Novel roles for the radial spoke head protein 9 in neural and neurosensory cilia

Irina Sedykh1,2, Jessica J. TeSlaa1,2,3,4, Rose L. Tatarsky1,2, Abigail N. Keller1,2,4, Kimberly A. Troops4,5, Aparna Lakkaraju4,5, Molly K. Nyholm1,2,5, Marc A. Wolman1 & Yevgenya Grinblat1,2,5

Cilia are cell surface organelles with key roles in a range of cellular processes, including generation of fluid flow by motile cilia. The axonemes of motile cilia and immotile kinocilia contain 9 peripheral microtubule doublets, a central microtubule pair, and 9 connecting radial spokes. Aberrant radial spoke components RSPH1, 3, 4a and 9 have been linked with primary ciliary dyskinesia (PCD), a disorder characterized by ciliary dysmotility; yet, radial spoke functions remain unclear. Here we show that zebrafish Rsph9 is expressed in cells bearing motile cilia and kinocilia, and localizes to both 9 + 2 and 9 + 0 ciliary axonemes. Using CRISPR mutagenesis, we show that rsph9 is required for motility of presumptive 9 + 2 olfactory cilia and, unexpectedly, 9 + 0 neural cilia. rsph9 is also required for the structural integrity of 9 + 2 and 9 + 0 ciliary axonemes. rsph9 mutant larvae exhibit reduced initiation of the acoustic startle response consistent with hearing impairment, suggesting a novel role for Rsph9 in the kinocilia of the inner ear and/or lateral line neuromasts. These data identify novel roles for Rsph9 in 9 + 0 motile cilia and in sensory kinocilia, and establish a useful zebrafish PCD model.
respiratory infections, hearing impairment, infertility, situs inversus and hydrocephalus. To date, mutations in at least 31 genes have been identified as causative of PCD, accounting for about 70% of disease incidence. These genes encode cytoplasmic proteins important for ciliary assembly, as well as components of the dynein arms, the central microtubule pair, and radial spokes. Thus far, four proteins of the spoke head have been associated with PCD in humans: RSPH1, RSPH3, RSPH4A and RSPH9. Patients with spoke head mutations typically have ciliary transposition defects characterized by loss of the central pair and displacement of an outer microtubule doublet into the center of the axoneme. Airway cilia from these patients show partially penetrant deficits in beat frequency and waveforms, which range from normal (planar) to aberrant (rotational), suggesting that radial spokes are involved in setting the parameters of ciliary motility.

Due to their association with the central microtubule pair, radial spoke head protein function has been presumed to be restricted to +2 motile cilia. Support for this hypothesis was recently provided by Shinohara et al., who showed that mouse embryos with rsph4a mutations develop with aberrant airway cilia, but normal +2 motile cilia of the node. In this report, we use genetic and imaging tools to examine the role of the radial spoke head component Rsph9 in the +2 olfactory motile cilia, +0 neural motile cilia and in +2 immotile kinocilia.

Results
Radial spoke head component Rsph9 is expressed in motile ciliogenic cells in zebrafish embryos. In zebrafish, motile cilia are produced by Kupffer’s vesicle (KV, the zebrafish equivalent of the mammalian node), the pronephric ducts, the nasal and otic placodes, and ventral spinal cord. As in other vertebrates, most motile cilia have the canonical +2 axonemes with the exception of those in the spinal cord, where +0 cilia predominate. Since expression of radial spoke proteins has not been described in zebrafish, we isolated cDNA clones that encode Rsp9 and Rsp4a (see Materials and Methods) and determined their expression patterns using whole mount in situ hybridization (WISH). Rsp9 was expressed in the ciliated structures, namely, KV (Fig. 1A), pronephric ducts, otic placodes, and ventral spinal cord (Fig. 1B,C). Rsp4a was expressed in a similar pattern (Fig. 1D). Rsp9 was also expressed in the ventral midline of the midbrain primordium (Fig. 1C,E), as was foxj1a (Fig. 1F), a key transcriptional regulator of motile ciliogenesis.

We next asked if zebrafish Rsp9 protein localizes to the ciliary axonemes, using an antibody that recognizes a conserved epitope in Chlamydomonas reinhardtii and human RSP9 proteins (see Methods).
Figure 2. Rsph9 protein is enriched in cilia. Wildtype embryos were stained with an anti-Rsph9 antibody (magenta) and acetylated α-tubulin (yellow). (A) Pronephric duct. (B) Ventral spinal cord. (C) Ventral midbrain. (D) Olfactory pit. Nuclei are visualized with DAPI (cyan). All images are shown with anterior to the left. (A,B) Are lateral mounts (dorsal at the top).

Co-immunostaining with acetylated tubulin, which marks ciliary axonemes, revealed Rsph9 reactivity along the length of most or all motile ciliary axonemes, including cilia of the pronephric ducts, the spinal canal, the ventral midbrain and olfactory pits (Fig. 2A–D, Supp. Fig. 1).

Olfactory cilia require Rsph9 for normal motility. To ask if Rsph9 function is required for ciliary motility in zebrafish, we used CRISPR-Cas9 targeted mutagenesis to generate mutations in exon 2 of the Rsph9 locus (see Materials and Methods for details). Two mutant alleles were isolated that contain, respectively, an 8 bp deletion (rsph9208) or an indel with a net gain of 2 bp (rsph9212) at the CRISPR target site. Both mutations are predicted to result in a frame shift and a truncated protein (Fig. 3A; Supp. Fig. 2). Western blot analysis confirmed the presence of a 30 kD band recognized by Rsph9 antibody in +/+ and rsph9208/+ embryos, and the absence of this band in rsph9208/− rsph9208 siblings (Supp. Fig. 2). Homozygous mutant embryos also lack Rsph9 immunoreactivity in the olfactory cilia, further confirming absence of full-length Rsph9 (Fig. 3C,D).

The olfactory epithelium contains multiciliated cells with 9 + 2 motile cilia8,10 and is easily accessible for live imaging. Rsph9 homozygous zebrafish formed normal olfactory structures by day 4 dpf (Fig. 3C). Motility of the olfactory cilia was visualized by high-speed (240 fps) live imaging in a group of rsph9208 homozygous and heterozygous siblings, genotyped post-hoc (Supp. Movies OC1-4, Supp. Fig. 3). This analysis, summarized in Fig. 3D and Supp. Table 1, revealed robust, coordinated ciliary motility in all tested rsph9208/+ larvae (11 total) and greatly diminished motility in 8 out of 9 rsph9208/− rsph9208 larvae. These results are consistent with a functional requirement for zebrafish Rsph9 in 9 + 2 cilia with radial spokes.

Rsph9 is required for motility of 9 + 0 cilia in the ventral spinal cord. We next asked if Rsph9 is also required for the motility of ventral spinal cord cilia, previously reported to have 9 + 0 axonemes8. Rsph9208 homozygotes with fluorescently labeled spinal cord cilia were produced by crossing Tg(3-actin:Arl13b-GFP)22 into the rsph9208 mutant background. Embryos derived from a cross between rsph9208/+ rsph9208 males and rsph9208/+ females were selected for GFP fluorescence. Five embryos were imaged live using high-speed confocal microscopy, with multiple individual cilia scored for motility in each (see Methods, Supp. Movies SC1-5, and Supp. Table 2). Heterozygous siblings contained a mixed population of cilia that moved vigorously in a regular waveform (Fig. 4A), in an irregular pattern (Fig. 4B), or remained largely immotile, with a slight motion that we described as vibrational (Fig. 4C). In contrast, only the vibrational, largely non-motile cilia were observed in the spinal cords of homozygous mutants (Fig. 4D). Taken together with the previously reported 9 + 0 axoneme structure and ciliary localization of Rsph9 in the ventral spinal cord (Fig. 2), these findings indicate a novel role for Rsph9 in 9 + 2 motile cilia.

Rsph9 is required for structural integrity of 9 + 0 and 9 + 2 motile cilia. In PCD patients with RSPh mutations, respiratory epithelial cilia show characteristic ultrastructural defects, namely, loss of the central microtubules and transposition of one of the outer doublets to the center of the axoneme23,28–32. To determine if Rsph9 is similarly required for ciliary structure in zebrafish, we examined rsph9 mutant embryos using transmission electron microscopy (TEM).

We first asked if 9 + 2 cilia in the pronephric ducts form aberrantly in rsph9 homozygous zebras. These embryos develop without overt phenotypic deficits and are adult viable. Nonetheless, pronephric ducts of rsph9 homozygotes formed cilia with a variety of aberrant axonemes (7 aberrant out of 23 total, Fig. 5A). Next, we assayed ciliary structure in the ventral neural tube of rsph9 mutant embryos at 1 dpf. In the spinal cord, 7 out of 8 cilia had the expected 9 + 0 axonemes (Fig. 5B), while one had an aberrant 8 + 1 structure (Fig. 5C). In the anterior neural tube (ventral midbrain), rsph9 homozygotes also contained a mixture of normal 9 + 0 and aberrant 8 + 1 axonemes (2 aberrant out of 8 total; Fig. 5D). To corroborate specificity of this aspect of the mutant phenotype, we examined ciliary structures in embryos depleted of Rsph9 by antisense morpholino injection (Supp. Fig. 4).
Neural tube cilia in Rsph9 morphants presented with a mixture of normal $+0$ axonemes and aberrant $+1$ configurations (Supp. Fig. 5). Likewise, ~ half of the morphant pronephric duct axonemes were aberrant (Supp. Fig. 5). Since to date motile neural cilia have been characterized only in the posterior neural tube, i.e. spinal cord, we wished to confirm the presence of $+0$ cilia in the anterior neural regions during normal development. Cilia in the dorsal midline of the brain primordium (midbrain level) were uniformly short, as expected for non-motile primary cilia (Fig. 6A; Supp. Fig. 6). In contrast, cilia produced at the ventral midline, which expresses rsph9 and foxj1a, were substantially longer and continued to elongate in the course of development (Fig. 6B; Supp. Fig. 7). A range of axonemal structures was revealed by TEM analysis of ventral midbrain cilia in 1 dpf wildtype embryos, with many lacking the central microtubule pair (34 of 40; Fig. 6C,D). Outer dynein arms were visible in $+0$ cilia, consistent with putative motility (arrows in Fig. 6C,D). Pronephric ducts contained only the typical $+2$ axonemes (Fig. 6E,F), indicating that we were able to consistently visualize $+2$ axonemal structures. Collectively, these data strongly suggest that Rsph9 function contributes to the structural integrity of ciliary axonemes in both $+2$ and $+0$ cilia, and that structural ciliary defects in rsph9 mutants have functional consequences for $+0$ ciliary motility.

Behavioral assays identify a role for Rsph9 in acoustic sensory reception. rsph9 is expressed in the otic primordium, where it localizes to kinocilia (Supp. Fig. 8). Hair cells of rsph9 mutant larvae form kinocilia (Supp. Fig. 9) and contain functional mechanotransduction channels, indicated by the ability of hair cells to take up the vital dye FM1-43 (Supp. Fig. 9). To test function of hair cells that lack Rsph9, we measured the ability of rsph9 mutant larvae to perform a startle response to acoustic stimulation using a behavioral platform that provides rigorous quantitative assessment of initiation and execution of this highly stereotyped behavior\(^3,4,4\). Individual larvae derived from an rsph9$^{212}$/rsph9$^{212}$ incross were exposed to a series of 20 non-habituating acoustic stimuli of low or high intensity and then genotyped post-hoc. rsph9$^{212}$/rsph9$^{212}$ larvae showed reduced initiation of both the short latency and long latency startle responses to high-intensity stimuli (Fig. 7A,B and Supp. Table 3) as well as to low intensity stimuli (data not shown). A similar deficit was observed in rsph9$^{208}$/rsph9$^{208}$ larvae (Fig. 7A,B and Supp. Table 3). Importantly, homozygous mutant larvae that did initiate a startle response performed it normally (Fig. 7C,D), indicating that spinal motor components of the startle circuit are functional in.
rsph9 homozygotes. Moreover, reduced excitability of hindbrain interneurons, such as the Mauthner neuron, result in deficits in the short, but not the long-latency startle responses\textsuperscript{43}. Since the rsph9 homozygous mutants show reduced initiation of both the short and long-latency startle response, the behavioral impairment is likely due to a sensory impairment, rather than a defect in the startle circuitry. Collectively, these results argue that the behavioral deficit in rsph9 mutant larvae lies upstream of the motor circuits which coordinate startle response performance, and are likely attributable to acoustic sensory impairment.

Figure 4. Motile spinal cord cilia require Rsph9 function. (A–C) schematics illustrating the three observed motility patterns: regular (A) irregular (B) and vibrational (C). (D) Neural ciliary motility is impaired in homozygotes (n = 15/19 cilia, 2 embryos) vs. heterozygous siblings (n = 54/55 cilia, 3 embryos). Bars = standard deviation.

Figure 5. Ultrastructural defects in rsph9 mutant cilia. (A–C): Representative TEM images of cilia from rsph9\textsuperscript{208}/rsph9\textsuperscript{208} embryos at 1 dpf. Both the normal 9 + 2 and aberrant 8 + 2 axonemes are present in the pronephric ducts (A). Normal 9 + 0 (B) and aberrant 8 + 1 (C) cilia are found in the ventral spinal cord. (D) Summary of ciliary structures observed by TEM in rsph9\textsuperscript{208}/rsph9\textsuperscript{208} embryos surveyed at kidney level (5 embryos, 2 experiments), midbrain level (3 embryos, 1 experiment), and spinal cord level (4 embryos, 2 experiments). PD: pronephric duct; SC: ventral spinal cord.
RspH9, a structural component of radial spokes found in motile 9 + 2 cilia, is mutated in a subset of PCD patients. Here we take advantage of the accessibility of embryonic zebrafish to examine rshp9 function in 9 + 0 vs 9 + 2 motile cilia in vivo. Our data demonstrate that zebrafish Rshp9 protein localizes to both types of motile cilia, and that depletion or absence of Rshp9 causes structural defects in neural 9 + 0 and pronephric 9 + 2 cilia. Remarkably, these defects closely resemble those reported in 9 + 2 cilia on respiratory epithelia from PCD patients with RSPH9 mutations. While radial spokes are known to interact with the central microtubule pair in 9 + 2 cilia, their involvement in 9 + 0 motile cilia was unexpected. A plausible explanation consistent with our data is that, even in the absence of central microtubules, motile cilia contain a modified central sheath complex that interacts with radial spokes. As in 9 + 2 cilia, we hypothesize that this interaction is necessary for keeping...
the outer microtubules properly aligned, and for the correct motility of 9 + 0 cilia. Testing this hypothesis, diagrammed in Fig. 8, will require identification of additional central complex components and determining their functions in motile ciliary subtypes in accessible model organisms.

It is possible that the absence of the central pair in neural cilia is an artifact of tissue processing that fails to preserve this structure. In this case, the electron-dense center visible in many 9 + 0 axonemes may be a remnant of the central pair. This concern is partially alleviated by our ability to see perfectly preserved central microtubule pairs in pronephric duct cilia, as well as centrally located microtubule singlets in aberrant neural cilia. Nonetheless, the definitive test will require additional ultrastructural examination of 9 + 0 cilia with more sensitive methods, e.g. the recently developed cryo-electron tomography.45

Figure 7. Initiation of acoustic startle response is impaired in Rsph9 mutants. (A) Initiation of the short-latency curve (SLC) maneuver in response to high intensity stimuli is reduced in rsph9208/rsph9208 and in rsph9212/rsph9212 larvae compared to homozygous wild type and heterozygous siblings. (B) Initiation of the long latency C-bend (LLC) escape maneuver is reduced rsph9208/rsph9208 and in rsph9212/rsph9212 larvae compared to siblings. (C) Head turn angle during ASR to high-stimulus is not dependent on the rsph9 genotype. (D) Turn duration (in milliseconds) during ASR to high-stimulus is not dependent on Rsph9 genotype. Error = SEM. N larvae at bottom of bar. *p < 0.001 ANOVA vs wildtype siblings.

Figure 8. Schematic comparison between motile 9 + 2, motile 9 + 0 and primary (immotile) 9 + 0 ciliary structures. The model diagrammed here suggests a close structural relationship between motile 9 + 0 cilia and motile 9 + 2 cilia. Specifically, 9 + 0 motile cilia are proposed to contain radial spokes that interact with a putative complex of proteins in the center. This complex may be related to the protein sheath that surrounds the central microtubule pair in 9 + 2 cilia.
Presumptive motile cilia are present on the apical surface of the neuroepithelium even before the lumen opens and begins to fill with cerebrospinal fluid (CSF) at ~24 hpf, and well before the CSF–producing choroid plexus forms at 48 hpf. The physiological significance of motile cilia at this early stage is unclear. It is widely assumed that motile cilia move fluid through the neural lumen similar to the way they mediate directional fluid flow in the pronephric duct. However, 9 + 0 cilia in the embryonic neural tube do not appear to beat coordinately and are unlikely to generate long-range directional flow. Instead, they may be important for local re-distribution of CSF components, i.e. growth factors. Later in development, motile ciliary dysfunction is linked to hydrocephalus, a debilitating symptom of PCD that is caused by excess cerebrospinal fluid (CSF) in the brain lumen. Excess CSF can result from overproduction of CSF, impaired reabsorption of CSF, or blockage of one of the narrow foramina connecting the ventricles (frequently the cerebral aqueduct). The underlying mechanisms that lead to CSF accumulation in PCD patients are poorly understood. Dnah5 mouse models have dysmotile cilia and reduced CSF flow velocity, which is thought to cause occlusion of the cerebral aqueduct and fluid buildup57,68. In contrast, ciliary dysmotility in zebrafish rsph9 homozygotes and PCD patients with RSPH mutations is not associated with hydrocephaly and does not preclude normal development, likely due to the fact that Rsp9-deficient cilia retain some motility.

The bulk of ciliary structure-function studies have been performed in the alga _Chlamydomonas reinhardtii_, whose cilia are exclusively 9 + 2. Based on these studies, radial spokes are thought to transduce signals from the central pair to the outer doublets, where asymmetrical dynein activity is responsible for driving ciliary beating49,50. Radial spokes are also thought to be involved in controlling the waveform, which in 9 + 2 cilia is usually planar. There are exceptions to this rule, e.g. in the zebrafish Kupffer’s vesicle (KV), where both 9 + 2 and 9 + 0 cilia that beat with a rotational waveform have been described51–53. Proper motility of KV cilia is required for correct L/R asymmetry during zebrafish embryogenesis, yet rsph9212 and rsph2 heterozygotes do not exhibit L/R deficits (data not shown). This observation suggests that Rsp9 function is not absolutely required for KV cilia motility, but does not rule out a role for Rsp9 in modulating motility. How radial spokes contribute to controlling motility in KV cilia, particularly to the choice of waveform, is an important outstanding question, and the zebrafish rsph9 mutants will be instrumental in arriving at the answer.

Respiratory dysfunction, which includes neonatal respiratory distress and severe sinusitis, is the most prominent clinical presentation in patients with Rsp9-linked PCD. Nasal biopsy scrapings are commonly used as an accessible proxy for respiratory passage lining to assess ciliary motility in PCD patients54. While zebrafish lack an airway epithelium, their non-sensory olfactory epithelium is also multi-ciliated and bears 9 + 2 motile cilia that beat in a coordinated fashion, creating directional flow of the mucus that leads to odorant exchange between the olfactory pit and the environment50,51,57. Zebrafish olfactory epithelium is superficially located, accessible to high-resolution live imaging in intact animals, and eminently suitable for use in high-throughput genetic and small molecule screens.

Zebrafish process high-frequency auditory input that activates acoustic startle response primarily through the sacculae of the inner ear; importantly, this machinery is already in place by 5 dpf, when our behavioral assays were conducted57. Acoustic response deficits in larvae that lack full-length Rsp9 are consistent with a requirement for radial spokes in the inner ear, specifically in the immotile 9 + 2 kinocilia produced by the hair cells. PCD patients with radial spoke defects suffer from hearing deficits thought to be caused by impaired mucosal clearance in the middle ear57; consequently, the potential role for radial spokes in kinocilia of the inner ear has not been addressed.

Kinocilia play essential, conserved roles in organizing stereociliary bundle formation58–60. Kinocilia also have direct mechanosensory functions in some contexts, e.g. the nascent hair cells in the zebrafish lateral line. The zebrafish inner ear contains an additional ciliary subtype with a 9 + 2 axoneme: motile cilia that form adjacent to kinocilia at the poles of the otic placode. These cilia play an auxiliary role in otolith biogenesis61–63. rsph9 mutants develop with normal otolith numbers, position and morphology (data not shown), implying that Rsp9 is not required for motility of otic cilia. Together with the apparently normal otolith morphology, acoustic deficits in rsph9 mutant larvae point to a potential novel function for Rsp9 in hair cell kinocilia. The robust startle response defect in rsph9 mutant larvae, combined with our ability to perform high throughput, behavior-based small molecule screens in zebrafish64, constitute a powerful tool for discovery of drugs that attenuate sensory impairment in an in vivo PCD model.

There is still much to understand about how radial spokes regulate ciliary motility during vertebrate embryogenesis, particularly in the developing neuroepithelium, where 9 + 0 motile cilia predominate. The rsph9 mutant zebrafish described here represent a novel tool for dissecting the mechanisms of radial spoke head function in a range of ciliary subtypes. This mutant line is also ideally suited for testing therapeutic approaches to alleviate mechanosensory and motility deficits that contribute to the severity of PCD, e.g. small molecule screening or mRNA therapeutics, an active area of research in respiratory disease treatment64.

**Materials and Methods**

**Zebrafish strains and embryo manipulation.** Adult zebrafish were maintained according to established methods65. All experimental protocols using zebrafish were approved by the University of Wisconsin Animal Care and Use Committee, and carried out in accordance with the institutional animal care protocols. Embryos were obtained from natural matings and staged according to ref. 66. Transgenic Tg(3-actin:ArL13b-GFP) zebrafish, kindly provided by Brian Ciruna, were used to generate embryos with fluorescently labeled cilia62. Gene-specific antisense oligonucleotide morpholinos were purchased from GeneTools (Philomath, OR) and included Rsp9 translation-blocking morpholino (rsph9MO, Castleman et al., 2009), p53 MO and standard control MO (conMO). 1–2 nl of Rsp9 or control MOs (2–4 ng/ml) were injected singly or in combination with p53 MO (4–6 ng/ml) in HEPES/KCl buffer67 into 1–2 cell stage embryos. For testing hair cell mechanotransduction, larvae were incubated in E3 with 3 uM FM1-43 for 45 seconds, rinsed twice for 5 minutes and imaged using a Leica stereoscope.
Immunohistochemistry, histology and in situ hybridization (ISH). Embryos for immunohistochemistry were fixed in 4% paraformaldehyde in PBS, subjected to antigen retrieval according to ref. 68 except that TritonX-100/goat serum were used in place of Tween/sheep serum. For Western analysis, individual embryos were genotyped from larval tailclips and protein lysates were extracted at 5 dpf according to ref. 69. The following antibodies were used: rabbit anti-Rsp9 (1:200 for IHC, 1:250 or 1:430 for Western blot, Sigma HPA031703), mouse anti-b-actin (1:5,000, Sigma A1978), mouse anti-acetylated tubulin (1:400, Sigma T6793), anti-gamma tubulin (1:1000, Sigma), mouse anti-GFP (1:500, Chemicon), rabbit anti-GFP (Life Technologies), and anti-trRFP (1:500, Evrogen). For IHC, primary antibodies were detected fluorescently with Alexa-labeled goat anti-mouse or goat anti-rabbit secondary antibodies (1:1000, Molecular Probes). For Western analysis, goat anti-rabbit secondary HRP conjugated antibodies were used (1:2,500, Promega W401B) and goat anti-mouse HRP (1:10,000, ThermoScientific 31430). Nuclei were counterstained with DAPI (Invitrogen). Embryos were mounted in VectaShield and imaged on an Olympus IX81 inverted confocal microscope with the Fluoview 1000 confocal package, using a 60x water immersion objective (NA 1.10) or 60x oil immersion objective (NA 1.35).

Transmission electron microscopy. Yolk cells of segmentation-stage embryos were injected with 6–8 nl of 40 mM AMP-PNP and yolk was expelled several minutes later using tweezers and a fine-gauge needle (adapted from). Embryos were then transferred to a glass petri dish with glutaraldehyde fix in 200 mM phosphate buffer, dissected in two just behind the otic placode and processed for TEM according to Jaffe et al., 2010.

CRISPR mutagenesis and high-resolution melt analysis (HRMA). The target site in exon 2 of rsp9 was selected using the ZiFiT Targeter website (http://zifit.partners.org/) with the criteria: 5’GG(N)18NG3’.

Sequencing and PCR genotyping of rsp9 mutant alleles. PCR fragments identified as mutant by HRMA were subcloned via TA cloning into pGEMT-Easy (Promega) and sequenced to characterize the mutant alleles. Subsequently, to efficiently genotype individual embryos and adult fish, PCR (see above for primer sequence) followed by Metaphor gel electrophoresis were used to identify rsp9208 allele. A digest with MboII (NEB) was added before electrophoresis to identify rps9232 allele (see Supp. Fig. 2).

Imaging ciliary motility in the spinal cord. Live imaging of spinal cord cilia was performed on the Revolution XD spinning-disk microscopy system (Andor, Belfast, UK) equipped with the Yokogawa CSU-X1 confocal spinning disk head (Yokogawa Electric Company, Tokyo, JP); Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY); iXon x3 897 EM-CCD camera (Andor); Andor laser combiner with four solid-state lasers at 405, 488, 561, and 640 nm and corresponding band-pass filter sets (Sutter, Novato, CA); and ASI motorized stage with piezo-Z for rapid Z-stack acquisition (Applied Scientific Instrumentation, Eugene, OR). Images were acquired using either the 60x/1.4 NA Plan Apo VC or 100 x/1.49 NA Apo TIRF objectives (Nikon, Melville, NY) for 50–150 frames at ~20 frames per second. During image acquisition, the same laser power, exposure and electron-multiplying gain settings were kept for each animal. Andor IQ3 software (Andor) was used for image acquisition and Imaris × 64 v. 7.1 (Bitplane, Zurich, CH) for conversion to movies. Imaris software (Bitplane) was used for background subtraction (using the background-subtraction algorithm and identical automatic threshold for all images), smoothing (Gaussian algorithm with identical threshold settings for all images), and conversion of the images to movies. 20 fps is just above the reported beat frequency of spinal cord cilia, ~12 beats per second. It is insufficient for rigorous quantification but allows qualitative assessment of ciliary motility. Individual cilia were scored qualitatively by two of the authors independently and in a genotype-blind manner, i.e. before embryonic genotypes were determined.

Olfactory cilia imaging. For direct visualization of ciliary motility, 4 dpf embryos were mounted in 3% methylcellulose and imaged on the upright Nikon Eclipse E600 microscope with a 60x NA0.95 objective, using an Apple iPhone 6 in slo-mo mode (240fps), mounted with a iDu professional iPhone 6 microscope adapter with built-in 30 mm 10x WF lens. Ciliary movement in the nasal pits was recorded for ~10s without digital zoom, then
for ~10s with digital zoom. Movies (Supp. Movies OC1–19) were played back at 24 fps (0.1x of the original speed) and analyzed qualitatively for normal versus reduced ciliary motility, blind to genotype, i.e. prior to identifying homozygous mutants by PCR.

**Behavioral testing.** All behavioral testing was conducted in a custom-built, computerized programmable system consisting of (1) a grid, where 16 individually housed larvae are tested simultaneously; (2) a vibrational mini-shaker, which delivers vibrational/acoustic stimuli of defined strength (1000 Hz) to the grid; (3) a high-speed camera that records the behavioral responses at 1,000 frames/second, and (4) a computer with custom software, which allows customized testing regimes to be programmed. Acoustic stimuli produced by the system range from low, subthreshold ones that do not elicit a response to robust, above-threshold levels that evoke an explosive startle response. Optical stimuli are delivered by briefly turning off the light (“dark flash”). After recording behavioral responses, movement tracks of each individual larva are analyzed frame by frame, automatically reconstructed over time, and the kinematic parameters of the response are calculated to describe the behavior.

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Y. G. analyzed the data. M. W. oversaw behavioral assays and analyzed the resulting data. Y. G., J. J. T., M. K. N. and A. N. K. generated zebrafish.

The study was designed by M. K. N., J. J. T. and Y. G. I. S., J. J. T. and R. L. T. carried out the bulk of the experiments.

Author Contributions

The study was designed by M. K. N., J. J. T. and Y. G. I. S., J. J. T. and R. L. T. carried out the bulk of the experiments. A. N. K. generated zebrafish rshp9 mutants. Confocal live imaging was performed by K. A. T. and A. L. I. S., J. J. T. and Y. G. analyzed the data. M. W. oversaw behavioral assays and analyzed the resulting data. Y. G., J. J. T., M. K. N. and I. S. wrote the manuscript. All authors edited and approved the manuscript prior to submission.
