Target-of-rapamycin complex 1 (Torc1) signaling modulates cilia size and function through protein synthesis regulation

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The cilium serves as a cellular antenna by coordinating upstream environmental cues with numerous downstream signaling processes that are indispensable for the function of the cell. This role is supported by the revelation that defects of the cilium underlie an emerging class of human disorders, termed "ciliopathies." Although mounting interest in the cilium has demonstrated the essential role that the organelle plays in vertebrate development, homeostasis, and disease pathogenesis, the mechanisms regulating cilia morphology and function remain unclear. Here, we show that the target-of-rapamycin (TOR) growth pathway modulates cilia size and function during zebrafish development. Knockdown of *tuberous sclerosis complex 1a* (*tsc1a*), which encodes an upstream inhibitor of TOR complex 1 (Torc1), increases cilia length. In contrast, treatment of embryos with rapamycin, an inhibitor of Torc1, shortens cilia length. Overexpression of *ribosomal protein S6 kinase 1* (*S6k1*), which encodes a downstream substrate of Torc1, lengthens cilia. Furthermore, we provide evidence that TOR-mediated cilia assembly is evolutionarily conserved and that protein synthesis is essential for this regulation. Finally, we demonstrate that TOR signaling and cilia length are pivotal for a variety of downstream ciliary functions, such as cilia motility, fluid flow generation, and the establishment of left-right body asymmetry. Our findings reveal a unique role for the TOR pathway in regulating cilia size through protein synthesis and suggest that appropriate and defined lengths are necessary for proper function of the cilium.

Chlamydomonas | mammalian target of rapamycin | Kupffer's vesicle | laterality | flagella

The cilium, a cell surface organelle, has emerged as a key center of sensation, signal transduction, growth, and motility for the vertebrate cell (1). Although previous studies have provided insight into many core components of cilia biogenesis, the regulation of cilia structure and function has remained poorly understood (2). However, the relevance of this regulation has grown in importance, as ciliary defects have been linked to an increasing number of human disorders. Additionally, the dramatically different yet stereotypic length of cilia in varying cell types suggests active regulation. However, the mechanisms that regulate cilia size and the functional consequences of aberrant cilia size, particularly that of abnormally long cilia, remain poorly understood.

Given the importance of cilia to core biological processes, it is not surprising that recent findings have linked cilia length defects with human disorders. Loss of tuberous sclerosis complex (TSC) proteins in zebrafish and mice result in cystic kidneys and abnormally long renal cilia (3, 4). Additionally, proteins associated with Meckel-Gruber syndrome and retinitis pigmentosa also influence cilia length (5, 6). Case studies of children with primary ciliary dyskinesia have revealed an association between abnormally long cilia in the lung airway and recurrent lung infections, suggesting that elongated cilia fail to remove contaminants in the airway (7). Furthermore, abnormally long cilia have been found in the renal epithelium during early tissue repair in humans and mice with acute tubular necrosis (8).

The first clues that signaling pathways may be involved in cilia length regulation came from the ciliated green alga, *Chlamydomonas reinhardtii*. Four long flagella mutants have been identified from genetic screens, with two encoding for kinases (9). Genetic analysis of these mutants has suggested that some of these proteins may be involved in a common signaling cascade (10); however, their function has not been elucidated. In vertebrates, the FGF pathway, the Notch pathway, and cAMP signaling have been shown to regulate cilia length (11–13). Actin dynamics and endocytic vesicular trafficking have also been implicated in this process (14). Combined, these findings suggest that the cilium is a dynamic structure that is subjected to regulation by diverse and broad signals. Furthermore, the seemingly unrelated and heterogeneous nature of these signals emphasize that our knowledge of the mechanisms regulating ciliary size is currently limited.

In a previous study, we found that zebrafish embryos deficient in *tsc1a*, one of two *TSC1* homologs, yielded abnormally elongated cilia in the pronephric duct and otic vesicle (3). *TSC1* and *TSC2* are causal genes of TSC, which is a rare, autosomal-dominant genetic disorder that is characterized by the growth of benign tumors in the brain, kidney and other vital organs (15). The gene products of *TSC1* and *TSC2* form a complex that prevents the activation of target-of-rapamycin complex 1 (TORC1), a major protein complex that executes TOR signaling (16). TORC1 integrates numerous environmental and upstream signaling inputs to function as a key controller of cell size, proliferation and metabolism (16). Prompted by these findings, we investigated TOR signaling as a potential modulator of cilia length and function during zebrafish development.

Results

Knockdown of *tsc1a* Induces Cilia Elongation. We chose to analyze a ciliated epithelial organ called Kupffer's vesicle (KV) because of the ease of quantifying cilia length and the availability of biological readouts for analyzing cilia function (Fig. S1). In a process analogous to the mouse node, motile cilia located in the KV generate a directional fluid flow that is believed to trigger early bilateral asymmetry in zebrafish (17, 18). We observed that the *tsc1a* translational blocking morpholino (MO) induced an abnormally long KV cilia phenotype in a dose-responsive manner (Fig. 1 *A*–*C*), expanding our previous finding in the otic vesicle and the pronephric duct (3). Additionally, there was not a significant change in the size of the KV or the number of cilia in the KV of *tsc1a* morphants (embryos microinjected with a MO) (Table S1). To address whether TSC signaling functions cell-autonomously in KV cells to regulate cilia length, we generated

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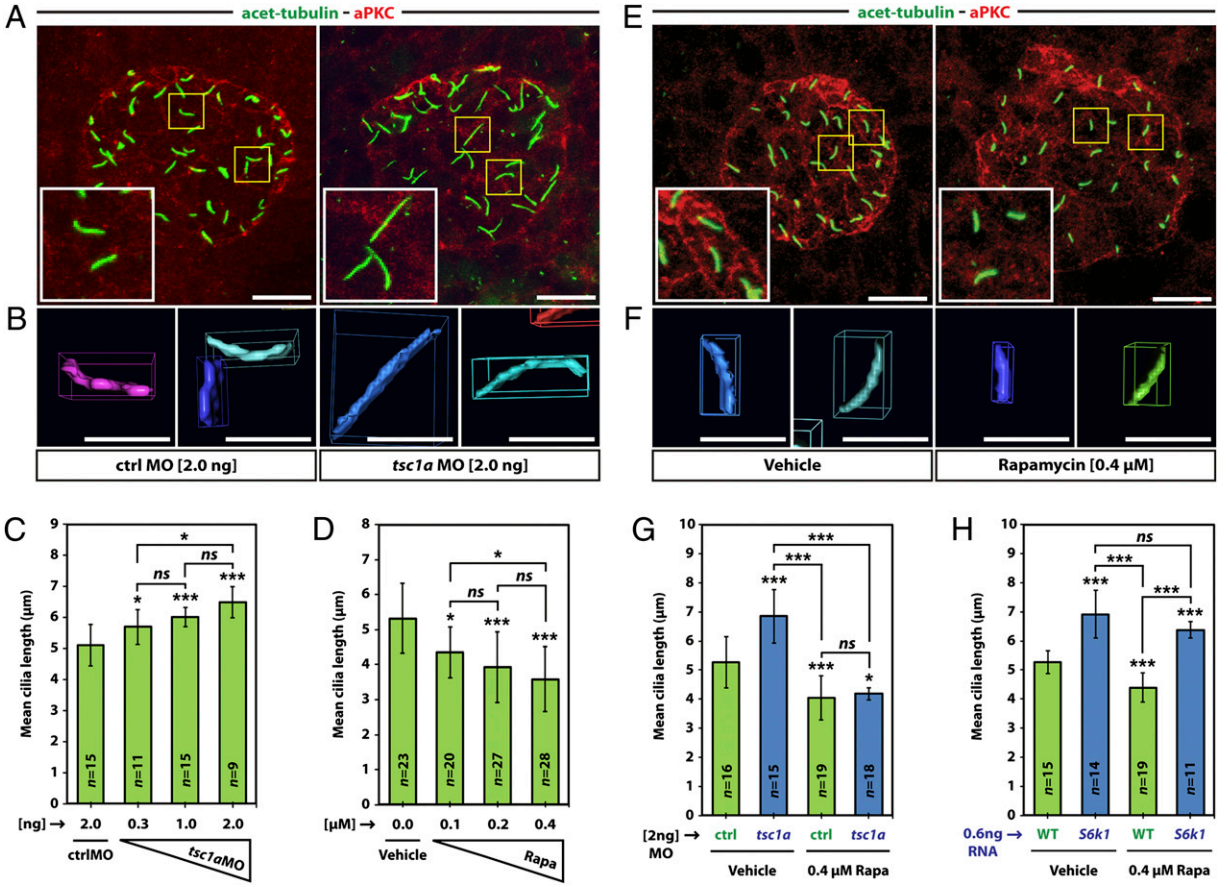

Fig. 1. Torc1 signaling modulates cilia length. (*A* and *E*) Representative confocal images of the KV from *tsc1a* MO and rapamycin-treated embryos at the six-somite stage stained with antibodies against acetylated α-tubulin (green, cilia) and aPKC (red, apical KV membrane). White-border box is a magnification from the background image. Yellow-border boxes indicate cilia that are rendered in *B* and *F*. (Scale bars, 10 μm.) (*B* and *F*) Three-dimensional volumetric rendering of individual KV cilia. (Scale bars, 5 μm.) (*C*) *tsc1a* morphants displayed significantly longer KV cilia (2.0 ng MO: $n = 9$ embryos, 385 total cilia), in a dose-responsive manner, relative to control morphants ($n = 15$ embryos, 585 total cilia, $P < 0.001$). (*D*) Embryos pretreated with rapamycin (0.4 μM rapamycin: $n = 28$ embryos, 1211 total cilia) had shorter cilia, in a dose-responsive manner, in comparison with vehicle-treated control embryos ($n = 23$ embryos, 1,035 total cilia, $P < 0.001$). (*G*) Rapamycin treatment shortened cilia length in *tsc1a* morphants ($n = 18$, 748 total cilia) compared with vehicle-treated *tsc1a* morphants ($n = 15$ embryos, 556 total cilia, $P < 0.001$) and control morphants ($n = 16$, 589 total cilia, $P = 0.024$). There was no significant difference in cilia length between rapamycin-treated *tsc1a* and rapamycin-treated control morphants ($n = 19$ embryos, 689 total cilia, $P = 0.974$). (*H*) Vehicle-treated *S6k1* OE displayed significantly elongated cilia ($n = 14$ embryos, 707 total cilia) in the KV relative to vehicle-treated wild-type embryos ($n = 15$ embryos, 721 total cilia, $P < 0.001$). Rapamycin treatment of *S6k1* OE did not significantly alter cilia length ($n = 11$ embryos, 418 total cilia, $P = 0.354$) compared with vehicle-treated *S6k1* OE. Cilia length was increased in rapamycin-treated *S6k1* OE compared with rapamycin-treated wild-type embryos ($n = 19$ embryos, 836 total cilia, $P < 0.001$). Asterisks above brackets denote ANOVA comparisons between samples indicated by the bracket, and asterisks above a single sample denote ANOVA comparisons between that sample and the control. $*P < 0.05$, $***P < 0.0005$. Data in *C*, *D*, *G*, and *H* are mean cilia length of embryos ± SD.

chimeric DFC$^{tsc1a\ MO}$ morphants in which *tsc1a* is knocked down solely in the dorsal forerunner cells (DFC, KV precursor cells) and the yolk (17). Similar to embryos with global knockdown of *tsc1a*, DFC$^{tsc1a\ MO}$ morphants exhibited long cilia in a dose-responsive manner compared with DFC$^{control\ MO}$ embryos (Fig. S2 and Table S2). In addition to DFC$^{control\ MO}$ morphants, chimeric yolk$^{tsc1a\ MO}$ embryos with yolk-specific knockdown of *tsc1a* served as an additional negative control and displayed no alterations in cilia length. These results reveal that cell-autonomous TSC signaling in KV precursor cells is crucial for the proper establishment of cilia length.

Tsc1a Modulates Cilia Length Through Torc1 Signaling. To define a mechanism by which the Tsc complex controls ciliary size, we explored whether Tsc1a could be acting through Torc1, a known downstream target (3). Wild-type embryos treated with 0.4 μM rapamycin, a widely-used inhibitor of Torc1, displayed significantly shortened cilia in the KV (Fig. 1 *D*–*F*). The number of cilia and the size of the KV in rapamycin-treated embryos were not significantly altered (Table S3). To confirm that Torc1 is downstream of Tsc1a in cilia length regulation, we performed an epistatic analysis. Knockdown of *tsc1a* in embryos followed by treatment with rapamycin resulted in short KV cilia in comparison with *tsc1a* and control morphants treated with vehicle alone (Fig. 1*G* and Table S4). Importantly, KV cilia of rapamycin-treated *tsc1a* morphants were of similar length to rapamycin-treated control morphants, indicating that Torc1 is indeed epistatic to Tsc1a for cilia length control.

Torc1 Regulates S6k1 Activity to Influence Cilia Length. TORC1 influences cellular growth by phosphorylating ribosomal protein S6 kinase 1 (S6K1), a serine/threonine kinase that regulates S6 ribosomal protein and other members of the translational machinery (3, 16). To assess whether S6k1 is required for Torc1-mediated cilia length control, we overactivated S6k1 by mRNA overexpression and analyzed for ciliary length defects. Strikingly, embryos overexpressing *S6k1* displayed abnormally long cilia in the KV (Fig. 1*H* and Fig. S3 *A* and *B*). KV size and cilia number were not significantly changed in *S6k1* overexpressants (OE; embryos microinjected with and overexpressing mRNA) compared

with controls (Table S5). Interestingly, *S6k1* OE also displayed long cilia and expanded lumens in the pronephric duct (Fig. S3 *D–I* and Tables S6 and S7), a phenotype commonly associated with ciliary defects (3, 11). Similar to embryos with global overexpression of *S6k1*, chimeric DFC$^{S6k1\ OE}$ embryos produced longer cilia in a dose-responsive manner (Fig. S3*C* and Table S8). Chimeric yolk$^{S6k1\ OE}$ embryos with yolk-specific overexpression of *S6k1* served as an additional negative control and displayed no alterations in cilia length. Thus, the regulation of cilia length by *S6k1* behaves in a cell-autonomous manner in DFCs. To determine whether the effect of S6k1 on cilia regulation is downstream of the TSC-TOR pathway, we performed an epistatic analysis between S6k1 and Torc1. Overexpression of *S6k1* followed by rapamycin-treatment did not significantly alter the long cilia phenotype compared with *S6k1* OE treated with vehicle alone (Fig. 1*H* and Table S5), suggesting that S6k1 acts downstream of Torc1 to regulate cilia length.

In accordance with these findings, immunoblots of *tsc1a* morphants and *S6k1* OE showed significantly increased levels of S6k1 phosphorylation at Thr389 compared with control samples (Fig. S4 *A* and *B*). Additionally, immunoblot analysis of embryos treated with rapamycin showed significantly decreased levels of phosphorylated S6k1 (Fig. S4*C*).

Gsk3b-Mediated Cilia Elongation Acts Through Torc1 Signaling. Inhibition of glycogen synthase kinase 3β (GSK3B) by lithium treatment induces a rapid and reversible long cilia phenotype in the unicellular green alga *C. reinhardtii* (19). Consistently, overexpression of *GSK3B*^{K85A}, a dominant-negative mutant of GSK3B (20), yielded a long cilia phenotype in zebrafish (Figs. S3 *D–I*, and S4 *E–H* and Tables S6, S7, S9, and S10). Because S6K1 is capable of directly inhibiting GSK3B through phosphorylation of Ser9 (21), TORC1 could regulate cilia length through GSK3B. Alternatively, GSK3B may regulate cilia length through TORC1 signaling, as GSK3B is known to repress TORC1 by directly activating TSC2 (22). In support of the latter mechanism, immunoblots from embryos overexpressing *GSK3B*^{K85A} demonstrated elevated levels of activated S6k1 (Fig. S4*D*), but the level of Gsk3b phosphorylation at Ser9 was not altered by disruptions in Torc1 signaling caused by either rapamycin treatment or *S6k1* overexpression (Fig. S4 *B* and *C*). Furthermore, overexpression of *GSK3B*^{K85A} followed by rapamycin treatment resulted in significantly shortened cilia compared with vehicle-treated *GSK3B*^{K85A} OE and wild-type controls (Fig. S4*I* and Table S11). Importantly, cilia of rapamycin-treated *GSK3B*^{K85A} OE were of similar length to rapamycin-treated wild-type embryos, indicating that Torc1 is indeed epistatic to Gsk3b in regard to cilia length

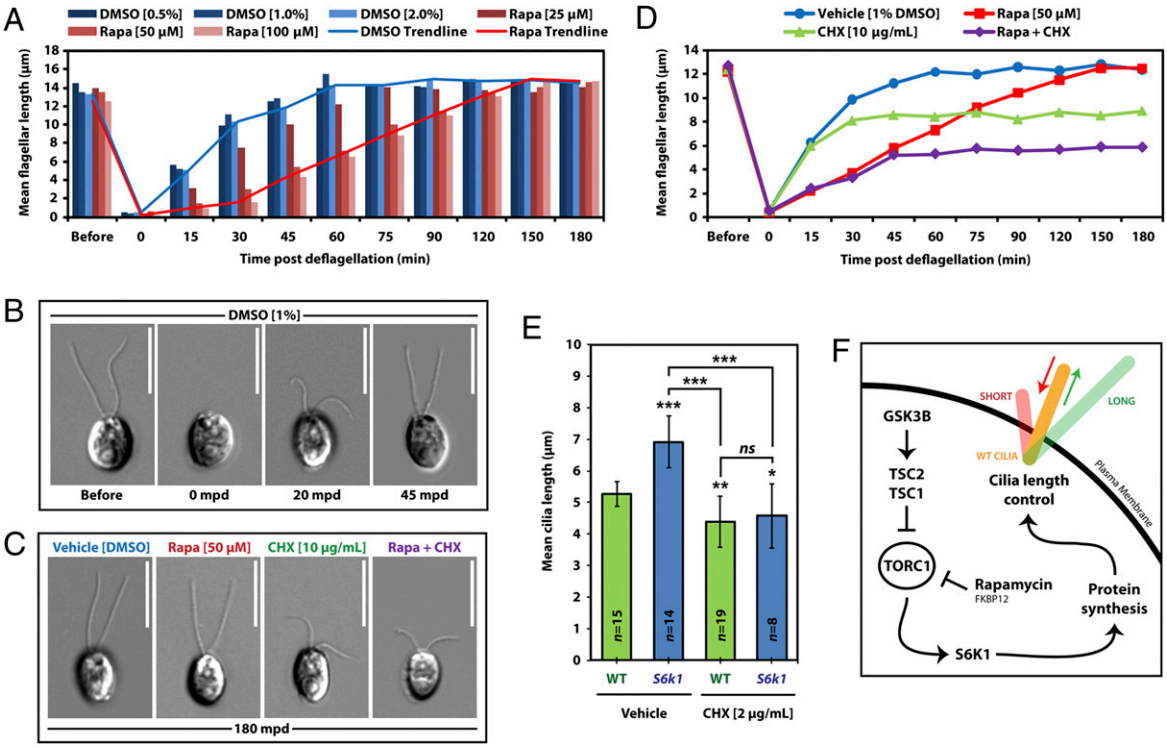

Fig. 2. Cilia length control by Torc1 is conserved and requires protein synthesis. (*A*) Rapamycin treatment of wild-type *cc-125 C. reinhardtii* cells delayed assembly of full-length flagella, in a dose-responsive manner, following deflagellation. $n = 50$ cells per condition and timepoint. Trendlines represent fitted curves for 2.0% DMSO and 100 μM rapamycin, respectively. (*B*) Representative DIC images of flagellar regeneration in vehicle-treated wild-type cells before and after deflagellation. (Scale bars, 10 μm.) (*C*) Representative DIC images of flagellar length recovery in vehicle-only, rapamycin-only (rapa), cycloheximide-only (CHX), or rapamycin- and CHX-treated cells at 180 mpd. (Scale bars, 10 μm.) (*D*) The shortened steady-state flagellar length induced by CHX treatment is exacerbated by rapamycin treatment. CHX-only treated cells showed a truncated steady-state length (8.2 ± 0.6 μm at 180 mpd) compared with vehicle-treated control cells (12.5 ± 0.3 μm at 180 mpd, $P = 0.006$). Rapamycin-only treatment severely delayed overall assembly kinetics but did not affect the final steady-state length (12.2 ± 0.6 μm at 180 mpd, $P = 0.359$). Rapamycin and CHX-treated cells produced a delayed recovery and maintained a severely truncated steady-state length (5.8 ± 0.1 μm at 180 mpd) compared with vehicle alone ($P < 0.001$), rapamycin-only ($P = 0.002$), and CHX-only treated samples ($P = 0.003$). $n = 50$ cells per condition and timepoint. Data represents mean flagellar length of cells ± SD. (*E*) KV cilia length (shown as mean cilia length of embryos ± SD) was shortened in CHX-treated *S6k1* OE ($n = 8$ embryos, 343 total cilia) compared with vehicle-treated *S6k1* OE ($n = 14$ embryos, 711 total cilia, $P < 0.001$) and wild-type embryos ($n = 15$, 738 total cilia, $P = 0.017$). There was no significant difference in cilia length in CHX-treated *S6k1* OE and CHX-treated wild-type embryos ($n = 19$ embryos, 777 total cilia, $P = 0.935$). Asterisks above brackets denote ANOVA comparisons between samples indicated by the bracket, and asterisks above a single sample denote ANOVA comparisons between that sample and the control. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$. (*F*) Activation of Torc1 signaling results in elongated cilia, and inhibition of Torc1 results in shortened cilia. Additionally, Gsk3b modulates cilia length through Torc1 signaling. Protein synthesis may represent a mechanism by which the TOR pathway regulates cilia length.

changes. Combined, these findings implicate Torc1 signaling as a mechanism for Gsk3b-mediated cilia elongation and further support the role of the TOR pathway in cilia length control (Fig. 2*F*).

Torc1 Signaling Regulates Cilia Assembly by Control of Protein Synthesis and This Mechanism Is Evolutionarily Conserved. A hallmark function of TORC1 signaling through S6K1 is to promote mRNA translation, raising the possibility that modulations in protein synthesis rates may represent a mechanism by which TORC1 controls cilia length. As numerous components of the TORC1 pathway are conserved in *C. reinhardtii* (23, 24), we adapted a flagellar regeneration assay to address the role of protein synthesis in cilia assembly. Amputation of flagella (deflagellation) results in the immediate assembly of new, full-length flagella following a deceleratory elongation kinetic (25). The addition of cycloheximide, a inhibitor of protein synthesis, at a concentration of 10 μg/mL during amputation immediately blocks new protein synthesis and results in the assembly of approximately half-length flagella, thus demonstrating that full-length assembly of flagella requires the incorporation of ciliary precursors from two sources: a preexisting flagellar precursor pool that is synthesized before amputation and precursors that are newly synthesized after amputation (Fig. S5*B*) (25). As TORC1 inhibition results in decreased translation, we treated algae cells with rapamycin and examined for flagellar assembly defects. When *cc-125* wild-type cells were deflagellated, rapid reassembly of flagella to wild-type lengths occurred within 60 min postdeflagellation (mpd) (Fig. 2 *A* and *B*). However, cells subjected to rapamycin treatment 24 h before amputation showed a significantly delayed and linear-like recovery by taking ~90–180 mpd, in a dose-responsive manner, to assemble full-length flagella. The slow reassembly of flagella in rapamycin-treated cells, rather than shortened flagella, may indicate that rapamycin treatment diminishes both the preexisting precursor pool and the synthesis of new precursors after amputation, although only partially. A plausible explanation for this incomplete blockage is the decreased potency of rapamycin in *C. reinhardtii* (23). Alternatively, rapamycin treatment is known to increase autophagy in the green alga (23, 24), which may be able to compensate for the inhibition of translation.

We further reasoned that by blocking new protein synthesis completely, cycloheximide treatment would reveal the specific impact of rapamycin treatment on the preexisting precursor pool. Consistent with previous findings, cycloheximide-only treatment of *cc-125* wild-type cells resulted in a mean flagellar length of 8.2 ± 0.6 μm after 180 mpd, and rapamycin- or vehicle-treated control cells recovered to a mean steady-state length of 12.2 ± 0.6 μm or 12.5 ± 0.3 μm, respectively (Fig. 2 *C* and *D*). Strikingly, cells treated with both rapamycin and cycloheximide displayed a substantially shortened mean flagellar length of 5.8 ± 0.1 μm after 180 mpd, representing a statistically significant decrease compared with cells treated with cycloheximide alone. This further shortened flagellar length indicates that rapamycin diminished the preexisting pool of precursors. Combined with the delayed but eventual recovery of full-length flagella in rapamycin-only treated cells, these data suggest that the inhibition of TORC1 by

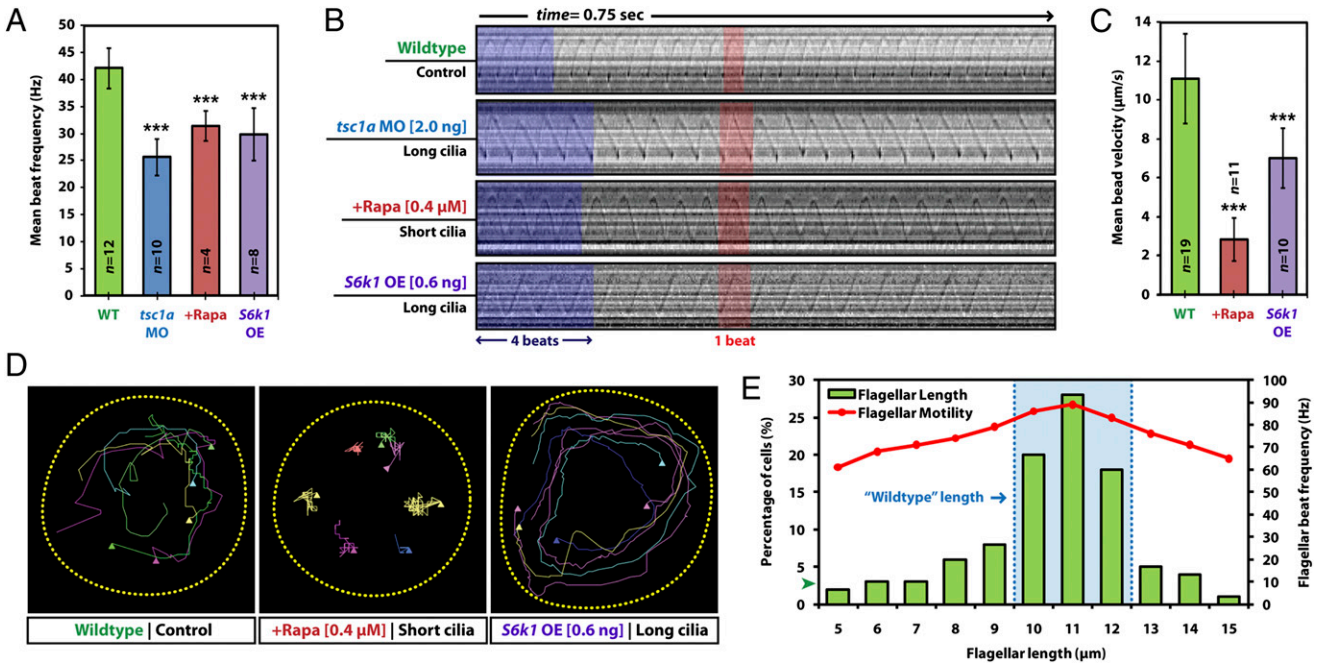

Fig. 3. Aberrant Torc1 signaling reveals a cilia length and function correlation. (*A*) Cilia from *tsc1a* MO (n = 10 embryos, 153 total cilia, $P < 0.001$), rapamycin-treated (n = 4 embryos, 69 total cilia, $P < 0.001$) and *S6k1* OE (n = 8 embryos, 107 total cilia, $P < 0.001$) embryos displayed significantly lower beat frequencies compared with wild-type embryos (n = 12 embryos, 177 total cilia). ***$P < 0.0005$. (*B*) Kymograph analysis of individual beating KV cilia from high-speed video recordings. Total time-lapsed is 1 s. Blue and red boxes highlight four and one complete beat cycles, respectively, of each cilium shown. (*C*) Rapamycin-treated (n = 11 embryos, 138 beads total, $P < 0.001$) and *S6k1* OE (n = 10 embryos, 121 beads total, $P < 0.001$) embryos showed significantly slower fluid-flow velocities in the KV lumen compared with wild-type embryos (n = 19 embryos, 297 beads total). ***$P < 0.0005$. (*D*) Directional KV fluid-flow was absent in rapamycin-treated embryos (n = 11 embryos, 138 beads total), but *S6k1* OE (n = 10 embryos, 121 beads total), and wild-type embryos (n = 19 embryos, 297 beads total) displayed a counter-clockwise flow. Triangles indicate the start of an individual bead trajectory. Dotted lines indicate the boundary of the KV as determined by DIC image overlays. (*E*) Flagellar length and beat frequency distribution of individual *cc-125 C. reinhardtii* cells in a synchronous wild-type population. Sixty-six of 100 analyzed cells showed a flagellar length of 10–12 μm (defined as "wild-type," highlighted by blue shade), with an average beat frequency of 86.1 ± 7.8 Hz. Arrowhead indicates percentage of nonciliated cells. Data represent three independent experiments. Asterisks above a single sample denote ANOVA comparisons between that sample and the control. Data in *A* is mean cilia beat frequency of embryos ± SD, and *C* is mean bead velocity of embryos ± SD.

rapamycin inhibits flagellar assembly by diminishing both sources of flagellar precursors.

To verify that protein synthesis is also crucial for cilia length and assembly regulation by Torc1 in the zebrafish KV, we treated *S6k1* OE or wild-type embryos with cycloheximide and examined for ciliary length defects. Wild-type embryos treated with cycloheximide exhibited short KV cilia in comparison with vehicle-treated *S6k1* OE and wild-type embryos (Fig. 2*E*, Fig. S5 *D* and *E*, and Table S12). Importantly, *S6k1* OE treated with cycloheximide displayed short cilia that were not significantly different compared with cycloheximide-treated wild-type embryos. Thus, protein synthesis is essential for cilia assembly and length regulation in diverse ciliated organisms. Collectively, these findings support a conserved mechanism by which TORC1 signaling modulates cilia length via translational control of ciliary precursors (Fig. 2*F*).

Disruption of Torc1 Signaling and Cilia Length Result in Abnormal Cilia Motility. To gain understanding of the relationship between cilia length and function, we first interrogated whether alterations in cilia length by the Torc1 pathway affect cilia motility in the zebrafish KV. As motile cilia may beat up to ~80 cycles per second (26), we used ultra high-speed videomicroscopy to image cilia in vivo at 1,000–2,000 frames per second (fps) with large resolutions using differential interference contrast (DIC) techniques. In wild-type control embryos, motile cilia beat in a circular motion at a mean frequency of 42.1 ± 3.8 Hz (Fig. 3 *A* and *B* and Movie S1). Surprisingly, the abnormally long motile cilia of *tsc1a* morphants also beat in a circular motion but at a significantly slower mean frequency of 25.6 ± 3.4 Hz (Movie S2). Similarly, the abnormally long cilia of *S6k1* OE also exhibited a drastically slower mean beat frequency (29.8 ± 4.9 Hz) (Movie S3). Interestingly, short motile cilia of rapamycin-treated embryos beat at a moderately slower beat frequency (31.4 ± 2.7 Hz) (Movie S4). Thus, abnormally long and short cilia that result from aberrant Torc1 signaling display cilia motility defects compared with wild-type cilia. Consistently, in *C. reinhardtii* cells, we also observed correlations between flagellar length and swimming speed during flagellar regeneration, regardless whether the cells are treated with vehicle alone, rapamycin, cycloheximide, or rapamycin together with cycloheximide (Fig. S5 *A* and *C*).

To analyze for a potential association between flagellar length and motility under less perturbed conditions, we measured flagellar length and motility of individual *C. reinhardtii* cells in a synchronous wild-type population. Wild-type *cc-125* cells synchronized by alternating light and dark cycles under minimal medium, whereby growth occurs primarily during the light phase and division during the dark phase (27), were recorded at 2,000 fps during the G1 growth phase of the light cycle. A flagellar length vs. beat-frequency histogram of the population indicates a Gaussian-like distribution of length, with the center of the peak between 10 and 12 μm (defined as the mean wild-type length) (Fig. 3*E*). Interestingly, cells within the mean wild-type length range displayed the maximal beat frequency of 86.1 ± 7.8 Hz (Movie S5). As cells deviated from the wild-type length range, flagellar motility decreased accordingly. Together, these experiments suggest the existence of an integral and conserved association between cilia length and motility that is in part regulated by the Torc1 pathway in zebrafish.

Torc1 Signaling Is Essential for Cilia-Directed Fluid Flow. Motile cilia of the KV are believed to drive a directional fluid flow that is crucial for the breaking of early bilateral symmetry (11, 17, 18). To assess whether the regulation of cilia size and motility by the Torc1 pathway can disrupt directional fluid flow in the KV, fluorescent beads were microinjected into the KV lumen and tracked by fluorescent videomicroscopy (11, 18). Wild-type embryos displayed a persistent counter-clockwise bead trajectory path with a mean velocity of 11.1 ± 2.3 μm/s (Fig. 3 *C* and *D* and Movie S6). Strikingly, *S6k1* OE also exhibited a circular, counter-clockwise bead motion but with a slower mean velocity of 7.0 ± 1.5 μm/s (Movie S7). In contrast, rapamycin-treated embryos displayed a stagnant trajectory that lacked persistent directional movement with a mean bead velocity of 2.8 ± 1.1 μm/s (Movie S8). These findings allude to the importance of appropriate cilia length for proper function of the organelle; cilia that are too long or short result in decreased motility and disruption of fluid flow.

Torc1 Signaling Is Crucial for the Establishment of Left-Right Asymmetry. Cilia defects are commonly associated with aberrant left-right body axis development in vertebrates (11, 17, 18). In zebrafish, *tsc1a* is required during early development for proper establishment of bilateral asymmetry (3), raising the possibility that Tsc1a acts through Torc1 signaling to influence left-right patterning by modulating ciliary size and function in the KV. To investigate this and the biological consequence of abnormal cilia length, we analyzed the left-right asymmetry of zebrafish embryos with disrupted Torc1 signaling. Although vehicle-treated embryos displayed normal left-sided expression of *cardiac myosin light chain 2* (*cmlc2*), a heart marker (28), embryos treated with rapamycin exhibited a significant increase in centrally-shifted *cmlc2* expression (Fig. S6 *A* and *B*). Similarly, *S6k1* and *GSK3B*^{K85A} OE embryos exhibited a significant increase in centrally shifted and right-sided expression of *cmlc2* (Fig. S6*C*). Hence, Torc1 signaling is essential for left-right body patterning. We further analyzed the expression domain of *southpaw* (*spaw*), which is a homolog of mouse *Nodal* and is one of the earliest known laterality markers in zebrafish (29). In control embryos, *spaw* is expressed in the left lateral plate mesoderm (Fig. 4). In contrast, rapamycin-treated embryos displayed a significant increase in bilateral expression of *spaw* (Fig. 4 *A* and *B*). Similarly, *S6k1* and *GSK3B*^{K85A} OE produced a significant increase in bilateral *spaw* expression (Fig. 4*C*). Thus, the function of Torc1 during the development of left-right body laterality precedes the asymmetric expression of *spaw*, a feature often linked to ciliary functions in the KV (3, 11, 17, 18).

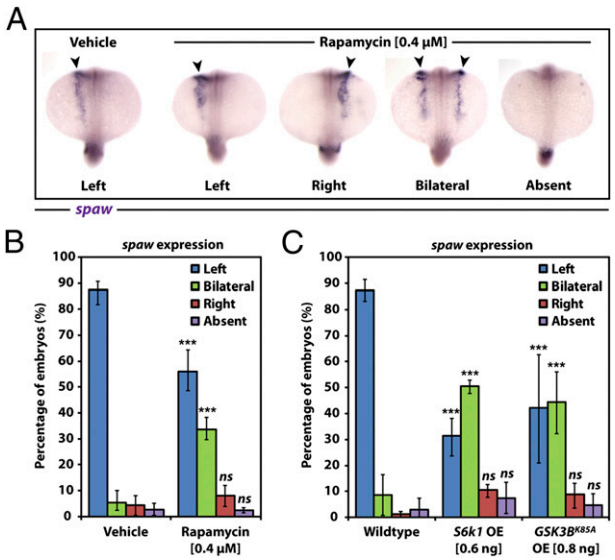

Fig. 4. Torc1 signaling is required for left-right body patterning. (*A*) Representative images of whole-mount *spaw* in situ expression in vehicle and rapamycin-treated embryos at 18–20 somite stage, viewed dorsally. Arrowheads point to the lateral plate mesoderm. (*B*) Rapamycin treatment produced a significantly greater number of embryos showing bilateral *spaw* expression ($n \geq 60$ embryos per treatment, $P < 0.001$). Data represent three independent experiments. (*C*) Overexpression of *S6k1* and *GSK3B*^{K85A} resulted in a significantly larger number of embryos showing bilateral *spaw* expression ($n \geq 75$ embryos per condition, $P < 0.001$). Data represent three independent experiments. Asterisks above a single sample denote ANOVA comparisons between that sample and the control. ***$P < 0.0005$. Data in *B* and *C* are mean embryo percentage ± SD.

Discussion

The TSC-TOR pathway has a well-established role in cell size control. Here, we report an additional role for this pathway in modulating cilia size and function (Fig. S7). We further show that this function of the TOR pathway is evolutionarily conserved and that protein synthesis plays an important role in this regulation. It is possible that a global up-regulation of proteins critical for ciliogenesis, such as intraflagellar transport components and tubulin (2), is responsible for cilia elongation in response to TOR pathway activation. Alternatively, TOR-mediated translation may regulate the abundance of cargos that are shuttled by intraflagellar transport into the cilium for assembly and maintenance. As the identity and function of such cargo proteins are largely unknown (2), it is challenging to test this hypothesis directly. Equally possible, the translation of a yet to be identified key regulator of cilia length control may trigger this response.

Given the central role of the TOR pathway in cellular responses to multiple environmental and intracellular signals, our results suggest that cilia length is dynamically regulated and may be responsive to multiple inputs. Furthermore, as diverse signaling cascades have thus far been associated with cilia-length defects, it is plausible that TOR represents one of many pathways in a complex network that senses and relays the need for moderating ciliary size and function.

Our results further illustrate the importance of cilia size for executing function. We showed that in the KV, cilia of wildtype length beat faster and are more efficient in generating directional flow than both abnormally long and short cilia. An alternative explanation for these phenotypes is that our genetic manipulations induce additional ciliary defects that are more directly responsible for the observed functional consequences. However, our studies show that in a wild-type population of synchronized *C. reinhardtii* cells grown from the same strain, the natural variation of cilia length also correlates with beating frequency, supporting the notion that cilia length is closely associated with cilia function. We therefore propose an intimate relationship between ciliary structure and function, whereby small changes in cilia size may result in profound functional and developmental disturbances. Although our study focuses on the impact of cilia length on cilia motility, it is tempting to speculate that the TOR pathway modulates cilia size as a method to regulate motility, sensation and signal transduction by cilia in response to diverse intra- and extracellular conditions, such as nutritional status and cellular growth.

Intriguingly, recent reports have indicated that the cilium may also regulate TORC1 signaling, as ciliary signaling can inhibit TORC1 activity (3, 30). Combined, these data suggest a potential reciprocal relationship between the cilium and the TOR pathway, whereby TOR relays multiple cues to regulate cilia length, which in turn enable the cilium to best perform its function: reporting extracellular information to the TOR signaling pathway, among others, to orchestrate cellular responses.

The dissection of a ciliary size-control network and the underlying mechanisms remain as challenging but crucial areas of study. A greater understanding of the regulation of ciliogenesis has become increasingly pivotal as ciliary defects continue to be linked to numerous disease pathologies and core biological processes. Given the implications of ciliary function to human disorders, we propose additional investigation into TOR-related diseases for ciliary biogenesis disruptions. Furthermore, because TSC-TOR signaling has been associated with disease etiologies, our findings suggest the existence of a previously unexplored subclass of ciliopathies based upon defects of cilia size.

Methods

All zebrafish methods and materials, such as husbandry, MO sequences, and antibodies, used in this study are listed in *SI Methods*. This includes a detailed description of embryo microinjection, DFC chimeric embryo production, immunohistochemistry, cilia length measurements, videomicroscopy, and in situ hybridization. Further, *C. reinhardtii* assays and statistical methods are also elaborated in *SI Methods*.

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