Zeta-Tubulin Is a Member of a Conserved Tubulin Module and Is a Component of the Centriolar Basal Foot in Multiciliated Cells

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Supplemental Figures:

Figure S1. Zeta-tubulin is expressed and can be specifically detected via immunoblot and immunofluorescence. Related to Figure 1.

A) Zeta-tubulin is expressed in adult tissues of *X. tropicalis*, with highest expression in the testis. The number of transcripts of zeta-tubulin per million...
reads for two biological replicates are shown for each tissue. B) Protein samples were probed for zeta-tubulin, with gamma-tubulin as a loading control. Zeta-tubulin antibody recognizes a single band in both A6 cell lysate and egg extract that is of equal size to in vitro translated zeta-tubulin. Molecular weights in kilodaltons are indicated on the left. Dividing line indicates joint between lanes on the same membrane. C) Identically loaded protein samples were probed with zeta-tubulin antibody incubated with the peptide used for antibody production (+ peptide) or without (- peptide). All bands recognized are specific to the antigen. Sample entitled “peak fraction” represents the most concentrated fraction of zeta-tubulin from a 10-40% sucrose gradient (as in Figure 1D). Molecular weights in kilodaltons are indicated on the left. D) U2OS cells were transfected with GFP-zeta-tubulin and stained for GFP (green), zeta-tubulin (red), and DAPI (blue). Prior to staining, zeta-tubulin antibody was incubated with the peptide used for antibody production (+ peptide) or without (- peptide). Zeta-tubulin antibody not incubated with peptide recognizes GFP-zeta-tubulin, whereas peptide-blocked zeta-tubulin antibody no longer recognizes GFP-zeta-tubulin. Scale bar, 5 µm.
Figure S2. Endogenous zeta-tubulin is a cytoplasmic protein in A6 cells and localizes to basal feet in multiciliated cells. Related to Figure 2.

A) A6 cells were fractionated into cytoplasm (1) and nucleus/centrosome (2) fractions. Fractions were probed for markers of cytoplasm (alpha-tubulin), nucleus (histone H3), and centrosome (gamma-tubulin). Zeta-tubulin is present only in the cytoplasmic fraction. Molecular weights in kilodaltons are indicated on the left. B) A6 cells were fixed in formaldehyde, quenched with borohydride, and stained for zeta-tubulin (green), centrin (red), and DAPI (blue). Zeta-tubulin does not localize to centrioles. Scale bar, 5 µm. C) A6 cells were permeabilized, fixed in methanol, and stained for zeta-tubulin (green), alpha-tubulin (red), and DAPI (blue). Zeta-tubulin does not co-localize with the spindle or spindle poles. Scale bar, 5 µm. D) Serum-starved A6 cells were fixed in methanol, postfixed in
acetone, and stained for zeta-tubulin (green), acetylated tubulin (red), and DAPI (blue). Zeta-tubulin does not localize to the primary cilium. Scale bar, 5 µm. E) Transmission EM of oviduct tissue stained with zeta-tubulin antibody and 10 nm gold-conjugated secondary antibody or secondary antibody alone were quantified. Two equal-sized areas of zeta-tubulin-labeled or control sections were assessed for the localization of gold particles. The total number of gold particles per localization type is shown. Zeta-tubulin specifically labels basal feet.
Figure S3. Zeta-tubulin precipitation reveals TRiC/CCT and GFP-zeta-tubulin is cytoplasmic in frog and mouse tissue culture cells. Related to Figures 2 and 4D.

A) GFP nanobody-conjugated beads were used to affinity purify GFP-zeta-tubulin from GFP-zeta-tubulin stable cells, and GFP nanobody-conjugated beads were incubated separately with A6 cell lysate as a control. The most abundant
co-purifying proteins as identified by mass spectrometry are shown, relative to their respective peptide counts. Hits include zeta-tubulin (blue), all 8 subunits of TRiC/CCT (green), heat shock or protein folding factors (pink), and proteasome-associated proteins (yellow). B) Wild-type A6 cells and the GFP-zeta-tubulin stable line were fixed in methanol and stained for GFP (green), gamma-tubulin (red), and DAPI (blue). GFP was only detected in the stable cells, and GFP-zeta-tubulin labels cytoplasm and does not co-localize with centrosomes. Scale bar, 5 µm. C) 3T3 mouse fibroblasts were infected with GFP-zeta-tubulin lentiviruses and fixed in methanol. Cells were stained for GFP (green), poly-glutamylated tubulin (red), and DAPI (blue). Inset shows a magnified image of the cilium (3x). Zeta-tubulin does not localize to microtubule-based structures in mouse cycling cells. Scale bar, 5 µm.
Figure S4. Controls for zeta-tubulin morpholino specificity and phenotype. Related to Figures 3 and 4.

A) Whole embryos injected with MO1 or no morpholino (control) were collected at stage 31 and lysed. Lysates were probed for zeta-tubulin, with alpha-tubulin as a loading control. Translation-blocking MO1 reduces zeta-tubulin protein.
Densitometry measures for zeta-tubulin signal normalized to the loading control are given under blot, and molecular weights in kilodaltons are indicated on the left. B) Early embryos were injected with MO2 or no morpholino (control) and animal caps were isolated. Animal caps were collected at stage 25 and lysed. Lysates were probed with zeta-tubulin, and GAPDH as a loading control. Splice-blocking MO2 reduces zeta-tubulin protein levels. Densitometry measures are given under blot, and molecular weights in kilodaltons are indicated on the left. C) Early embryos injected with 0, 20, 40, or 60 ng of MO2 were collected between stages 24-27 and lysed. Whole embryo cDNA prepared from two independent experiments was subjected to RT-PCR using zeta-tubulin and beta-actin primers, where beta-actin was a control for cDNA concentration. MO2 reduces zeta-tubulin mRNA levels in whole embryos. Negative controls (-RT) did not receive reverse transcriptase during generation of cDNA. Size standards for DNA fragments shown on the left in base pairs (bp). D) The number of basal bodies in each tadpole epidermal multiciliated cell was counted. The numbers of cells, embryos, and basal bodies as are in Figure 3E. Depletion with MO1 increased the number of basal bodies in multiciliated cells slightly (p = 0.023, Mann-Whitney), while MO2 decreased the number of basal bodies slightly (p=0.002, Mann-Whitney) E) Confocal image of a live Xenopus tadpole epidermal multiciliated cell expressing mem-RFP (red). The length and number of cilia are markedly reduced in morphants. Scale bars, 10 µm. F) Normalized LAP-ODF2 fluorescence intensity is not affected by zeta-tubulin morpholinos in tadpole epidermal multiciliated cells (ns = not significant, Mann-Whitney). G) The number of subapical actin foci (as seen in Figure 4B) per basal body in tadpole epidermal multiciliated cells were quantified. The number of subapical actin foci per basal body is significantly reduced in MO1-treated multiciliated cells, indicating that depletion of zeta-tubulin disrupts the apical actin cytoskeleton in these cells (***p < 0.0001, Mann-Whitney). H) Epidermis that is mosaic for zeta-tubulin MO3 or zeta-tubulin Mismatch MO2, stained with Alexa Fluor 568 phalloidin. Tadpole epidermal multiciliated cells without morpholino (open arrowhead) and cells with morpholino (filled arrowhead) are shown; MO3 reduces phalloidin intensity whereas Mismatch MO2 does not (ns = not significant). Scale bars, 5 µm. I) Zeta-tubulin depletion significantly reduces phalloidin staining intensity in tadpole epidermal multiciliated cells receiving any of three morpholinos tested relative to neighboring control cells (***p<0.0001 Mann-Whitney). Cells receiving Mismatch MO2 (MM-MO2) are statistically similar to controls (ns = not significant). Number of cells counted is shown, and the total number of embryos is in parentheses.
Table S1. Tubulin family conservation reveals modularity of ZED tubulins. Related to Figure 1.
The tubulin families present in each species or group of organisms are noted. In cases where a particular tubulin was missed by previous analysis, the database and sequence identifier are listed.

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Supplemental Materials and Methods:

Evolutionary analysis

The tree showing loss of ZED tubulins in eukaryotes (Figure 1A) was made by aligning the C-termini of cytochrome C from each organism in Clustal Omega and built by the neighbor-joining method employed by NJ Plot and visualized using Unrooted [S1]. Alignment of tubulin sequences for Figure 1B was performed using the same method as above.

Sequences used for the tubulins are as follows:

- H. sapiens alpha-tubulin NP_116093.1
- H. sapiens beta-tubulin AAH20946.1
- H. sapiens epsilon-tubulin NP_057346.1
- H. sapiens gamma-tubulin NP_001061.2
- H. sapiens delta-tubulin NP_057345.2
- M. domestica alpha-tubulin XM_007506231.1
- M. domestica beta-tubulin XP_001368521.1
- M. domestica gamma-tubulin XP_001363035.1
- M. domestica delta-tubulin XP_001362515.1
- M. domestica epsilon-tubulin XP_001379228.2
- M. domestica zeta-tubulin ENSMODT00000029350
- C. reinhardtii alpha-tubulin XP_001691876.1
- C. reinhardtii beta-tubulin XP_001693997.1
- C. reinhardtii gamma-tubulin XP_001691292.1
- C. reinhardtii delta-tubulin AAB71840.1
- C. reinhardtii epsilon-tubulin XP_001703303.1
- C. reinhardtii zeta-tubulin XP_001702996.1
- X. laevis alpha-tubulin NP_001165669.1
- X. laevis beta-tubulin NP_001080566.1
- X. laevis gamma-tubulin P23330.1
- X. laevis delta-tubulin AAL27450.1
- X. laevis epsilon-tubulin NP_001082338.1
- X. laevis zeta-tubulin NP_001082326.1

RNA-Seq Analysis

For tissue-specific expression analysis, we downloaded previously published RNA-seq data of six tissues from X. tropicalis adults (brain, heart,
kidney, liver, and testis, with two biological replicates) [S2] from the European Nucleotide Archive (Study # SRP015997) and mapped against the X. tropicalis longest transcripts database (Ensembl version 72) using Bowtie1 [S3]. The zeta-tubulin transcript is annotated as XB-GENE-5900222 (Ensembl transcript ID ENSXETT00000051467) in this database. As an estimate of the expression level, we report the TPM (transcripts per million reads) for zeta-tubulin using eXpress [S4].

**Plasmid constructs**

An EST encoding X. laevis zeta-tubulin (cryptic tubulin) cDNA was obtained from Open Biosystems (catalog # EXL1051-640940; accession # CA982622). This construct was sequenced and found to vary from the NCBI-annotated sequence at base 908 (T>C), which results in a coding change (L>S). However, the closely related species X. tropicalis has a serine at the same position, and analysis of other available ESTs confirmed that the expressed mRNA concurs with the plasmid sequence and not with the NCBI reference. We concluded that the correct sequence has a serine at that position. The above zeta-tubulin sequence was used to create pTS3210, a tag-less form of zeta-tubulin coding sequence (CDS) cloned into a derivative of pCS2 with a modified MCS (pCS10R, Wallingford Lab) using 5'-EcoRI and 3'-NotI sites. Zeta-tubulin CDS was also cloned with 5'-BspEI and 3'-XhoI sites into pEGFP-C1 to produce an N-terminally tagged GFP-zeta-tubulin construct (pTS2843). GFP-zeta-tubulin was excised from pTS2843 using 5'-NheI and 3'-EcoRI and cloned into pCS10R digested with 5'-Spel and 3'-NotI sites (destroys Spel site, pTS2849). Zeta-
tubulin CDS with a stop codon was cloned into pDONR221 (pTS1950) via Gateway Technology (Invitrogen) as pTS2766. pTS2766 was used in a Gateway LR reaction into pTS3517 (lentiviral N-terminal GFP-tag destination vector) to produce pTS3755, into pTS1951 (N-terminal GFP-tag destination vector) to produce pTS2769, and into pTS1956 (N-terminal 6xMyc-tag destination vector) to produce pTS2768. pTS3210 was used as a template to amplify zeta-tubulin and insert it into pENTR/D-TOPO (Life Technologies). A Gateway LR reaction was then used to insert zeta-tubulin into pCS CHERRY DEST (a gift from Nathan Lawson’s lab, Addgene # 13075) to generate pCS-mCherry-zeta-tubulin.

*X. tropicalis* epsilon-tubulin was ordered from Open Biosystems (catalog # MXT1765-99236012). The CDS was PCR-amplified with a stop codon and cloned into pDONR221 (pTS1950) via Gateway BP reaction as pTS3058, and then cloned into the N-terminal GFP destination vector pDEST53 (Invitrogen, pTS1951) by Gateway LR reaction as pTS3065. The CDS from pTS3065 were amplified by PCR and inserted into pENTR/D-TOPO, then cloned into pCS EGFP DEST (a gift from Nathan Lawson’s lab, Addgene # 13071) through a Gateway LR reaction to generate pCS-GFP-epsilon-tubulin.

Centrin (Unigene accession no. Xl.50473) was fused to the N-terminus of RFP to generate centrin-RFP (Addgene # 26753) [S5, S6]; this plasmid was used as a template to generate centrin-BFP by Michinori Toriyama of the Wallingford lab. CLAMP (Unigene accession no. Xl. 26316) was N-terminally fused to GFP as described [S6]. This plasmid was used to insert CLAMP into the CS107-3stopRFP (courtesy of E. Brooks). EMTB-3XGFP was a gift from William Bement
mem-RFP was a gift from Scott Fraser. Human ODF2, specifically the Cenexin isoform that localizes to the mother centriole, was donated by the lab of Maxence Nachury as a Gateway donor vector (pTS2148) and cloned by Gateway LR reaction into pTS2046 to generate LAP-ODF2 (pTS2149).

**In Vitro Translation**

Zeta-tubulin was made in reticulocyte lysate using the TNT Quick Coupled Transcription/Translation system (Promega catalog # L2080) according to the manufacturer’s instructions. Lysates were translated for 90 min in the presence of protease inhibitors (Roche catalog # 11836170001) and diluted 1:4 prior to adding sample buffer. For testing antibody specificity, 5 µL of the resulting sample was loaded (represents ~1 µL of the original reaction volume).

**Cell and MTEC Culture**

*X. laevis* A6 kidney epithelial cells (ATCC catalog # CCL-102) were cultured in 70% L15, 15% FBS, and 1% penicillin-streptomycin at 25 °C. A6 cells were induced to ciliate by incubation in serum-reduced media (70% L15, 2.5% FBS, 1% penicillin-streptomycin) for 24 h. A6 cells were transfected using JetPrime (VWR catalog # 89129-922), as directed. Mouse 3T3 fibroblasts and U2OS cells were maintained in DMEM, 10% FBS, and 1% penicillin-streptomycin at 37 °C and 5% CO₂. Mouse and human cells were transfected using
Lipofectamine LTX (Life Technologies), as directed. Mouse tracheal epithelial cells (MTECs) were isolated and cultured as previously described [S7].

**Antibody generation and purification**

Antibodies were generated in rabbits against a peptide corresponding to the C-terminal 20 residues of *X. laevis* zeta-tubulin, synthesized with an N-terminal cysteine residue for coupling to resin (N-CEAFHTLQSVADYSLGEP-C). Antibodies were affinity-purified on a column of antigenic peptide conjugated to SulfoLink Coupling resin (Thermo Scientific catalog # 20401), according to manufacturer’s directions. Fractions containing antibody were concentrated using Centricon columns (Millipore) and supplemented to 10% glycerol for storage.

**Antibodies**

The antibody raised against zeta-tubulin was used at 1:2000 (approximately 0.5 µg/µL) for immunoblots. For immunostaining of endogenous zeta-tubulin in A6 cells, the antibody was used at 1:500. Staining of oviduct multiciliated cells was done at 4 °C overnight with zeta-tubulin antibody at 1:5000. Immunofluorescence experiments also used antibodies against alpha-tubulin (clone DM1A, Sigma) at 1:4000, gamma-tubulin (clone GTU88, Sigma) at 1:1000, centrin3 (clone 3E6, Abnova) at 1:1000, acetylated tubulin (clone 6-11-B, Sigma or Invitrogen) at 1:5000-1:15,000 (optimized by batch), and GFP (generated as previously described [S8]) at 1:1000. Immunoblotting experiments used antibodies against gamma-tubulin (GTU88) at 1:10,000, CCT2 (gift from Judith
Frydman) at 1:1000, and histone H3 antibody (Millipore catalog # 04-928) at 1:1000. Alexa488- or Alexa594-conjugated secondary antibodies against rabbit IgG or mouse IgG isotypes 1, 2a, and 2b (Life Technologies) were used at 1:1000 for immunofluorescence.

**Immunoblotting**

Samples were loaded onto SDS-PAGE gels and run at 200 V for approximately 1 hour. Gels were transferred in 1x transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) ethanol) for at least 1 hour at 100 V onto nitrocellulose membrane (BioRad). Membranes were stained with Ponceau Red, incubated in 5% milk in TBST for at least 30 min, and then incubated in primary antibody overnight. Membranes were washed three times, 5 min each, then incubated in secondary antibody solution (donkey anti-rabbit IRDye800CW, goat anti-mouse IRDye680RD, donkey anti-goat IRDye800CW, and donkey anti-mouse IRDye680RD (LI-COR Biotechnology), all used at 1:20,000) for at least 30 min. Membranes were washed again three times, 5 min each, and scanned on a LI-COR Odyssey scanner. Some blots were visualized by chemiluminescent exposure of film. For those, the secondary antibody was HRP-conjugated (Jackson Immunotech, anti-mouse catalog # 715-035-150 and anti-rabbit catalog # 111-035-144) and the developing reagent was SuperSignal Pico Western (Fisher Scientific catalog # PI-34080).
**Immunofluorescence of cells and MTECs**

Cells were grown on poly-L-lysine coated coverslips and fixed in either 100% -20 °C methanol or 4% formaldehyde (v/v) for 10 min. MTEC filters were excised using a razor blade and fixed in 100% -20 °C methanol for 10 min. Formaldehyde-fixed samples were quenched with 1 mg/mL sodium borohydride for 10 min. Where indicated, permeabilization was performed by incubation in BRB80-Triton (80 mM mM PIPES pH 6.8, 50 mM EGTA, 1 mM MgCl₂, 0.05% Triton X-100) for 1 min immediately prior to fixation. Where indicated, acetone postfix was performed directly after 10 min incubation in 100% -20 °C methanol by incubation for 1 min in 100% -20 °C acetone. All samples were washed in PBS after fixation and blocked in PBS-BT (1X PBS, 3% BSA, 0.1% Triton X-100, 0.02% azide) for at least 30 min. Samples were then incubated for at least 30 min in primary antibody prepared in PBS-BT. Samples were washed three times with PBS-BT followed by incubation with secondary antibodies prepared in PBS-BT. Samples were briefly incubated in 1 µg/mL DAPI, washed 3 times with PBS-BT, and then mounted in MOWIOL/DABCO. Cells were imaged using an Axio Observer microscope (Zeiss) with a confocal spinning disk head (Yokogawa, Japan), PlanApoChromat 63x/1.4 NA objective, and a Cascade II:512 EMCCD camera (Photometrics). All localizations for cells and MTECs were performed at least twice, with same result.

*Xenopus* oviduct was harvested from an adult female frog and washed in PBS. The oviduct was fixed for 10 min at room temperature in 4% formaldehyde (v/v), placed in PBS at 4 °C for several days, then vortexed to separate
dissociated cells. Dissociated cells were centrifuged onto coverslips in a Sorvall HB-6 rotor in 15 mL Corex tubes with coverslip adaptors for 5 min at 1000 rpm, 4 °C. Cells were then stained as above, except the primary antibodies were incubated overnight at 4 °C. Localization of zeta-tubulin in dissociated oviduct multiciliated cells was performed twice, and a peptide block was used the first time to confirm specificity of signal.

**Peptide block**

Peptide was added to a final dilution of 1.25 µM in approximately 2.7 nM antibody solution. For immunoblotting, the peptide and antibody were incubated together in milk, whereas the control antibody was incubated without peptide. For immunofluorescence, antibody and peptide were mixed in PBS-BT (1x PBS, 3% BSA, 0.1% Triton X-100, 0.02% azide), whereas the control antibody was incubated without peptide. All solutions were mixed for 3 h at room temperature prior to use. Peptide blocks were performed once for each new sample on which zeta-tubulin antibody was applied.

**Cytoplasmic/Nuclear Fractionation**

Fractionation was performed after the method in Meylan, et al. (2009) [S9]. Cells were washed in cold PBS, scraped into lysis buffer A (20 mM HEPES pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 0.1% NP-40, LPC, PMSF), and incubated for 10 min. Lysates were layered over 30% sucrose in lysis buffer A and centrifuged at 5000 x g for 10 min in a
microcentrifuge at 4 °C. The supernatant was recovered as the cytoplasmic fraction. Pellet containing nuclei was washed 3 times with lysis buffer A. The pellet was lysed with lysis buffer B (20 mM HEPES pH 7.6, 20% glycerol, 500 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 0.1% NP-40, LPC, PMSF) and incubated 30 min on ice. Lysate was cleared at 13,000 rpm in a microcentrifuge at 4 °C for 15 min, and the supernatant was recovered as the nuclear fraction. Fractionation of A6 cells in this manner was performed once (see Figure S2A), however result concurred with a centrosome prep of *Xenopus* tissue culture cells showing no zeta-tubulin in the centrosome fraction.

**Sucrose Gradients**

Thin-wall tubes for the Beckman TLS-55 rotor (Beckman-Coulter catalog #347356, ~2.2 mL capacity) were prepared by coating in 1% gelatin and washing 5 times with distilled water. 10% and 40% sucrose solutions (w/v) were prepared in buffer XB (100 mM KCl, K-HEPES pH 7.6, 0.1 mM CaCl₂) and cast as a continuous 2 mL gradient into the prepared tubes. Frog egg extract was prepared as described [S10]. Egg extract was diluted 1:10 in XB + 0.2% Triton X-100 supplemented with LPC and PMSF and cleared for 5 min at 100,000 rpm in a TLA100 rotor. A6 cells were lysed in the same buffer and centrifuged at 16,000 x g in a microcentrifuge for 5 min at 4 °C prior to being cleared in the same manner as above. Whole *Xenopus* oviduct was harvested from an adult female frog and washed twice in PBS, followed by three consecutive washes over 15 min in 2% cysteine in PBS to reduce the mucous layer prior to lysis. The oviduct was then
flash-frozen in liquid nitrogen and ground with a mortar and pestle to a fine powder. The powder was dissolved in XB + 0.2% Triton X-100, then tumbled for 5 min at 4 °C before adding to 1 mM DTT to further reduce mucous. The lysate was tumbled again for 5 min at 4 °C and centrifuged for 5 min at 16,000 x g in a microcentrifuge at 4 °C. The lysate was further clarified by centrifugation in a TLA100.3 rotor for 5 min at 100,000 rpm prior to use. 200 µL of each of the above lysates was loaded on top of the gradients, with a separate gradient containing molecular weight standard proteins (Sigma catalog # MWGF1000). The gradients were centrifuged at 55,000 rpm in a TLS-55 rotor for 2.5 h at 4 °C and then separated into to 150 µL fractions. Each fraction was supplemented with 50 µL of 4x SDS sample buffer with DTT, boiled, and equal volumes of each fraction were loaded onto gels. The standards were analyzed by Coomassie staining and the lysates analyzed by SDS-PAGE and immunoblotting with zeta-tubulin, gamma-tubulin, and CCT2 antibodies. The signal for each protein on immunoblots or Coomassie stain was determined using Image Studio software (LI-COR Biotechnology), then plotted and fitted with a second-degree polynomial. The derivative of each distribution of protein was used to determine the actual peak of the distribution. Peaks for each of the standard proteins were determined, and those with sufficiently clear Coomassie staining to be suitable for densitometry were plotted and fitted to a linear function. The equation of that standard curve was used to determine the S-value of zeta-tubulin and gamma-tubulin in each gradient. All gradients were performed at least twice with similar results, and approximate S-values were calculated from one replicate.
Cell line generation and affinity pulldown of zeta-tubulin

A6 cells were seeded in 10 cm tissue culture dishes and transfected once a day for three consecutive days with pTS2849. Medium was changed the day after the final transfection, with the addition of 0.5 mg/mL G418. Cells were split to low confluence and maintained for approximately one month in 20% conditioned medium (derived from confluent A6 cell culture)/80% fresh medium with 0.5 mg/mL G418 while sparse, then fresh medium with 0.5 mg/mL G418 thereafter. The cells were trypsinized and washed in 1% BSA in PBS and filtered through a 40 µm cell strainer (Fisher catalog # 08-771-1). Cells were sorted for GFP expression to single colonies (12% of the culture was GFP positive by FACS). Clones were grown for approximately 2 months in conditioned medium (1:4 with fresh medium as above). Resultant clones were lysed and immunoblotted for zeta-tubulin to show both endogenous and overexpressed zeta-tubulin, of which 64% retained GFP-zeta-tubulin expression. One cell line that had approximately equal levels of endogenous and GFP-tagged zeta-tubulin and normal morphology and growth rate was used for affinity purification of zeta-tubulin.

Twelve confluent dishes each of GFP-zeta-tubulin-expressing cells and wild-type A6 cells were trypsinized and washed in PBS. Cells were then lysed for 20 min at 4 °C in XBT (100 mM KCl, K-HEPES pH 7.6, 0.1 mM CaCl₂, 0.2% Triton X-100, LPC and PMSF). Lysates were cleared at 3500 rpm in a JS5.3 rotor for 15 min at 4 °C and then incubated for 3 h with 50 µL beads conjugated to GFP nanobody [S11]. Beads were washed five times with 1 mL XBT before
elution in 100 µL 0.1 M glycine-Cl pH 2.5. Samples were immediately placed on ice and neutralized with 10 µL 2 M Tris-Cl pH 8.5. Samples were analyzed by MUD-PIT at the Stanford University Mass Spectrometry Core.

**Immunoelectron microscopy:**

An oviduct from an adult *X. laevis* female was removed and placed directly into 4% EM-grade formaldehyde (v/v). The oviduct was sectioned in fixative in <20 min and transferred to Karlsson-Schultz salt solution (0.1 M sodium phosphate, 90 mM sodium chloride, pH 7.3). The fixed pieces of oviduct were dehydrated in a series of increasing ethanol concentrations followed by infiltration and embedding in Lowicryl HM20. Embedded oviduct was then sectioned and stained. Sections were stained with 1:300 dilution of zeta-tubulin antibody at 4 °C overnight followed by 1:50 of Goat anti-Rabbit secondary antibody conjugated to 10 nm gold particles (Ted Pella catalog # 15726) for one hour at room temperature. The technical control was stained at the same time with the same secondary antibody solution, but no primary antibody was used. Grids were contrast-stained for 3 min in a 1:1 mixture of 3.5% uranyl acetate and 50% (v/v) acetone followed by staining in 0.2% lead citrate for 3-4 min. Sections were imaged with a JEOL JEM-1400 electron microscope at 120 kV and images were taken using a Gatan Orius 4k x 4k digital camera.
Xenopus embryos

Adult X. laevis females were injected with human chorionic gonadotropin (Merck) to induce ovulation. Eggs were collected the following day by manual manipulation and fertilized in vitro. Embryos were raised in 1/3X Marc’s Modified Ringer’s solution (MMR) and dejellied in 3% cysteine at the two-cell stage. Embryos were raised at either room temperature or 18 °C and collected at stage 31-34 for most experiments. Exceptions include analysis of ciliogenesis (stages 26-28) and morpholino efficiency (various stages, see below).

Morpholino oligonucleotide and mRNA injections

Capped RNA was synthesized using mMessage mMach in kits (Life Technologies AM1340). Morpholinos were ordered from GeneTools, LLC.

Zeta MO1 (translation-blocking): CCACCTGGAGCCAAACCAACGACAT
Zeta MO2 (splice-blocking): AATTAGAGGAACCACACACCTCT
Zeta Mismatch MO2 (MM-MO2): AATaAcAAGcAACCaAgAgACCCTCT
Zeta MO3 (splice-blocking): TACCTAAGGAGGAAGTAACAGGACT

Zeta MO2 targets the Exon2/Intron2 junction; Zeta MO3 targets the Intron5/Exon6 junction. For imaging experiments, 15-20 ng of MO1, 20-30 ng of MO2, 30ng of MM-MO2, and 30ng of MO3 were injected. For immunoblotting, 20-40 ng of MO1 or 30 ng MO2 was used.
For microinjection, 4-cell stage embryos were placed in 2-3% Ficoll and the ventral 2 blastomeres were injected with 10 nL of mRNA and morpholino solution using an Oxford universal micromanipulator. For immunoblotting and RT-PCR, embryos were injected with the above solution into all 4 blastomeres. For mosaic experiments, embryos were either injected with the above solution into 1 ventral blastomere in 4-cell stage embryos, or 7 nL into 2 ventral blastomeres at the 8-16-cell stage.

**Staining and imaging of Xenopus embryos**

Injected embryos were anesthetized with benzocaine and mounted between two coverslips and secured with silicone vacuum grease. Embryos were selected for analysis on the basis of a healthy appearance, correct and consistent stage, and where appropriate, expression of the co-injection marker as assessed using a stereomicroscope. Embryos were imaged in Attofluor Cell Chambers (Life Technologies A-7816) or custom chambers as described [S12] using a Zeiss LSM700 inverted confocal microscope, with the exception of high-speed capture of ciliary beating. For those experiments, embryos were mounted as described above, and multiciliated cells on the dorsal or ventral edge of the embryo (oriented roughly parallel to the coverslip) were identified. High speed movies were taken on a Nikon Eclipse Ti inverted confocal microscope equipped with a resonance scanner. For phallloidin staining, embryos were fixed in MEMFA (100 mM MOPS pH 7.4, 2 mM EGTA, 1 mM MgSO₄, 3.7% (v/v) formaldehyde) overnight at 4 °C or for 2 hours at room temperature, washed 3 times with PBST.
or PBS, and stained with 3% Alexa Fluor 568 phalloidin (Life Technologies, A12380) or CruzFluor 405 phalloidin (Santa Cruz Biotechnology, sc363790) in PBST for 4 h at room temperature or overnight at 4 °C, washed 3 times in PBST and mounted and imaged as above.

Images shown in Figures 2C (11 cells, 4 embryos, one experiment), 2D (8 cells, 3 embryos, two experiments), and 4C (9 cells, 3 embryos, 1 experiment) were consistent across all cells imaged. Images shown in Figure 3A are representative of two independent experiments each for MO1 (control: 36 cells, 9 embryos; MO1: 34 cells, 8 embryos) and MO2 (control: 22 cells, 6 embryos; MO2: 21 cells, 6 embryos). MO3 showed similar basal body spacing defects, while MM-MO2 was similar to wild type, though basal body orientation was not assessed for these treatments. Figures 3B and 3C are the quantified results from three of those experiments, two for MO1 and one for MO2. Figures 3D and 3E were quantified from all four of those experiments and an additional experiment using MO1 (control: 10 cells, 4 embryos; MO1: 9 cells, 3 embryos), excluding a few cells that could not be quantified for this purpose due to drift during imaging.

Figure 4A is representative of results from two independent experiments using MO1 (control: 21 cells, 7 embryos; MO1: 23 cells, 8 embryos). Figure 4B is representative of results from two independent experiments (control: 27 cells, 8 embryos; MO1: 27 cells, 8 embryos MO1). Similar actin phenotypes were observed for MO2- and MO3-treated embryos when imaged for actin intensity analysis, while MM-MO2 cells appeared similar to controls (Figures S4H and S4I; see below for quantification methods and embryo numbers). Images of cilia in
Figure S4E are representative of two independent experiments (control: 61 cells, 9 embryos; 30 ng MO2: 60 cells, 8 embryos). Movies of ciliary beating (Movies S1 and S2) are representative of two independent experiments using either MO1 or MO2 (control: 29 cells, 6 embryos; MO1: 20 cells, 3 embryos; MO2: 21 cells, 4 embryos).

**Analysis of morpholino efficiency**

For MO1, whole embryos were collected at stage 31 and lysed in Mammalian Protein Extraction Reagent (Life Technologies catalog # 78501); result shown in Figure S4A is representative of 3 independent experiments. For MO2, animal caps were collected at stage 25 and lysed as above; result shown in Figure S4B was performed once in that manner, but concurs with corresponding RT-PCR data in Figure S4C (both replicates of this experiment are shown). Lysate was centrifuged at 14,000 rpm in a microcentrifuge for 15 min at 4 °C, and the supernatant collected and, if necessary, stored at -80 °C.

Protein lysate was mixed with sample buffer and boiled for 10 min, separated on 4-20% Precise Protein Gels (Thermo Scientific) and transferred to nitrocellulose membranes (GE Water & Process Technologies, # EP4HY0010). Membranes were incubated for 1 h at room temperature on a shaker in blocking solution (3-5% milk in PBST). Membranes were then incubated in primary antibody in blocking solution overnight at 4 °C. Anti-zeta-tubulin was used at 1:5000; loading controls were either anti-alpha tubulin (Abcam ab4074, 1:500-1000) or anti-GAPDH (Cell Signaling Technology 14C10, 1:1500-2000). The next
day membranes were washed 3 times in PBST and incubated with secondary antibody in blocking solution for 1 h at room temperature (Jackson ImmunoResearch catalog # 711-035-152, 1:1000). Blots were developed using either SuperSignal Pico Western (Thermo Scientific, #34077) or SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, #34075) and assessed using a GE ImageQuant LAS 4000. Densitometry was performed in Fiji [S13].

For RT-PCR, embryos were collected at stages 24-27 in Trizol reagent (Ambion). Lysate was centrifuged at 12,000 g for 10 min at 4 °C, supernatant was collected and extracted with chloroform, and precipitated with isopropanol to collect RNA. cDNA was generated using M-MLV (Invitrogen catalog # 28025) and random hexamer primers according to the manufacturer’s instructions. Zeta-tubulin RT-PCR was run for 35 cycles, and beta-actin for 30 cycles. Note that a reverse primer targeting zeta-tubulin intron 2 only gave a band at the 60 ng morpholino dose, suggesting that the splice-blocked mRNA is highly unstable (data not shown).

Primers used are as follows:

Zeta-tubulin forward: AATGCCATCTGTGTGGACAGTGAG
Zeta-tubulin reverse: GTGGAGCCACAGCTACTGAC
Beta actin forward: ATGGAAGACGATATTGCCGC
Beta actin reverse: GCATGTGGGTAGAGCACATAGCC
Quantification of basal body orientation, basal body distribution, and fluorescence intensity

To quantify rootlet orientation, vectors from the tip of the rootlet to the basal body were drawn manually in Fiji [S13] using maximum intensity projections. Cells were only omitted from this analysis if they moved out of frame during the course of imaging, were not mounted flat to glass, or had large apical curvature such that the apical focal plane was variable. Basal bodies were only omitted if directionality of the rootlet could not be clearly determined. The vector length, mean angle, and statistical significance of differences were determined using the CircStat MATLAB Toolbox (Philip Berens, 2009), and Compass plots were generated in MATLAB. Data presented (Figures 3B and 3C) are pooled from two independent experiments using MO1 and one experiment using MO2 (7651 basal bodies, 44 cells, 11 embryos), and controls pooled from all three independent experiments (8331 basal bodies, 47 cells from 12 embryos).

To measure the distance between neighboring basal bodies, maximum intensity projections of the apical-most optical sections were generated and the position of each basal body manually marked in Fiji. The distance between each basal body and its nearest neighbor was calculated in MATLAB (code available upon request) and the average distance for individual cells determined. To generate the histogram in Figure 3D, near neighbor distances were binned, then normalized against the total number of basal bodies, yielding the percentage of basal bodies in each bin for each cell. Individual cell results were then averaged, yielding the mean percentages and standard error for all bins in each
experimental group. Data shown are the pooled results of three independent experiments using MO1 (7749 basal bodies, 43 cells, 11 embryos) and two independent experiments using MO2 (3035 basal bodies, 20 cells, 6 embryos); no morpholino control results were pooled from all five independent experiments (11,059 basal bodies, 65 cells, 19 embryos). Cells were only omitted from this analysis if they moved out of frame during the course of imaging, were not mounted flat to glass, or had large apical curvature such that the apical focal plane was variable. These data were also used to assess the number of basal bodies per cell.

To quantify actin intensity, centrin-BFP and/or membrane-GFP was used as a co-injection marker to identify multiciliated cells receiving morpholino in mosaic tissue, and images were taken with expressing and non-expressing multiciliated cells in the same frame. The area just inside the cell cortex was manually traced in maximum intensity projections in Fiji to quantify the average actin intensity quantified for each multiciliated cell. To generate a normalized value, the actin intensity of an expressing cell was divided by the actin intensity of a non-expressing cell(s) in the same frame. If more than one non-expressing cell was present, the mean actin intensity of all non-expressing cells within the frame was calculated and used for analysis. Results represent 3 independent experiments using MO1 (68 expressing cells, 11 embryos), 5 independent experiments using MO2 (112 expressing cells, 16 embryos), 2 independent experiments using MO3 (51 expressing cells, 6 embryos), 2 independent experiments using MM-MO2 (54 expressing cells, 6 embryos), and results pooled from all 5 independent control
experiments (86 expressing cells, 15 embryos). At least two frames per embryo were used for analysis. Subapical actin foci per cell were counted and divided by the number of basal bodies in each cell. The data shown are the results of two independent experiments (control: 27 cells, 8 embryos; MO1: 27 cells, 7 embryos). MO2 and MO3 exhibited a similar phenotype but were not quantified. All cells imaged were included in the analysis with the exception of cells exhibiting the criteria listed above.

To quantify ODF2 intensity, embryos were injected with centrin-(BFP or RFP) and LAP-ODF2, and maximum intensity images of single cells were generated. In single multiciliated cells, centrin and ODF2 foci were identified and the mean intensity of each focus measured in Fiji [S13] (3D Objects Counter Plug-In). The intensity measurements for all foci within the cell were averaged to generate the centrin and ODF2 intensities. For each cell, the ODF2 value was normalized to the centrin value. Results shown are the result of one experiment (0 ng: 11 cells, 3 embryos; MO1: 11 cells, 3 embryos; MO2: 9 cells, 3 embryos). A second experiment using MO1 gave similar results (MO was statistically similar to controls), but is not plotted here because different amounts of ODF2 and centrin were injected.

Compass plots and the Watson-Williams significance test were performed in MATLAB. All graphs and tests for significance were performed using Prism software.
Lentivirus production and infection

Lentiviruses were produced in HEK293T cells. Cells were seeded so as to be 70% confluent at the time of transfection (1.1 x 10^7 cells/15 cm dish if seeded the day before). Cells were transfected with Polyethylenimine (PEI) (3:1 ratio of 1 mg/ml PEI in µL to DNA in µg) with a 2:2:1 ratio of the lentiviral transgene vector, psPAX2 (Trono Lab), and pMD2.G (Trono Lab) (20:20:10 µg in a 15 cm dish). PEI/DNA mix in 1 ml OPTI-MEM was vortexed for 10 s and incubated for 30 min prior to adding to cells. The medium was changed the day after transfection and viral supernatants were collected three days after transfection. Lentivirus was concentrated by centrifugation in a SW-41 rotor at 20,100 rpm for 140 min at 20 °C. Supernatant was decanted and the tube was inverted to allow the pellet to dry. 100 µL PBS was added to the pellet and allowed to dissolve overnight at 4 °C. The resulting virus supernatant was centrifuged at 2600 rpm for 5 min in a microcentrifuge to pellet debris and then frozen at -80 °C for storage. MTECs were infected with 10 µL concentrated virus per 24-well filter, and mouse 3T3 cells were infected two times with 10 µL of concentrated lentivirus in a 6-well microtiter dish before being split onto coverslips for immunofluorescence.
Supplemental References:


