Figure S1. Validation of the newly synthesized glycylated peptides and the purified gly-pep1 antibody. (A–E) Synthetic β2-tubulin C-terminal–tail peptides were analyzed by tandem mass spectrometric analysis to verify their sequence and glycylation status. A 0.1 mM stock solution of each of the synthetic peptides (A, control-pep; B, gly-pep1; C, gly-pep2; D, bi–gly-pep1; and E, bi–gly-pep2) was prepared from lyophilized powder by dissolving in a 50:50 (vol/vol) solution of acetonitrile/water. For mass spectrometric analysis, the stock solution was further diluted to 1 µM with the same solvent supplemented with 1% formic acid. The peptide solutions were then introduced into a mass spectrometer (Synapt HDMS G2-Si; Waters) by direct infusion at a flow rate of 7 µl/min. Peptide ions were generated by electrospray (microspray) in standard ion source conditions for peptidic analytes. The double-charged ions were quadrupole selected as precursor ions, and the collision energy was increased to 20–25 V. Collision-induced dissociation tandem mass spectra were recorded. After in silico fragmentation of each peptide with massXpert (Rusconi, 2009), the theoretical data were matched with the observed mass extracted from the mass spectra using the mMass tool (Strohalm et al., 2010). All the peaks that defined the peptide sequence were labeled, and the peaks that unambiguously defined the glycylation modification are detailed in insets, indicating that all the peptides had the modification at the expected sites. From the mass spectrometry analysis, we found that the theoretical mass/charge (m/z) values matched the observed m/z values for each of the modified peptides (as seen in the insets of each mass spectrogram), confirming the sequence and site of modification for each of the peptides. (F and G) The rabbit polyclonal antibody gly-pep1 was purified on an affinity column, which was generated by coupling the antigenic peptide (gly-pep1) to a HiTrap N-hydroxysuccinimide–activated HP affinity matrix (17-0716-01; GE Healthcare). 2.5 mg of the peptide was resuspended in 1 ml of coupling buffer (200 mM sodium bicarbonate buffer, pH 8.3, and 500 mM NaCl) and incubated at room temperature for 5 min. Meanwhile, the column was washed with 6 ml of ice-cold 1 mM HCl. The peptide was then quickly loaded onto the column, sealed, and incubated for 30 min at room temperature. After the coupling, the column was connected to the AKTA purifier 10 (GE Healthcare) and washed with 30 ml PBS. The free N-hydroxysuccinimide–reactive groups of the resin were then deactivated using a series of washing steps as per the manufacturer’s protocol. 20 ml gly-pep1 serum was centrifuged at 66,000 g for 30 min at 4°C to remove any cellular debris. The supernatant was filtered through a 0.22-µm filter and then loaded onto the prequilibrated [with PBS] affinity column at a flow rate of 0.2 ml/min overnight at 4°C. The flow-through was collected, and the column was washed with 40 ml PBS at a flow rate of 1 ml/min, followed by 20 ml PBS with 1 M NaCl, and again with 20 ml PBS. The specifically bound antibodies were eluted with elution buffer (100 mM glycine/HCl, pH 2.3) in 500-µl fractions. (F) Elution profile of the antibody (absorbance at 280 nm). The pH was immediately adjusted to pH 8.0 by adding 50 µl of 1 M glycine, pH 9.0, to each fraction. The fractions were pooled and dialyzed three times against 1 liter of PBS at 4°C for 6 h. (G) 10 µg of the purified antibody was visualized by SDS-PAGE and Coomassie brilliant blue staining. Note a single band for the antibody heavy chain at ~50 kD and multiple bands for the light chains at ~25 kD. The purified antibody was adjusted to 1 mg/ml of final concentration and stored at −20°C.
Figure S2. **Extended data for Fig. 5 (A–D): analyses of TTLL3 knockdown in MDCK cells.** (A) Expression of TTLL3 was analyzed using quantitative RT-PCR (qRT-PCR). RNA was isolated from MDCK cells 24 h after transduction with lentiviruses coding for shRNAs (scramble, TTLL3_478, TTLL3_556, and TTLL3_994) with TRizol reagent according to the manufacturer’s protocol (Thermo Fisher Scientific). The quality of the RNA was determined by an agarose gel electrophoresis, and RNA was quantified with a spectrophotometer (Nanodrop; Thermo Fisher Scientific). qRT-PCR was performed under standard conditions using the SYBR green master mix kit on the ABI Prism 7900 Sequence Detection System. The relative mRNA expression levels of TTLL3 were expressed as the N-fold difference in their expression relative to the TBP gene. Purified cDNA from dog testes (CliniSciences) was used as a positive control for the qRT-PCR. Data from two independent experiments are shown. Each column represents one individual experiment. Primers used are as follows. For TTLL3, we used forward primer 5′-GGT TCG GGG CTC TCT TAT TCT CAT-3′ and reverse primer 5′-GCG CAC AGA GCT TCA TCC ACA-3′. For TBP, we used forward primer 5′-AAG CTT GAC CTA AAG ACC ATT GCA CT-3′ and reverse primer 5′-GGT TCG GGG CTC TCT TAT TCT CAT-3′. (B) Immunofluorescence images of 3-d-starved MDCK cells transduced with lentiviruses containing shRNA for TTLL3 (TTLL3_478 and TTLL3_556). Cilia were stained for glycylation (red) and ac-tubulin (green). The images for scramble and TTLL3_994 shRNA treatment are shown in Fig. 5 A. (C) Extended quantification of the ciliary length distribution as in Fig. 5 B, including data for the TTLL3_478 and TTLL3_556 shRNA treatment. (D) Extended quantification of the percentage of glycylated cilia per length category as in Fig. 5 C, including data for the TTLL3_478 and TTLL3_556 shRNA treatment. (E) Extended distribution of ciliary lengths and statistics as in Fig. 5 D, including data for TTLL3_478 and TTLL3_556 shRNA treatment. Data are represented as a scatter plot with a line indicating the median (value indicated) and whiskers at interquartile ranges (25th and 75th percentiles). P-values were calculated by one-way ANOVA. Individual experiments are shown in Fig. S3 B. For the number of individual measurements per data point, see Table S1 (n > 1,000). (F) Immunofluorescence images of 3-d-starved MDCK cells transduced with lentiviruses containing scramble and TTLL3_994 shRNA. Cilia were stained for glycylation (red), ac-tubulin (green), and glutamylation (gray). Bars, 10 µm.
Figure S3. Extended data for Fig. 4 D, Fig. 5 (D and G), and Fig. S2 E: ciliary length distributions—statistical analyses of individual experiments. (A) Ciliary length measurements from individual experiments for each experimental condition shown in Fig. 4 A are represented as scatter plots with a line indicating the median (value indicated) and whiskers at interquartile ranges (25th and 75th percentiles). P-values were calculated by one-way ANOVA (combined data: Fig. 4 D). For the number of individual measurements per data point, see Table S1 (n > 100). (B) Ciliary length measurements from individual experiments for each experimental condition shown in Fig. 5 A and Fig. S2 B are represented as scatter plots with a line indicating the median (value indicated) and whiskers at interquartile ranges (25th and 75th percentiles). P-values were calculated by one-way ANOVA (combined data: Fig. S2 E). For the number of individual measurements per data point, see Table S1 (n > 300). (C) Ciliary length measurements from individual experiments for each experimental condition shown in Fig. 5 E are represented as scatter plots with a line indicating the median (value indicated) and whiskers at interquartile ranges (25th and 75th percentiles). P-values were calculated by one-way ANOVA (combined data: Fig. 5 G). For the number of individual measurements per data point, see Table S1 (n > 300).
Table S1 is provided as an Excel file and presents source data for quantification.

References
