (**b**) Polyglutamylation defects at the ciliary axoneme by knockdown with *Cep41* siRNA. IMCD3 cells transfected with #1 siRNA recapitulated the phenotype of human *CEP41* mutant cells: proper generation of cilia (stained with ARL13B), but defective ciliary axonemal glutamylation (stained with GT335). Arrows mark the cilium with glutamylation defects and insets magnify the stained cilia. Scale bars= 5 μm. (**c**) Graph shows quantification after comparisons of staining with either ARL13B or GT335 between the control scrambled (Scr.) siRNA- and *Cep41* siRNA #1- transfected cells. **$P < 0.001$ and error bars show s.e.m.

**Supplementary Table 1. Clinical and molecular data from families with heterozygous mutations in *CEP41***

| **Demographic Information** | | | | | |
|---|---|---|---|---|---|
| **Family ID** | **COR-130** | **MTI-109** | **COR-157** | **MKS-1012** | **COR-210** |
| Country of orgin | Germany | Spain | Italy | France | Switzerland |
| Patient (sex) | M | F | M | N/A | F |
| Death | N | N | N | Y, ToP | N |
| Documented Consanguinity | N | N | N | N | N |
| **Neurological signs** | | | | | |
| Hypotonia/Ataxia | Y | Y | Y | N/A | Y |
| Psychomotor Delay | Y | Y | Y | N/A | Y |
| Mental Retardation | Y | Y | Y | N/A | Y |
| OMA | Y | N | Y | N/A | Y |
| Breathing Abnormalities | Y | N/A | Y | N/A | N |
| Head Circumference | N/A | N/A | 25%ile | N/A | N/A |
| **Ocular Signs** | | | | | |
| Retinopathy | N | N | N | N/A | N |
| Other abnormalities | N | N | N | N | N |
| Coloboma | N | N | N | N | N |
| **Renal signs** | | | | | |
| NPHP/UCD | N | N | N | N/A | N |
| Kidney ultrasound | N | N | N | N/A | N |
| **Other organs** | | | | | |
| Liver abnormalities | N | N | N | N | N |
| Polydactyly | N | N | N | N | B preaxial |
| Other abnormalities | N | N | N | Exencephaly | N |
| **MRI reading** | | | | | |
| MTI | Y | Y | Y | N/A | Y |
| Other abnormalities | N | Abnormal clefting of cortex | N | Cleft palate | |
| ***CEP41* mutation analysis** | | | | | |
| exon(s) | 7 | 3 | 2 | 5 | 11 |
| Hetero/Homozygous | Heterozygous | Heterozygous | Heterozygous | Heterozygous | Heterozygous |
| Nucleotide change | c.536A>G | c.107T>C | c.83C>A | c.265C>G | c.1078C>T |
| Amino acid change | p.179R>H | p.36M>T | p.28S>Stop | p.89Q>E | p.360R>C |
| Amino acid consequence | Basic to basic | Neutral to neutral | Nonsense | Neutral to acidic | Basic to neutral |
| **Comprehensive JSRD Genetic Screening** | | | | | |
| Linkage | N/A | N/A | N/A | N/A | N/A |
| Additional gene with PDSV | *KIF7* | *CC2D2A* | None | None | *CC2D2A* |
| exon | 3 | 25 | | | 35 |
| Hetero/Homozygous | Heterozygous | Heterozygous | | | Heterozygous |
| Nucleotide change | c.811delG | c.3217C>T | | | c.4340A>C |
| Amino acid change | p.E271RfsX51 | p.1049R>Stop | | | p.1447E>A |
| Amino acid consequence | Truncating | Nonsense | | | Acidic to neutral |
| In NCBI SNP Database | No | No | | | No |

Supplemental Table 1. Abbreviations. AG: Ambiguous genitalia, B: Bilateral, F: Female, GHD: Growth hormone deficiency, M: Male, MP: Micropenis, N: No, N/A: Not available/Not applicable, NPHP: Nephronophthisis, OMA: oculomotor arpaxia, PDSV: potentially deleterious sequence variant, UCD: Urinary concentration defect, U: Unilateral, Y: Yes

**Supplementary Table 2. Summary of the *Cep41*$^{GT/GT}$ mice embryos at E10-E13**

| | WT | *Cep41*$^{+/Gt}$ (n=47) | *Cep41*$^{Gt/Gt}$ (n=60) |
|---|---|---|---|
| **Normal development** | 16 | 44 | 45 |
| **Abnormal development (Mortality)** | 0 | 1 (2) | 11(4) * |

**Abnormal development includes the phenotype of exencephlay, hemorrhage, or dilated pericardium.**
***P* < 0.05 in comparision between abnormal heterozygous and homozygous *Cep41* mice emrbyos.**

**Supplementary Table 3. Primers for *CEP41* mutation screening**

| Primer name | Sequences | Anneal Temp |
|---|---|---|
| Human *CEP41*-1F | 5'-CTAGGGTTAGAGGCAACCCG-3' | 60℃ |
| Human *CEP41*-1R | 5'-TGGCTGCTCTTCCCTGC-3' | |
| Human *CEP41*-2F | 5'-TCCTGACTTTACCTTATCGTTATCC-3' | 60℃ |
| Human *CEP41*-2R | 5'-ATGTGGGGTTGCTTTCTGTC-3' | |
| Human *CEP41*-3F | 5'-CAGGATAGGAAAGCTTAGGGG-3' | 60℃ |
| Human *CEP41*-3R | 5'-CACCTGTGGAACACAATTGTTAG-3' | |
| Human *CEP41*-4F | 5'-AAAGACCACGTCACTTGACAAC-3' | 60℃ |
| Human *CEP41*-4R | 5'-AACTCCTCTGCAAACTTTCCC-3' | |
| Human *CEP41*-5F | 5'-TGATGTTTGTTGTTCCCTCG-3' | 60℃ |
| Human *CEP41*-5R | 5'-CATCAAAGTCCCAGTCCCTG-3' | |
| Human *CEP41*-6F | 5'-TTCTTGACTTAATGTCAGTGGAAC-3' | 60℃ |
| Human *CEP41*-6R | 5'-CCAGTTCTGTTTCTAAGGCAAG-3' | |
| Human *CEP41*-7F | 5'-TCGACCTTTAAGCCACCAAC-3' | 60℃ |
| Human *CEP41*-7R | 5'-GTTCCAGCCTGCCTAGACTC-3' | |
| Human *CEP41*-8F | 5'-TTAAACCAGCTGGGAATTGAG-3' | 60℃ |
| Human *CEP41*-8R | 5'-TGATAACTTTGATTCTTACAATCCTC-3' | |
| Human *CEP41*-9F | 5'-TCGTAGTCTTGGGACATCAGC-3' | 60℃ |
| Human *CEP41*-9R | 5'-AGAAAAGGGGATGCAGGTG-3' | |
| Human *CEP41*-10F | 5'-AGACATGCAGGCTTCTTTCC-3' | 60℃ |
| Human *CEP41*-10R | 5'-TGAGGTGATATTCAAAGCTGC-3' | |
| Human *CEP41*-11F | 5'-CTGTACTTGGCAGATTGTCCC-3' | 60℃ |
| Human *CEP41*-11R | 5'-ACTTGGGAAATGCTCAGGG-3' | |

**Supplementary Table 4. siRNAs and primers for q-RT-PCR used in this study**

**siRNAs target sequences**

| siRNA | Sequences | Target |
|---|---|---|
| Mouse *Cep41* #1 | 5'-UAGACAAAGGGCUCGUAAA-3' | 452-460 of cds |
| Mouse *Cep41* #2 | 5'-GUUGAAGAUUAAAGAACGU-3' | 3' UTR |
| Mouse *Cep41* #3 | 5'-ACAAGAACGCCCACGGCAA-3' | 641-659 of cds |
| Mouse *Cep41* #4 | 5'-CAGAGACUGUUUCGGCAAA-3' | 3' UTR |

**Primers for quantitative real-time PCR**

| Primer name | Sequences |
|---|---|
| Mouse *Cep41* F | 5'-GACCAGCAGACAACCCTAGC-3' |
| Mouse *Cep41* R | 5'-AGCTGGTGGGCAGGTTCT-3' |
| Mouse β-*actin* F | 5'-GACCCAGATCATGTTTGAGACC-3' |
| Mouse β-*actin* R | 5'-GGCCATCTCTTGCTCGAAGTC-3' |