Calaxin is required for cilia-driven determination of vertebrate laterality

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Calaxin is a Ca^{2+}-binding dynein-associated protein that regulates flagellar and ciliary movement. In ascidians, calaxin plays essential roles in chemotaxis of sperm. However, nothing has been known for the function of calaxin in vertebrates. Here we show that the mice with a null mutation in Efcab1, which encodes calaxin, display typical phenotypes of primary ciliary dyskinesia, including hydrocephalus, situs inversus, and abnormal motility of trachea cilia and sperm flagella. Strikingly, both males and females are viable and fertile, indicating that calaxin is not essential for fertilization in mice. The 9 + 2 axonemal structures of epithelial multicilia and sperm flagella are normal, but the formation of 9 + 0 nodal cilia is significantly disrupted. Knockout of calaxin in zebrafish also causes situs inversus due to the irregular ciliary beating of Kupffer’s vesicle cilia, although the 9 + 2 axonemal structure appears to remain normal.
Motele cilia and flagella are organelles that have been conserved through evolution\textsuperscript{1-3}. They possess internal cytoskeletal structures, axonemes, that are composed of nine outer doublet microtubules and two central singlet microtubules (9 + 2 structure)\textsuperscript{4,5}. Two types of projection extend from each microtubule doublet, the outer and inner dynein arm (ODA and IDA), both of which are large, multi-subunit complexes consisting of heavy, intermediate, and light chains. Dynein heavy chains (HCs) are motor subunits that hydrolyze ATP to convert chemical energy into mechanical energy for microtubule movement. The intermediate and light chains (ICs and LCs) assemble and regulate the motor subunits.

Genetic defects of the dynein components cause primary ciliary dyskinesia (PCD), a human ciliopathy disease\textsuperscript{6-8}. PCD is characterized by defects in the motility of cilia and flagella in a variety of cells, including sperm, and in tissues of the trachea, ependyma, and embryonic node. By utilizing both mice and zebrafish as model systems, it is possible to acquire important insights into the phenotypes and mechanism of PCD\textsuperscript{9-11}.

PCD is most often caused by defects in a subunit of the ODA, including HCs (DNAH5 and DNAH11), ICs (DNAI1, DNAI2 and TXNDC3/NME8), LCs (DNAI1 and TECTE3), components of the docking complex (CCDC114, TTC25), and ODA-associated proteins (CCDC151, CCDC103, and ARMC4)\textsuperscript{12-22}. These mutations result in the complete or partial absence of the ODA, leading to PCD\textsuperscript{23}. An exception to this is patients with mutations in DNAH11 (previously termed, Left-Right Dyscin, LRD)\textsuperscript{24}. Like PCD patients with mutations in other ODA-coding genes, those with DNAH11 mutations have respiratory defects, situs abnormalities and are infertile. However, their ciliary structures are normal with normal ODAs\textsuperscript{24,25}.

The motility of cilia and flagella is modulated in response to several extracellular stimuli\textsuperscript{26-28}. The most critical intracellular factor mediating these changes is Ca\textsuperscript{2+}. Calaxin is a neuronal calcium sensor protein first described in the sperm of the ascidian Ciona intestinalis\textsuperscript{29-31}. It directly binds to the β-type heavy chain (orthologous to Chlamydomonas y heavy chain\textsuperscript{32}) of the ODA in a Ca\textsuperscript{2+}-dependent manner and regulates the propagation of the asymmetric flagellar wave. It is also necessary for changes in swimming direction during sperm chemotaxis\textsuperscript{30} (Fig. 1a). In sea urchin embryos, calaxin is a critical regulator for the coordinated movements of monocilia and is a prerequisite for the establishment of ciliary orientation\textsuperscript{32} (Fig. 1a), which is generally thought to be determined by the planar cell polarity. Calaxin is a Ca\textsuperscript{2+} sensor that has evolved in the opisthokont (animal fungi) lineage\textsuperscript{3}; however, it has not been widely studied, particularly in vertebrates.

The initial aim of this study was to elucidate the role of calaxin in vertebrate male fertility, particularly how sperm chemotaxis contributes to the success of vertebrate internal fertilization. We generated knockout mice lacking the gene encoding calaxin, Efcab1. Both male and female Efcab1\textsuperscript{-/-} mice were unexpectedly fertile. However, many Efcab1\textsuperscript{-/-} mice showed hydrocephalus and visceral inversion, both of which are typical features of ciliopathy, without apparent changes in 9 + 2 axonemal structures. Intriguingly, calaxin knockout caused a drastic loss of nodal cilia, whereas other cilia and flagella were normally formed.

**Results**

Calaxin knockout mice exhibit PCD but are fertile. To elucidate the physiological function of calaxin in vertebrates, we generated a knockout mouse in which exon 4 of Efcab1, the gene encoding calaxin, was genetically disrupted by homologous recombination (Supplementary Fig. 1a–c). No calaxin expression was observed in the sperm, trachea or ependyma of Efcab1\textsuperscript{-/-} mice either at mRNA (Supplementary Fig. 1c) or at protein level (Fig. 1b, c, Supplementary Fig. 1d). Notable phenotypes of postnatal Efcab1\textsuperscript{-/-} mice were hydrocephalus and situs inversus in 35% and 49% of offspring, respectively (Fig. 1d, e), with both phenotypes present in 16% of offspring (Fig. 1f).

In Ciona, calaxin is essential for chemotaxis of the sperm to the egg; however, both male and female Efcab1\textsuperscript{-/-} mice were fertile, although litter sizes when either or both parents were Efcab1\textsuperscript{-/-} were significantly lower compared with litters from wild-type parents (Fig. 1g). The litter size from Efcab1\textsuperscript{-/-} males and females was 1–2, whereas that from wild-type mice was 7–8. However, Efcab1\textsuperscript{-/-} sperm showed the same in vitro fertilization rate as wild-type sperm (Fig. 1h). The number of Efcab1\textsuperscript{-/-} embryos was almost the same as that of wild-type embryos at embryonic day 8 (E8) but after E14 the number of Efcab1\textsuperscript{-/-} embryos declined (Fig. 1i). Efcab1\textsuperscript{-/-} male and female offspring were born at non-Mendelian frequency with a deficit of Efcab1\textsuperscript{-/-} mice (Supplementary Table 1), indicating homoyogotic embryonic lethality. Efcab1\textsuperscript{-/-} mice showed both cardiac hypertrophy (Fig. 1j) and enlargement of brain ventricles (Fig. 1k, l). Hydrocephalus emerges after birth and is not always lethal\textsuperscript{33}; therefore, the cause of embryonic lethality is most likely to result from cardiac defects that often accompany PCD\textsuperscript{34}. Surviving Efcab1\textsuperscript{-/-} mice showed a similar survival rate to wild-type mice (more than 10 weeks) (Fig. 1l). A bacterial artificial chromosome containing the entire Efcab1 gene (Supplementary Fig. 2a, b) rescued the expression of calaxin protein (Supplementary Fig. 2c) and the PCD phenotypes of Efcab1\textsuperscript{-/-} mice (Supplementary Fig. 2d), clearly indicating the distinct roles of calaxin in ciliary function.

Cilia of Efcab1\textsuperscript{-/-} mice have morphologically normal 9 + 2 axonemes. Normal sperm flagella were observed by differential interference contrast (DIC) light microscopy (Fig. 1c) and scanning electron microscopy (Fig. 1a) in Efcab1\textsuperscript{-/-} male mice (Figs. 1c, 2a); similarly no abnormality was observed in the multicilia of tracheal or ependymal epithelia by scanning electron microscopy (Fig. 2b, c). Transmission electron microscopy of sperm flagella, tracheal cilia and ependymal cilia revealed that the ODAs were mostly intact in Efcab1\textsuperscript{-/-} mice (Fig. 2d–f); in rare cases a few ODAs were absent from some doublet microtubules (Supplementary Fig. 3).

Efcab1\textsuperscript{-/-} flagella and cilia show reduced motility and fluid flows. Efcab1\textsuperscript{-/-} mice were fertile, with Efcab1\textsuperscript{-/-} sperm showing the same degree of in vitro fertility as wild-type sperm (Fig. 1h), indicating that calaxin and its regulation of sperm motility is not essential for successful fertilization. To examine the detailed role of calaxin in the regulation of flagellar motility, we analyzed the flagellar waveform and its propagation in Efcab1\textsuperscript{-/-} sperm. The swimming velocities of Efcab1\textsuperscript{+/+} and Efcab1\textsuperscript{+/-} sperm were 109.2 ± 16.4 and 103.1 ± 15.7 μm/s, whereas that of Efcab1\textsuperscript{-/-} sperm was significantly decreased (74.9 ± 12.5 μm/s) (Fig. 3a). This decrease most likely resulted from transient arrest of post-hook bend propagation; Efcab1\textsuperscript{-/-} sperm showed transient arrest of pro-hook bend propagation (Fig. 3b; Supplementary Movies 1 and 2). Nonetheless, Efcab1\textsuperscript{-/-} sperm were fertile.

Tracheal cilia of Efcab1\textsuperscript{-/-} mice also exhibited active motility (Supplementary Movies 3 and 4). However, analysis of fluid flow in tracheal using fluorescent beads revealed that the flow velocity in Efcab1\textsuperscript{-/-} mice was decreased to almost half that of wild-type mice (Fig. 3c, d; Supplementary Movies 5 and 6). High-speed filming of the trajectories of fluorescent beads attached to the tips of cilia showed that the frequency of ciliary beating was...
Fig. 1 Generation and phenotypes of Efcab1 mutant mice. a Function of calaxin in the propagation of flagellar and ciliary waveforms. Typical waveforms of Ciona sperm flagella and sea urchin embryonic cilia in normal and calaxin-inhibited conditions are shown. Modified from previous publications30,32. b Immunoblot analysis of Efcab1+/− sperm by anti-Efcab1 and anti-acetylated-α-tubulin antibodies. c Immunofluorescence analysis of Efcab1+/− sperm flagella, trachea and ependymal cilia by anti-Efcab1 and anti-acetylated-α-tubulin antibodies. Scale bar, 50 μm (top) and 25 μm (bottom left and right). d Efcab1−/− mouse showing hydrocephalus (upper; head morphology, scale bar = 1 cm, lower; 5 μm paraffin section stained with hematoxylin-eosin) and e situs inversus. Lv, lateral ventricle; Lv, liver; Sp, spleen. f Percentage of Efcab1+/− mice with hydrocephalus and/or situs inversus. N = 130 (71; male, 59; female). g Average litter size from parents of different genotypes. h The rate of in vitro fertilization of eggs with epididymal sperm collected from Efcab1+/− and Efcab1−/− mice. Numbers in parentheses indicate the number of eggs examined from triplicate experiments. i Number of embryos at E8, E14 and E16 after mating with Efcab1+/− or Efcab1−/− male mice. j The ratio between heart and body weight in Efcab1+/− and Efcab1−/− mice. k Comparison of lateral ventricle area between Efcab1+/− and Efcab1−/− mice. l Survival rate at 10 weeks. N = 46 (Efcab1+/− and Efcab1−/−) and N = 198 (Efcab1−/−). Values indicate mean ± SEM. *p < 0.05, **p < 0.001 vs. Efcab1+/− (Student’s t-test).

significantly decreased in Efcab1−/− mice (Fig. 3c; Supplementary Movies 7 and 8). The velocity of the recovery stroke in Efcab1−/− cilia was drastically slower than that in Efcab1+/− cilia.

As with the case of trachea cilia, ependymal cilia of Efcab1−/− mice also showed active motility (Fig. 3f; Supplementary Movies 9 and 10). Analysis of the fluid flow being driven by ependymal cilia showed that the flow velocity was slightly decreased in Efcab1−/− mice compared with that in wild-type cilia (Fig. 3g; Supplementary Movie 11 and 12). This was most likely due to the cilia having a narrower range of effective stroke (Fig. 3h), as observed in the calaxin-knockdown embryos of sea urchins32.

Calaxin knockout causes a distinct defect in nodal cilia formation. The leftward fluid flow at the ventral surface of the

![Image](https://example.com/image.jpg)
embryonic node is critical for left-right asymmetry determination. This flow is generated by the rotary movement of monocilia. Bending of each cilium was clearly observed during the rotation (Supplementary Movie 13). To investigate the cause of situs inversus in Efcb1−/− mice, we observed the nodal cilia. Compared with wild type embryos at E7.5, the nodal cilia of Efcb1−/− embryos were strikingly sparse or completely absent in some cases and longer microvilli were more prominent on the cell surface (Fig. 4a). The number of motile cilia on the node was less than 15% of the wild-type number (Fig. 4b; Supplementary Movie 14). The fluid flow generated by nodal cilia was clearly leftward in wild-type mice, whereas that in Efcb1−/− mice was not detectable or became random in direction (Fig. 4c, d; Supplementary Movie 15 and 16). Fluorescent microbeads at the Efcb1−/− node showed a certain but random flow (Fig. 4d) and the velocity was significantly lower than that at the wild-type node (Fig. 4e).

Next, we recorded and analyzed the trajectories of nodal cilia tips. The nodal cilia of wild-type mice showed rotary movement (Fig. 4f); however, Efcb1−/− nodal cilia moved in an irregular, not rotary manner and often in planar trajectories (Fig. 4g). To check if this aberrant ciliary motility and fluid flow resulted in situs inversus, we examined the gene expression of Nodal and Lefty, both of which are expressed in the left plate mesoderm (LPM) and are key genes in the determination of left–right asymmetry35. Both genes were expressed in the LPM of wild-type E8.0 embryos but in Efcb1−/− embryos both genes were expressed on both sides (Fig. 4h).

Since two populations of cilia are known in mouse node, we examined the localization of Efcb1 by immunofluorescent staining. In wild-type embryos, the cilia in the central region of node were recognized with anti-Efcb1 antibody but no significant staining was observed in cilia of the peripheral region (Fig. 4i). As demonstrated by scanning electron microscopy (Fig. 4a, b), we could not detect cilia of the central node region in Efcb1−/− mice. However, significant numbers of cilia were observed in the peripheral region of these mice (Fig. 4i).

Calaxin-deficient zebrafish show situs inversus but normal cilia formation in Kupffer’s vesicle. To investigate the conservation of vertebrate calaxin function in the determination of body laterality, we carried out CRISPR/Cas9-mediated knockout of efcb1 in zebrafish. We identified an 11 bp-insertion in exon 2 of efcb1, including an in-frame stop codon (Fig. 5a, c), which resulted in the loss of Efcb1 protein expression (Fig. 5a–c). In zebrafish, cilia-directed flow in Kupffer’s vesicle (KV) has a critical role in establishing the left-right body axis36. In contrast to the drastic loss of nodal cilia formation in Efcb1−/− mouse embryos, efcb1−/− zebrafish showed normal formation of KV cilia (Fig. 5e); no significant difference was observed in the number of cilia between wild-type and efcb1−/− fish (Fig. 5f). However, many of the KV cilia in efcb1−/− zebrafish beat with an irregular cycle (Fig. 5g, h) in contrast to the smooth rotary movement in wild-type fish (Supplementary Movie 17). Detailed observation of ciliary movement revealed KV cilia of knockout fish to have abnormal helical movements with less ciliary bending (Supplementary Movie 18). These irregular movements were almost completely rescued by injection of efcb1 mRNA (Fig. 5g–i; Supplementary Movie 19). The abnormal ciliary movements in efcb1−/− fish induced laterality defects in embryos. In wild-type embryos, the heart ventricle loops toward the right and the atrium loops toward the left. However, in almost half of the efcb1−/− embryos the direction of the heart loop was reversed. The reversed loop was rescued by injection of efcb1 mRNA (Fig. 5j, k).

Discussion
Knockout of mouse calaxin, a Ca2+ sensor for ODAs, caused several defects commonly seen in PCD. Despite apparent normal
formation of epithelial cilia and sperm flagella, calaxin deficiency resulted in striking inhibition of nodal cilia formation, which could be one reason for the loss of nodal flow. However, induction of left side-specific gene expression requires at least two nodal cilia\(^{37}\). Therefore, although \(Efcab^{+/−}\) mice have a greatly reduced number of nodal cilia (−5 cilia on average) this cannot explain why half showed \textit{situs inversus}. Rather, we consider aberrant ciliary motility with planar beating, not smooth rotational movement, to be critical for the laterality defect. The common feature of structural normality of 9 + 2 cilia between calaxin knockout mice and PCD patients with DNAH11 (β-type HC of the ODA\(^{24}\)) mutations suggests a possible relationship between these two proteins.

Calaxin plays an important role in propagation of the asymmetric waveform in ascidian sperm flagella\(^{36}\). This is consistent with our results showing suppression of propagation of the pro-hook bend in the sperm flagella of calaxin-deficient mice. However, calaxin-deficient mice are fertile, indicating that calaxin-dependent regulation of the flagellar bend propagation might not be essential for successful fertilization, at least in mice, and that sperm with abnormal propagation of the pro-hook bend can penetrate the zona pellucida to achieve fertilization. This is in contrast to mutant mice lacking sperm-specific calcineurin (PPP3C/PPP3R2), which show defects in bend formation in the proximal region of the flagellum and are defective in zona penetration\(^{46}\).

Loss of calaxin resulted in a narrower spatial range of beating in the trachea and brain multicilia of mice. A quite similar effect is also observed in the monocilia of calaxin-deficient sea urchin embryos\(^{32}\), suggesting that a major role of calaxin in the regulation of ciliary beating is likely common between mice and sea urchins. In sea urchin embryos, any deficiency of calaxin causes
Fig. 4 Appearance and motility of nodal cilia in Efcab1−/− mice. a Scanning electron microscopy of E7.5 embryo and nodal cilia in Efcab1+/+ and Efcab1−/− mice. Left, whole embryo; middle, node area; right, magnified image of nodal cilia. b Number of motile cilia in the node. N (embryos) = 18 (Efcab1+/+) and N = 18 (Efcab1−/−). c, d Nodal flow in Efcab1+/+ (c) or Efcab1−/− (d) E7.5 embryos. Upper; Trajectories of fluorescent beads driven by nodal flow. Sixty images acquired at 0.25 s intervals are superimposed. Red and blue dots indicate the start and end points of the superimposition, respectively. Lower; direction and distance traveled by fluorescent beads in Efcab1+/+ or Efcab1−/− nodal flows. Arrow length represents bead path and distance for 5 s. A, anterior; P, posterior; R, right; L, left. e Flow velocity of fluorescent beads. Boxes correspond to the first and third quartiles, and whiskers extend to the full range of the data. N (beads) = 112 from four embryos (Efcab1+/+) and N = 46 from 10 embryos (Efcab1−/−). f, g Trajectories of fluorescent beads binding to the tip of nodal cilia in Efcab1+/+ (f) and Efcab1−/− (g) mice. h Whole-mount in situ hybridization analysis of Nodal and Lefty expression in E8.0 embryos. R, right; L, left. Scale bars: 200 μm (a, left), 10 μm (a, middle, c, d), 1 μm (a, right), 4 μm (f, g), 1 mm (h). i Immunofluorescent localization of Efcab1 in the central and peripheral regions of node in Efcab1+/+ and Efcab1−/− mice. Images with anti-Efcab1 (green) and anti-acetylated-α-tubulin (red) antibodies are shown. Scale bars: 10 μm. **p < 0.01, ***p < 0.001 vs. Efcab1+/+ (Student’s t-test)
disruption to their basal body orientation, leading to uncoordinated ciliary movement and aberrant swimming\textsuperscript{32}. However, despite clear changes in the ciliary waveform and beating of each cilium, the effects of calaxin-knockout on the epithelial fluid flow in mouse multicilia were not overly drastic. It is likely that weak beating of each cilium would not affect the ciliary orientation in multicilia, such as observed in sea urchin monocilia, as it has been suggested that the mechanical feedback of hydrodynamic force within multicilia would compensate for this\textsuperscript{39–41}.

Knockout of calaxin caused reduced motility in both the sperm flagella and epithelial multicilia, without any apparent changes in the $9 + 2$ structure. In rare cases, ODAs on the doublet 3, 5, and 8
in sperm flagella and the doublet 8 and 9 in trachea cilia were lost, suggesting that calaxin might play some role in the stability of ODAs, particularly for those on the doublets located either side of the plane of central pair complex which are strongly involved for flagellar or ciliary bending. In contrast to the apparently normal formation of sperm flagella and epithelial multicilia, calaxin mutants were deficient in the formation of nodal cilia, indicating a distinct mechanism for ciliary formation in the node.

Why is calaxin essential for the formation of nodal cilia only? It could be related to the type of cilia, i.e., mono-cilia or multi-cilia; however, knockdown of calaxin in sea urchin embryos results in no structural changes in ectoderm monocilia.32 Moreover, knockout of calaxin in zebrafish resulted in normal formation of KV monocilia.33 The most probable explanation for the specific effect on ciliary formation is that mouse node cilia have a 9 + 0 axonemal structure, unlike the 9 + 2 structure in other motile cilia. There have been conflicting reports as to whether the dynein species present in nodal cilia possess ODAs and/or IDAs; however, they most probably only possess ODAs.10,42 When ODAs are assembled in a 9 + 0 arrangement, calaxin might have a critical role in ciliogenesis. This could also indicate a novel mechanism where the Ca2+-dependent dynein sensor, calaxin, is involved in intraflagellar transport, docking axonemal components at the basal body/transition zone, or maintenance of axonemal integrity. Although intraflagellar transport is regulated by intracellular Ca2+ concentration,3 the possibility that Ca2+-binding axonemal proteins respond to the changes in intracellular [Ca2+] is yet to be identified. It is possible that Ca2+-binding axonemal proteins, such as ODA calaxin, IDA centrin, radial spoke and central apparatus, might be involved in the regulation of intraflagellar transport. This idea is consistent with zebrafish 9 + 2 KV cilia with ODAs, IDAs, radial spokes and central apparatus, being normally formed in calaxin-knockout embryos, although motility was sufficiently altered to induce a laterality defect. Two populations of nodal cilia, including those in central region with 9 + 0 and in peripheral region with 9 + 2 structures, have been observed in the mouse node.46,47 This is consistent with the fact that LRD is only localized in the cilia of central node region46 and suggests that calaxin is closely related to the function of LRD motor activity.

Asymmetry of intracellular Ca2+ dynamics in the node has been observed, but Ca2+-dependent regulation of nodal cilia motility is less understood. Attempts to image Ca2+ dynamics in the node have produced different conclusions regarding the necessity of Ca2+ dynamics in left-right asymmetry.10 Change in motility of KV cilia upon changes in intracellular Ca2+ has been observed in zebrafish embryos.48 However, it is not known how intracellular Ca2+ regulates ciliary waveforms. We found that knockout of calaxin causes the change from rotary movement with proper ciliary bending to planar movement with less bending, indicating that nodal ciliary motility depends on intracellular Ca2+-dependent regulation of calaxin. Ca2+-binding to calaxin appears to occur at the second EF-hand between concentrations of 10−7 to 10−6 M intracellular [Ca2+], which induces proper propagation of asymmetric flagellar bend in Ciona sperm.51,52. The resting [Ca2+] in a mouse node is reported to be ~300 nM, which would represent a threshold for Ca2+-binding to calaxin. Therefore, we suggest that Ca2+ would be bound to calaxin in the node and would regulate proper ciliary bending, resulting in the generation of leftward fluid flow.

Methods

Generation of Efcab1-deficient mice and genotyping. A knockout-first allele system targeting vector for mouse Efcab1, the ortholog of the Ciona calaxin gene, was obtained from the International Mouse Phenotyping Consortium (https://www.mousephenotype.org/). Chimeric mice were generated using EGR-G101 embryonic stem (ES) cells by embryo reaggregation of ES cells with ICR-derived 8-cell embryos, and crossed with C57BL/6N female mice to obtain F(1) mice with a conditional knockout allele (tm1a), or crossed with a CAG-Cre transgenic female mouse53 to remove the LoxP-flanked region (exon 4) and obtain the tm1b line. The tm1b line was used to obtain the Efcab1 knockout line. Efcab1 tm1a mice were deposited in the Riken Bioresource Center with the stock number; RRBC05968. Chimeric mice were generated using EGR-G101 embryonic stem (ES) cells by embryo reaggregation of ES cells with ICR-derived 8-cell embryos, and crossed with C57BL/6N female mice to obtain F(1) mice with a conditional knockout allele (tm1a), or crossed with a CAG-Cre transgenic female mouse53 to remove the LoxP-flanked region (exon 4) and obtain the tm1b line. The tm1b line was used to obtain the Efcab1 knockout line. Efcab1 tm1a mice were deposited in the Riken Bioresource Center with the stock number; RRBC05968.

Quantitative PCR. Total RNA was extracted from brain, testes or trachea using RNeasy mini kit (Qiagen) according to the manufacturer’s protocols. To remove genomic DNA contamination, extracted RNA was treated with Recombinant RNasefree kit (Takara). The total RNA was reverse-transcribed into cDNA using SuperScript III (Invitrogen). qSYBR Green mix (Bio-Rad) was used for PCR reactions carried out with the Thermal Cycler Dice Real Time system (Takara). Primer pairs used for PCR reactions were following:

| Primer (P5) | Primer (P3) |
|------------|-------------|
| EFCAB1  /  | 5-CAGCTGAGGCAGCCGTCGCT-3' |
| 5-TCCCAACTGAGCTGACTGAC-3' |

Knockout of zebrafish Efcab1. Zebrafish embryos and larvae were raised at 28.5 °C in 1/3 Ringer’s solution (39 mM NaCl, 0.97 mM KCl, 1.8 mM CaCl2, and 1.7 mM HEPES, pH 7.2). Genome-editing was performed according to a previously reported method54 with the target site, CGTGGCTTACGCGAAAGTG. Animal procedures were performed with the approval of The University of Tokyo.

Relative concentrations of Efcab1 mRNA were normalized with GAPDH Ct (cycle threshold) values.
Whole mount in situ hybridization (WISH). cDNA clones of Nodal and Lefty in pBlueScript SK were kindly provided by Dr. Hiroshi Hamada (Riken, Japan). The latter contained a highly conserved region between closely related Lefty1 and Lefty2. The plasmids were linearized by restriction enzymes and digoxigenin-labeled RNA probes were generated by T7/T3 RNA polymerases. The product was treated with RNA-free DNase and checked by agarose gel electrophoresis. Whole mount in situ hybridization was performed according to standard procedure.

For zebrafish, embryos were fixed with 4% paraformaldehyde (PFA) in PBS, and then stored in methanol at −20 °C. After rehydration with PBST, hybridization was performed overnight at 63 °C with digoxigenin-labeled RNA probes. Hybridized specimens were washed with SSC (saline sodium citrate) buffer, then treated with AP-conjugated anti-digoxigenin Fab fragments (1:4000 dilution) in PBST (Roche) in PBST at 4 °C overnight. After washing with PBST, signals were developed using BM-purple (Roche). When desired intensities of staining were obtained, reactions were stopped. Before observation, specimens were transferred to 80% glycerol/PBS to make them transparent. Images were taken using a stereoscopic microscope (MVX10; Olympus) and a CCD camera (DP73; Olympus).

In vitro fertilization. Folicular development and ovulation were induced by hCG injection of 8–12-week-old female mice. Mature oocytes were recovered 14–16 h after the hormone treatment. Mature spermatozoa were obtained from the cauda epididymis of 8-10-week-old male mice. Spermatozoa were suspended and pre-incubated in TYH culture medium for 2 h and added to the oocyte suspension at a ratio of 1:5. Mature spermatozoa were incubated at 37 °C, 5% CO2. After 30 min, male and female pronuclei were stained with Hoechst 33342 and counted under a fluorescence microscope.

Transmission electron microscopy. Samples were fixed in 2.5% glutaraldehyde, 5 mM MgSO4, 0.1 M cacodylate buffer, pH 7.4, for 1 h at room temperature. After washing with 0.1 M cacodylate buffer, pH 7.4, the samples were post-fixed in 1% OsO4 on ice for 1 h, dehydrated in a 30–100% ETOH series, substituted by propylene oxide and embedded in Epon 812 or Agar Low Viscosity Resin (LV-Resin). Sections (70 nm) were cut using an ultramicrotome (LEICA, Wetzlar, Germany) and mounted on uncoated carbon-coated grids. Samples were stained with 7% uranyl acetate for 20 min, followed by Reynolds lead staining for 2 min, and observed under a transmission electron microscope (JEM 1200EX, JEOL).

Recording of mouse sperm flagellar motility. Spermatozoa were collected from the cauda epididymis, suspended in TYH culture medium and incubated under mineral oil (Nacalai Tesque) at 37 °C in 5% CO2. Sperm motility was observed in a phase contrast microscope (BX51, Olympus) with a 10× objective on a BX51 phase contrast microscope (Olympus) and recorded at 500 frames per second (fps) through a HAS-D3 high-speed camera (DITECT, Japan). Velocity of sperm swimming was calculated from the trajectory for 0.5 s.

Recording of cilia motility in mouse trachea and brain. Tracheal cilia were observed according to a previously described method. Mouse trachea were removed by dissection and placed in DH10 culture medium containing 8.3 g/l DMEM powder (Sigma D5030), 25 mM HEPES-NaOH, pH 7.2, 4.5 g/l glucose, 0.11 g/l sodium pyruvate, and 50% rat IC serum. A 0.3 mm thick silicon sheet with a hole was set onto a glass slide and a node fragment was transferred in medium with the nodal cilia face up. The specimen was then covered with a coverslip and observed under a differential interference contrast microscope (BX51, Olympus) with a 40× or 60× objective.

Analysis of ciliary movement and fluid flow. Fluid flow was traced using 1 μm fluorescent beads (FluoSphere, F-8820, Invitrogen) and recorded at 200 fps by a fluorescence microscope (BX51, Olympus) equipped with a high-speed camera (HAS-220 (DITECT) and a 20× objective for tracheal and ependymal cilia or a 60× objective for nodal cilia. Flagellar and ciliary motility and the trajectories of beads were analyzed by motility analysis software, Bohboh. To analyze the trajectories of tracheal ciliary beating, fluorescent beads attached near the tips of cilia were recorded and traced by Bohboh. For nodal cilia, recorded images were processed to reduce the background and the positions of cilium tips were traced by Bohboh.

Kupfer’s vesicle cilia analysis. Embryos with a Kupfer’s vesicle were selected at 12 hpf and dechorionated before observation. For orientation, embryos were transferred into 0.08% low gelling agarose in 0.08% low gelling agarose. After fixation in cold methanol (−20 °C), dehydrated with PBS, and embedded in 8.3 g/l DMEM powder (Sigma D5030), 25 mM HEPES-NaOH (pH 7.5) or directly by the SDS-buffer for epithelial tissues or sperm, respectively, then separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membranes were blocked by PBST (PBS containing 0.1% Tween 20) containing 7.5% skimmed milk, followed by incubation with the anti-mouse Efcab1 antibody (1:2000) or by a mouse monoclonal antibody against tubulin-α (Clone DM1A) (Toro Systems Scientific, 1:10,000). Blots were then incubated with HRP-conjugated secondary antibodies (1:10,000), washed with PBST and developed with ECL-Prime enhanced chemiluminescence substrate kit (GE Healthcare).

Mature spermatozoa were collected from the cauda epididymis, suspended in phosphate-buffered saline (PBS) and immobilized on a poly-lysine coated glass slide. They were fixed in cold methanol (−20 °C), dehydrated with PBS, then incubated with T-PBS (0.1% Triton X-100 in PBS) containing 10% goat serum in T-PBS for 2 h. After blocking, samples were incubated in the blocking buffer containing a rabbit polyclonal antibody against mouse Efcab1 at 1:100 dilution for 1 h. After washing with T-PBS three times for 1 h, samples were treated with secondary antibodies (Alexa Fluor® 488-labeled secondary antibody against rabbit IgG, Invitrogen, and Alexa Fluor® 546-labeled secondary antibody against mouse IgG, Invitrogen) at 1:1000 dilution, washed with PBS and developed with ECL-Prime enhanced chemiluminescence substrate kit (GE Healthcare).

Antibodies, immunoblotting, and immunofluorescence microscopy. A cDNA fragment encoding mouse Efcab1 was PCR-amplified, subcloned into a pET-23d vector (Novagen) and expressed in E. coli BL21 (DE3). The recombinant protein was then purified using a His-tag affinity column. A polyclonal antibody against mouse Efcab1 was produced in 16 rabbits via immunization with the recombinant protein. For immunoblot analysis, proteins were extracted by 4 M urea, 1% CHAPS, 20 mM HEPES-NaOH (pH 7.5) or directly by the SDS-buffer for epithelial tissues or sperm, respectively, then separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membranes were blocked by PBST (PBS containing 0.1% Tween 20) containing 7.5% skimmed milk, followed by incubation with the anti-mouse Efcab1 antibody (1:2000) or by a mouse monoclonal antibody against tubulin-α (Clone DM1A) (Toro Systems Scientific, 1:10,000). Blots were then incubated with HRP-conjugated secondary antibodies (1:10,000), washed with PBST and developed with ECL-Prime enhanced chemiluminescence substrate kit (GE Healthcare).

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and observed using a fluorescence microscope (BX60; Olympus) and a CCD camera (ORCA-R2; Hamamatsu).

**Statistics and reproducibility.** Data were collected as follows. For sperm flagella, N (sperm) = 299 from four animals (Efcab1+/–), N = 466 from six animals (Efcab1+/+) and N = 465 from six animals (Efcab1–/–). For tracheal flow velocity, values are means ± SE. N (beads) = 140 from five animals (Efcab1+/–), N = 101 from three animals (Efcab1+/+) and N = 200 from eight animals (Efcab1–/–). For brain ependymal cilia, values are means ± SE. N (beads) = 221 from three animals (Efcab1+/+) and N = 155 from five animals (Efcab1–/–). For counting motile nodal cilia, values are means ± SE. N (embryos) = 18 (Efcab1+/–) and N = 18 (Efcab1–/–). For nodal flow, values are means ± SE. N (beads) = 112 from four embryos (Efcab1+/+) and N = 46 from 10 embryos (Efcab1–/–). The significance of differences between Efcab1+/+ and Efcab1–/– was calculated using a two-tailed Student’s t-test at the significance level P < 0.05. All experimental findings were reliably reproduced.

**Data access.** Other relevant information regarding data accession is described in the Supplementary information (Supplementary Note 1).

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Movies associated with the current study are available online at https://doi.org/10.4804/mixfig/er9c81218c89. All other data generated during and/or analyzed during the currently study are available from the corresponding author on reasonable request.

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Author contributions

K.I. designed the outline of the study. K.Sh., M.K., K.M., and K.I. designed the experimental strategy. Y.S. and M.I. made the knockout mice and T.B., A.N., N.K., and K.M. maintained the mice. K.Sa., D.S., M.N., R.Y, K.J., and K.I. performed electron microscopy. K.Sa, K.Sh, A.N., N.K, M.Mi., H.U., S.N., S.T., K.M., and K.I. performed other experiments in mice and analyzed the data. H.Y., M.Mo., M.K. contributed to experiments with knockout zebrafish. K.I. wrote and K.M., M.K., K.Sh. edited the manuscript. All authors agreed to the final version of the manuscript.

Additional information

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