Functional Characterization of *Cfap206* for Bivalve Ciliogenesis by RNAi and CRISPR/Cas9 Technologies

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Cilia are organelles located on the surface of eukaryotic cells and play important roles in numerous physiological and developmental processes. Cilia- and flagella-associated proteins (CFAP family) are well known due to their close relation to the assembly and functionalization of both cilia and flagella. In vertebrates, *Cfap206* is reported to function during motile cilia assembly, and its abolished expression could lead to abnormal ciliary beating and decreased sperm motility. However, there is scarcely any information on the function of *Cfap206* in marine invertebrates. Here, through using an ideal shellfish research model, the dwarf surf calm *Mulinia lateralis*, we explored the role of *Cfap206* during embryonic ciliogenesis as well as during male gametogenesis. In trochophore larvae, effective knocking down or knocking out of *Cfap206* was detected respectively through RNAi and CRISPR/Cas9 technology, showing an obviously decreased number of cilia. During the period of gonadal maturation, we managed to deliver the dsRNA of *Cfap206* to male individuals for 14 days through carrier vector feeding. Significant suppression of *Cfap206* was observed, together with impaired sperm motility and aberrant sperm tail assembly. The present study provided valuable information on gene function exploration methods in *M. lateralis* and further enriched the understanding of *Cfap206* on ciliogenesis in marine invertebrates. Furthermore, by revealing the relationship between *Cfap206* and spermatozoa flagellum, it is possible to generate male sterile populations in hermaphroditic marine organisms to improve crossbreeding efficiency.

Keywords: *Mulinia lateralis*, *Cfap206*, ciliogenesis, RNAi, CRISPR/Cas9, breeding

INTRODUCTION

Cilia are membranous organelles that protrude from the surface of most eukaryotic cells, contributing to multiple functions such as movement, sensation, and signal transmission (Pazour and Witman, 2003; Jakobsen et al., 2006; Verhey et al., 2011; Bangs and Anderson, 2017). The basic structure of cilia is highly conserved, including four parts: matrix, transition zone, axoneme, and ciliary membrane (Elliott and Brugmann, 2019). According to the differential axoneme microtubule composition, cilia could be divided into motile cilia and nonmotile cilia (known as primary cilia) (Verhey et al., 2011). The axoneme of motile cilia usually possesses nine doublet microtubules surrounding a central pair of singlet microtubules (Satir and Christensen, 2007). The microtubule doublets are interconnected with ciliary dynein motors, the activity of which is regulated through radial spokes (RS1, RS2, and RS3) to produce fluid and pulsatile motion. Thus, motile cilia could mediate the movement of cells and extracellular fluid, such as the movement of eggs and sperm, as well as the removal of foreign bodies in the respiratory tract (Afzelius and Eliasson, 1983; Lyons et al., 2006; Orhon et al., 2015). The axoneme of primary cilia lack the central pair of singlet microtubules, and there are a number of surrounding sensory membrane proteins that can assist in performing sensory, signal transduction, and coordination functions (Hua and Ferland, 2018; Tao et al., 2020). Primary cilia are closely related to the transduction of Hedgehog (Hh) pathway (Danwei et al., 2003), Wnt pathway (Ross et al., 2005), platelet-derived growth factor (PDGF) pathway, and Ca^{2+} signaling cascade (Orhon et al., 2015).

The cilia- and flagella-associated protein (CFAP) family is proven to be associated with the normal assembly of cilia or flagella (Tang et al., 2017). *Cfap39* and *Cfap65*, for example, are required for the assembly of the dynein regulatory complex in the generation of motile cilia (Lin et al., 2012; Beckers et al., 2020) and deletion of *Cfap43*, *Cfap44*, *Cfap65*, *Cfap69*, and *Cfap251* will lead to abnormal sperm flagellum assembly in mice or humans, resulting in male infertility (Tang et al., 2017; Dong et al., 2018; He et al., 2020; Li et al., 2020). In particular, *Cfap206* has recently been identified as a downstream target gene of *Foxj1*, a key transcription factor that has been proven to be closely related to the occurrence of mobile cilia in vertebrates (Stubbs et al., 2008; Thomas et al., 2010; Alten et al., 2012; Vij et al., 2012). Any mutation or deletion of them may lead to severe cilia diseases, such as primary ciliary dysmotility (PCD), hydrocephalus, retinitis pigmentosa, polycystic kidney disease (PKD), and multiple morphological abnormalities of the sperm flagella (MMAF) (Pazour and Rosenbaum, 2002; Satir and Christensen, 2007; Sironen et al., 2020). Moreover, CFAP206 protein was found to be located in cilia, which was required for establishing the dynamin motor RS2 (Beckers et al., 2020). It was reported that the stable assembly of calmodulin- and spoke-associated complex (CSC), which is considered to mediate the specific connection of radial spokes with adjacent structures in cilia, was regulated by *Cfap206* in a dependent manner (Heuser et al., 2012; Vasudevan et al., 2015; Toure et al., 2021). Although *Cfap206* is a key gene for ciliogenesis, it has only been studied in several model organisms, such as *Tetrahymena* and *Mus musculus*. For example, when *Cfap206* was knocked out, the cilia of

Tetraphymena cells were found to produce abnormal waveforms due to the abnormal RS2 assembly and showed reduced bending amplitude and weakened intertemporal coordination, but there was no significant difference in cilia number and length (Vasudevan et al., 2015). Interestingly, the loss of *Cfap206* also leads to abnormal patterns of bending or curling of sperm flagellum in mice; however, the *Cfap206*-KO mice did show sperm flagella shortening or loss (Shen et al., 2021). Bivalve mollusc is an ancient Lophotrochozoa group of marine invertebrates that possesses rich species diversity. Cilia play critical roles in important physiological activities of bivalve species, such as filter feeding, respiration, reproduction, as well as larval movement. However, knowledge of molecular modulation of ciliogenesis is quite limited in bivalves, and to the best of our knowledge, this is the first study on *Cfap206* to point to the regulation of both cilia and flagella. Notably, many bivalves, such as *Argopecten irradians* (Wei et al., 2021), are hermaphroditic, and a better understanding of the regulation of *Cfap206* on sperm flagellum may help to produce a male sterile population, thus improving artificially crossbreeding efficiency.

The dwarf surf clam (*Mulinia lateralis*) is a small, buried bivalve mollusc belonging to the family Mactridae, which is naturally distributed in estuaries and mudflats along the Atlantic coast from Canada to the Caribbean Sea (Walker and Tenore, 1984). They possess many appealing features for becoming a promising bivalve model, such as small adult size (15–20 mm), rapid development and early transparent shell, short generation time (~2 months), and convenient artificial cultivation (no attachment base) (Calabrese, 1970; Santos and Simon, 1980). In addition, *M. lateralis* represents the first bivalve with successful demonstration of transgene technology (Lu et al., 1996). At present, our team has established a standard laboratory artificial breeding system for *M. lateralis*, and we have successfully achieved gene knockdown through RNAi as well as gene knockout through CRISPR/Cas9 microinjection. Thus, we chose *M. lateralis* to explore the participation of *Cfap206* in both ciliogenesis and spermatogenesis in this study. Our study provides the functional characterization of bivalve *Cfap206* and assists in a better understanding of cilia/flagella regulation in marine invertebrates. Further investigation of the relationship between *Cfap206* and sperm flagellum formation may provide a possible method for the generation of male sterile individuals to improve the crossbreeding efficiency for hermaphroditic marine organisms.

MATERIALS AND METHODS

Identification, Phylogenetic and Spatiotemporal Expression Analysis of *Cfap206* in *M. lateralis*

The available CFAP206 protein sequence of 14 representative species were downloaded from NCBI and Uniprot databases (**Table 1** shows the accession numbers). These orthologous sequences were used as queries for whole-genome blast with the *E*-value threshold of 1*E*–05 in the *M. lateralis* proteome and genome database. To ensure the integrity and accuracy of *Cfap206*, its nucleic acid sequence was predicted using ORF

TABLE 1 | The accession numbers of CFAP206 proteins.

Species	Accession number
Homo sapiens	NP_001026913.1
Crassostrea gigas	XP_011442159.1
Xenopus tropicalis	XP_002938343.1
Macaca fascicularis	XP_005552462.1
Rattus norvegicus	XP_017449021.1
Mizuhopecten yessoensis	XP_021375351.1
Pomacea canaliculata	XP_025093619.1
Actinia tenebrosa	LOC116292837
Orbicella faveolata	XP_020624489.1
Exaiptasia diaphana	XP_020907483.1
Felis catus	XP_003986405.2
Ciona intestinalis	LOC100178966
Psetta maxima	F2P81_018558
Mus musculus	NP_001333991

Finder, and the amino acid sequence was submitted to the SMART database to verify the presence of the CFAP domain. The molecular weights and isoelectric points were calculated by using Compute PL/Mw tools. The genetic structure of *Cfap206* was mapped using the GSDS2.0 website. The Geneious Prime software and PHYRE2.0 website were used to predict the secondary structure and tertiary structure of CFAP206 protein.

CFAP206 protein sequences of 12 species were obtained from the NCBI and Uniprot databases, including *Homo sapiens, Mus musculus, Petromyzon marinus, Crassostrea gigas, Mizuhopecten yessoensis, Mulinia lateralis, Capitella teleta, Aplysia californica, Lingula anatina, Ciona intestinalis, Nematostella vectensis, and Lottia gigantea*. ClustalW (Larkin et al., 2007) and Genedoc Software (Nicholas, 1997) were used for multiple sequence alignment. The phylogenetic analysis of *Cfap206* was performed by MEGA7.0, and the establishment method of the tree was neighbor joining (NJ). The parameter was P-distance, and the value of bootstrap replications was set to 1,000. According to the

transcriptome data of *M. lateralis* obtained in our laboratory, the expression level of *Cfap206* in fourteen embryonic stages and seven adult tissues were plotted.

RNAi Based on Electroporation

Based on unpublished genome and transcriptome information from our lab, the exon and intron of *Cfap206* gene were annotated by MAKER2 (Version 3.01.03) to obtain cDNA sequence information. Also, the interference sites (110–594 bp) were identified using SiDirect Version 2.0. *Cfap206*-dsRNA was synthesized by MEGA Script RNAi kit (Thermo Fisher Scientific, America) (RNAi primers were shown in **Table 2**). Male and female *M. lateralis* with well-developed gonads were selected, then the fresh sperm and eggs were obtained under standard laboratory conditions. After extrusion of the first polar body (around 15 min after mixing the sperm and eggs), each group collected 6,000–7,000 fertilized eggs for electroporation. Bio-Rad Gene Pulser Xcell (America) was selected as the electroporation instrument, and the electric transfer parameters were set according to **Table 3**. We chose filtered seawater as the electroporation buffer: blank control group was not processed; electroporation-control group was given electric shock without dsRNA; and experimental group was added with dsRNA (concentration maintained at 250 ng/µl) and was given electric shock. After 30 min on ice, all groups were cultured at room temperature in a 6-well plate to trochophore larvae stage.

Deletion of *Cfap206* Gene Using CRISPR-Cas9 System

The functional domain of *Cfap206* was predicted using the SMART website. Single-guided RNAs (sgRNAs) were designed on the exons of this domain, and sgRNAs containing SNPs were excluded (sgRNA sequences are shown in **Table 2**). sgRNAs were synthesized using MEGAscript[TM] T7 Transcription Kit (Thermo

TABLE 2 | Primers used for RNAi, qPCR, sgRNA synthesis, and PCR analyses.

Name	Sequence
Cfap206-RNAi-F	TAAAGGCAGTAGTGTTGGACCCATC
Cfap206-RNAi-R	ATTGAAGAGTCTAATTCCCGTCACA
Cfap206-sgRNA-F1	GAAATTAATACGACTCACTATAGTTATGTGTTGATCGATTGAgttttagagctagaaatagcaagttaa
Cfap206-sgRNA-F2	GAAATTAATACGACTCACTATAGGAATTAACAATGATTGTGAgttttagagctagaaatagcaagttaa
Cfap206-sgRNA-F3	GAAATTAATACGACTCACTATACATTCATGTCACTAACAAAGgttttagagctagaaatagcaagttaa
Cfap206-sgRNA-F4	GAAATTAATACGACTCACTATAGAGTGGATTTCAAGATGAGgttttagagctagaaatagcaagttaa
Cfap206-sgRNA-F5	GAAATTAATACGACTCACTATATTGTAGTTGTTCCATACGAGgttttagagctagaaatagcaagttaa
Cfap206-sgRNA-F6	GAAATTAATACGACTCACTATACAGTAACATTTTAGCCAGTTgttttagagctagaaatagcaagttaa
sgRNA-R	AAAAAAAGCACCGACTCGGTGCCAC
Cfap206-verify-F1	AAATCTAGCAGAATTATGAGGTATG
Cfap206-verify-R1	CCAGACCTGAGGAGAACAGCGGA
Cfap206-verify-F2/3	TCGCTGTCGTGAGAGAGGCTA
Cfap206-verify-R2/3	ACATTCTGAGTTGTTGCAGGG
Cfap206-verify-F4/6	CCAATACCTGCCTACCTTGACTTA
Cfap206-verify-R4/6	AACACCACTTAAGAATGGAACCAA
Cfap206-verify-F5	GAGAAGATTCAGTCTGGAGATAGC
Cfap206-verify-F5	GTCAAGGTAGGCAGGTATTGGAAT
Cfap206-qPCR-F	AGATTCAGTCTGGAGATAG
Cfap206-qPCR-R	GTACAGTAGCTTGTAGTTG
RS23-qRCR-F	CCATCAAGATGGGTGAAAC
RS23-qRCR-R	CGAATCCAGCGACTAAGA

TABLE 3 | The electroporation parameters for *Cfap206*-dsRNA delivery.

Criteria	Parameter
Voltage (V)	100
Cuvette (mm)	4
Number of pulse	1
Pulse interval (s)	0
Pulse length (ms)	0.2

Fisher Scientific, America). The editing efficiency of sgRNAs was tested *in vitro* using the Guide-it sgRNA Screening Kit (Takara, Japan). sgRNAs and Cas9 protein in different concentrations were incubated at 37° C for 15 min (**Table 4**), and then, microinjection was performed using fertilized eggs of *M. lateralis*. The control and injected embryos were cultured in filtered seawater at 24°C. The injection volume of the Cas9 protein-sgRNAs mixture is about 0.1 nl. A single embryo was collected approximately 24 h after microinjection, and genomic DNA was extracted by the Chelex®-100 method (Li et al., 2021). The DNA fragment containing the target site was amplified using MightAmp™ DNA Polymerase Ver.3 (Takara, Janpan), and the corresponding verified primers are shown in **Table 2**. The PCR fragments were sequenced in Sangon Biotech.

Plasmid Construction and Induction of Interfering dsRNA

Following the relevant method developed in the Pacific oyster *Crassostrea gigas* (Feng et al., 2019), the interference fragment of *Cfap206* (110–594 bp) was inserted into the L4440 vector to construct the *Cfap206*-dsRNA-L4440 plasmid. The *Cfap206*-dsRNA-L4440 and L4440 plasmids were transfected into competent *Escherichia coli* cells HT115 for expression. The strain HT115 carrying the *Cfap206*-dsRNA-L4440 plasmid was inoculated in LB medium containing ampicillin (50 µg/ml) and tetracycline (12.5 µg/ml) at 37°C and 220 rpm to the logarithmic stage (OD: 0.4–0.6). In total, 0.8 Mm IPTG was added to a 40 ml bacterial solution and stirred at 37°C for 4 h to induce dsRNA. The bacteria of the induced group and the noninduced group were centrifuged at 5,500 rpm for 2 min. After the supernatant was removed, the total RNA was extracted by the Trizol method, and the presence of dsRNA was determined by 1.5% agarose gel.

RNAi by Ingested dsRNA-Expressing Bacteria

Three hundred male *M. lateralis* with healthy growth status, equal size, and immature gonads were selected and divided into three groups (blank-control group, L4440-control group, and *Cfap206*-dsRNA-L4440 group). Eight liters of filtered seawater was added to the glass tank, and each group was fed with a micropump. The water flow rate was controlled at 12 ml/min, and the water temperature was maintained at about 25°C. Each group was temporarily reared for 5 days without treatment to observe the mortality under natural growth state. During the interference process, *Chlorella pyrenoidesa* was fed twice a day from days 1 to 7, with 180 µl (1×10^{10}/ml) each time. In detail, normal algae were fed at 10 am, and normal algae or algae-bacteria were fed at 10 pm for different groups, and the sea water was changed once a day. From days 8 to 14, to speed up gonadal ripening to maturation, 180 µl of normal *C. pyrenoidesa* were fed at 10 am, and normal mixed algae (**Table 5**) or mixed algal-bacteria were fed at 10 pm, and egg yolk was fed continuously during these 2 weeks.

Quantitative RT-PCR

According to the phenol chloroform extraction method described by Hu et al. (2006), the total RNA of embryos was extracted. The Reverse Transcriptase M-MLV (Rnase H-) kit (Takara, Japan) was used to reverse transcription into cDNA, with a concentration of 20 ng/µl after inversion. Fluorescent real-time quantitative PCR was performed using the 2× ChamQ SYBR Color qPCR Master Mix (Vazyme, China). The $2^{-\Delta\Delta t}$ method is used to quantify the expression of *Cfap206*. Quantitative RT-PCR primers are shown in **Table 2**. All qPCR experiments were repeated twice, with two biological replicates and three technical parallels for each sample, using *RS23* as an internal control.

Immunofluorescence

Embryos were washed twice with 1×PBS to remove seawater and fixed overnight with 4%PFA at 4°C. The next day, after being centrifuged at 300 rcf at room temperature for 30 s, the embryos were washed with 1×PBS 3 times. After washing, the sample was dehydrated with 80% methanol and 20% DMSO at 4°C overnight. After washing with PBST (1×PBS+0.5%Triton X-100) 3 times, the sample was then added with 500 µl trypsin and incubated for 5 min. After repeated washing with PBST, the sample was incubated with 10% goat serum (NGS) at room temperature for 2 h. Rabbit antiacetylated tubulin antibody was then added to 10%NGS in a ratio of 1:1,000 and the samples were incubated overnight at 4°C. After washing with PBST three times, goat anti-rabbit FITC was added to 2%NGS/PBST at the ratio of 1:200, and the sample was incubated at room temperature for 2 h. Following washing with PBST, the sample

TABLE 4 | The editing efficiency and survival rate of trochophore larvae after injection.

Cas9 protein concentration (ng/µl)	sgRNA concentration (ng/µl)	Editing efficiency (%)		Survival rate (%)	
		sgRNA4	sgRNA6	sgRNA4	sgRNA6
0	0	0	0	90	90
800	0	0	0	23	20
800	800	43	60	20	18
800	1500	20	19	13	15
800	3000	25	43	5.6	8.3

TABLE 5 | Mixed algae used to ripen *M. lateralis*.

Species	Density (per/ml)	Dosage (μl/h)
Platymonas helgolandica	5×10^8	5
Isochrysis galbana	1×10^9	5
Chaetoceros muelleri	1×10^9	15

was added with phalloidin and DAPI in a ratio of 1:500 and stained for 30 min at room temperature. After being washed with PBS three times, the sample was mounted and the confocal images were taken.

Sperm Motility Test

After 14 days of feeding, sexually mature males were selected for artificial sperm stimulation for each group. Sperm motility was tracked using the CASAS-QH-III automatic sperm analyzer (Tsinghua Tongfang, China). The process was completed by the National Laboratory (Qingdao).

Microscopy

For confocal observation, the cellular morphology was observed with the A1R Ti-E confocal microscopy (Nikon, Japan). For SEM observation, the critical point-dried sample was coated in gold and the sample surface was observed through a Tescan™ Vega3 scanning electron microscope (Czechia).

RESULTS AND DISCUSSION

Identification of *Cfap206* in *M. lateralis* and Phylogenetic Analysis

We identified one *Cfap206* (*MlCfap206*) gene through whole-genome searching of *M. lateralis*, and its genomic structure showed 16 exons, 15 introns, a 5′UTR, and a 3′UTR (**Figure 1A**). The ORF length and protein length of *MlCfap206* were 1875bp and 624 amino acids, respectively. A similar protein structure was revealed for CFAP206 from various species. For example, in mice, *Cfap206* encodes 620-amino acids and possesses the conserved FAP206 domain (Shen et al., 2021) (**Figure 1B**). This may suggest the conservative function of *MlCfap206* related to cilia and flagella. The secondary structure of CFAP206 protein is shown in **Figure 1C**, which contained 27 α-helixes, 25 β-folds, 36 β-bends, and 35 random coils. Based on template d1mhyd (confidence: 87.5), the predicted tertiary ferritin-like structure within the CFAP206 domain (420aa-467aa) is shown in **Figure 1D**.
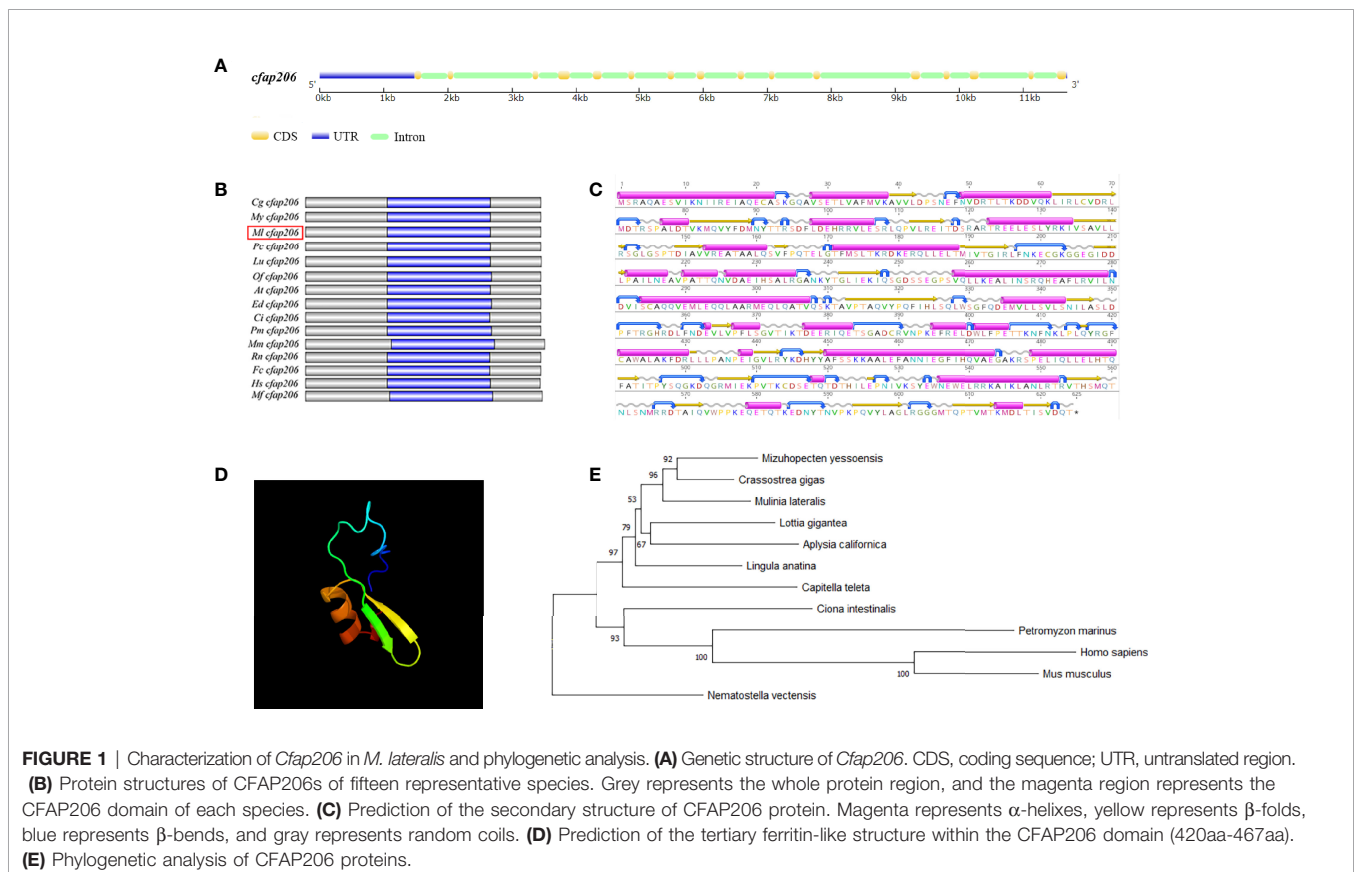
FIGURE 1 | Characterization of *Cfap206* in *M. lateralis* and phylogenetic analysis. **(A)** Genetic structure of *Cfap206*. CDS, coding sequence; UTR, untranslated region. **(B)** Protein structures of CFAP206s of fifteen representative species. Grey represents the whole protein region, and the magenta region represents the CFAP206 domain of each species. **(C)** Prediction of the secondary structure of CFAP206 protein. Magenta represents α-helixes, yellow represents β-folds, blue represents β-bends, and gray represents random coils. **(D)** Prediction of the tertiary ferritin-like structure within the CFAP206 domain (420aa-467aa). **(E)** Phylogenetic analysis of CFAP206 proteins.

Phylogenetic analysis of CFAP206 proteins was performed using the NJ method with 1,000 bootstrap pseudoreplicates (**Figure 1E**), from 12 selected animals spanning Cnidaria (including *N. vectensis*), Brachiopoda (*L. anatina*), Mollusca (including *C. gigas*, *M. yessoensis*, *M. lateralis*, *A. californica*, and *L.gigantea*), Annelida (including *C. teleta*), and Chordata (including *H. sapiens*, *M. musculus*, *C. intestinalis*, and *P. marinus*). Obviously, *Ml*CFAP206 was first grouped together with CFAP206 proteins from molluscs (such as *M. yessoensis* and *C. gigas*). These mollusc CFAP206s were then sequentially clustered with CFAP206s from brachiopods, annelids, and chordates. The clustering relationship of CFAP206 is consistent with the species' evolutionary relationship.
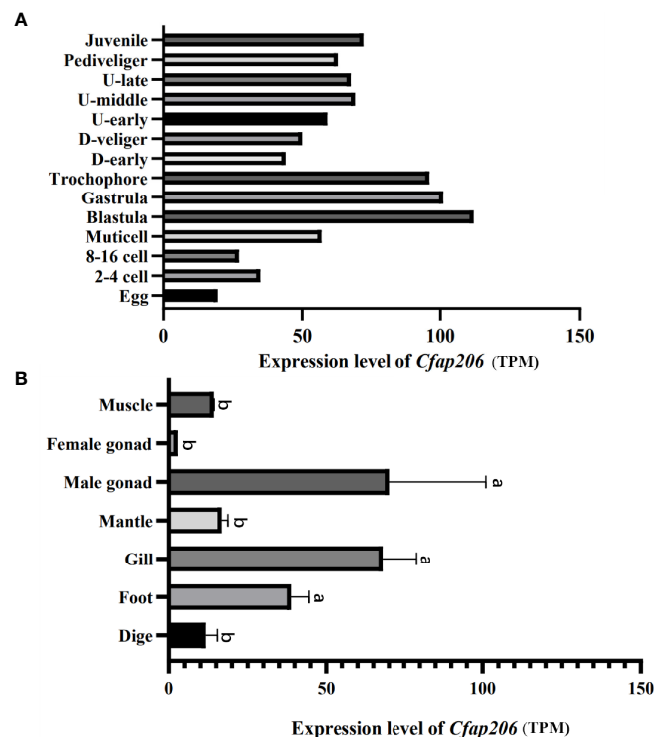
Spatiotemporal Expression Analysis

Spatiotemporal expression analysis of *MlCfap206* in the present study was performed based on our unpublished transcriptomic data. Transcripts per million (TPM) of *MlCfap206* in fourteen embryonic stages as well as in seven adult tissues was used to perform the expression level analysis (**Figure 2**). During development (**Figure 2A**; **Supplementary Table S1**), the high expression level of *Cfap206* is mainly observed in blastula, gastrula, and trochophore larvae, three key periods for embryonic motile cilia formation and development. A similar pattern could be observed in *M. yessoensis* based on the published transcriptome data (Hou et al., 2011), which suggests that *Cfap206* may be required for

embryonic ciliogenesis regulation. Among the seven adult tissues of *M. lateralis*, the highest expression of *Cfap206* was found in the male gonad, followed by foot and gill (**Figure 2B**; **Supplementary Table S2**). Functionalization of these three organs needs specialized cilia for sperm tail formation, byssus attachment, as well as for respiration, food intake, or waste removal through gill filaments (Ward et al., 1993; Andrade et al., 2015; Shen et al., 2021). Lines of evidence showed that *Cfap206* plays important roles in ensuring the stability of RS and CSC, participating in cilia and flagella formation regulation (Dubruille et al., 2002; Vasudevan et al., 2015). Similar to our findings, previous studies have found that *Cfap206* is massively overexpressed in human testicles, and the sperm of mice without the *Cfap206* gene shows abnormalities in the morphology, number, and length of flagella (Shen et al., 2021). To date, cilia regulation of gill has only been reported in marine invertebrates including *Ciona intestinalis* and *C. gigas*, mainly focusing on *calaxin*, *Meichroacidin*, *Tektin A1*, and *Tektin B1* (Shimazaki et al., 2006; Wang et al., 2015), and the regulatory genes of foot cilia have not been reported yet. Our results suggested that *Cfap206* may be involved in these organs' cilia regulation in marine bivalves.

RNAi to Suppress *Cfap206* Expression Affected Embryonic Ciliogenesis

The trochophore larvae possess apical tuft and cilia band which are the typical ciliated features and are responsible for sensation, movement, and feeding (Arenas-Mena, 2010). Moreover, the

FIGURE 2 | Spatiotemporal expression of *Cfap206* in *M. lateralis*. **(A)** The expression of *Cfap206* in fourteen embryonic stages. **(B)** The expression of *Cfap206* in seven tissues. SPSS28.0 was used for one-way ANOVA analysis of the data, and Duncan method was used to test the significance of the difference between the values of each group.

ciliated trochophore larvae are considered to be the most conserved phylogenetic stage of marine Lophotrochozoans, and several pieces of evidence show they share an ancient and common origin (Paps et al., 2015; Xu et al., 2016; Wang et al., 2020). Thus, we chose trochophore larvae to explore whether or not *Cfap206* participate in bivalve embryonic ciliogenesis. Firstly, we generated the *Cfap206*-dsRNA through *in vitro* transcription and the target bands were correctly located (**Figure 3A**). With optimized electroporation conditions and reasonable dose of *Cfap206*-dsRNA (~250 ng/μl), we detected significant suppression of *Cfap206* ($p < 0.01$) in *M. lateralis* trochophore larvae by qPCR (**Figure 3B**). Compared with the control group, the electroporation-blank group showed normal gene expression level, excluding that there might be side effects of electroporation itself on gene expression. Morphological observations of each group of larvae were then made by confocal microscopy and scanning electron microscopy (**Figures 3C, D**). Similar size as well as spindle-shaped body were observed for the larvae from three groups, indicating the normal speed of embryo development. However, obvious cilia reduction/abolishment showed up in the larvae when *Cfap206* was significantly suppressed, demonstrating the irreplaceable requirement of *Cfap206* during embryonic ciliogenesis of *M. lateralis*. Previous studies in model animals such as *Xenopus laevis* and *Tetrahymena* revealed that *Cfap206* is mainly involved with the regulation of cilia motility (Vasudevan et al., 2015). In *X. laevis*, moderate ciliary defect occurred during larva development when *Cfap206* gene was knocked out, leading to abnormal ciliary movement waveform and decreased motor ability. Similarly, the axoneme of the cilia of *Tetrahymena* with *Cfap206* knocked out were compressed, which swam at only 30% of the speed of the wild type (Vasudevan et al., 2015). However, the relationship between *Cfap206* and ciliogenesis is not clear in bivalves. According to our results, it was obvious that the downregulation of *Cfap206* expression in *M. lateralis* led to a significant decrease in the number of cilia. This phenomenon reminded us that there might be function divergence of *Cfap206* on cilia regulation among different species, or *Cfap206* is effective at different hierarchies during signal transduction which is worthy of further in-depth study.

CRISPR/Cas9 Knockout of *Cfap206* Affected Embryonic Ciliogenesis

The efficiency of sgRNAs plays a key role in the editing effect (Li et al., 2021). Thus, to generate *Cfap206*-KO larvae of *M. lateralis*, we first performed the *Cfap206*-sgRNA screening to avoid the influence of SNP and to perform *in vitro* efficiency test. Six sgRNAs (sgRNA1-6) were chosen (**Figure 4A**). Among them, it was found that *Cfap206*-sgRNA-2, *Cfap206*-sgRNA-4, and *Cfap206*-sgRNA-6 could work and cut the target DNA fragment into two pieces *in vitro*, and *Cfap206*-sgRNA-4 seems to be the most effective one (**Figure 4B**). Next, through microinjection together with Cas9 protein (800 ng/μl), we performed the *in vivo* editing efficiency test of these three effective *Cfap206*-sgRNAs. A previous study revealed that the concentration ratio of sgRNA-Cas9 was a key factor in inducing

effective gene mutation (Li et al., 2021). In the present study, three different concentrations of *Cfap206*-sgRNA (800/1,500/3,000 ng/μl) were tested and showed a different editing efficiency as well as survival rate. Unfortunately, after *Cfap206*-sgRNA-2/Cas9 microinjection, no positive editing results turned back. However, *Cfap206*-sgRNA-4 and *Cfap206*-sgRNA-6 still worked *in vivo* and 800 ng/μl was the optimal concentration for microinjection (**Table 4**). In comparison, under optimal concentration, the *in vivo* editing efficiency of *Cfap206*-sgRNA-6 (60%) was higher than *Cfap206*-sgRNA-4 (43%), and the corresponding positive editing results are shown in **Figure 4C**. In *M. lateralis*, the target gene mutations *in vivo* which were induced by *Cfap206*-sgRNA/Cas9 mainly included insertion or deletion mutations within 11 bp, similar to the editing results of other invertebrates (Lin and Su, 2016; Hiruta et al., 2018; Li et al., 2021). In addition, we noticed that the editing efficiency of different sgRNAs might be different *in vitro* or *in vivo*. This may be due to the influence of complex physicochemical factors in the internal environment on the sensitivity of sgRNA, leading to the difference in the efficiency of sgRNA *in vivo* and *in vitro*. Due to the limited number of positive-edited trochophore larvae, we performed bright field morphological observation (about 20–30 larvae were observed) which showed an obvious reduced cilia number after *Cfap206* knocked out (**Figure 4D**), consisting with the *Cfap206*-RNAi phenotype. It reinforced that *Cfap206* played a key role during embryonic ciliogenesis regulation in *M. lateralis*. In addition, CRISPR/Cas9 system has been widely used as a powerful gene editing tool in agriculture and animal breeding, so the CRISPR/Cas9 system we established in *M. lateralis* for mediating gene knockout may be beneficial for trait improvement in marine bivalves.

dsRNA-Expressing Bacteria Feeding Interfered With Adult Ciliogenesis

To further explore whether or not *Cfap206* also participates in the male gametogenesis, we managed to deliver the dsRNA of *Cfap206* to male individuals for 14 days feeding during the gonadal maturation period. We successfully induced *Cfap206*-dsRNA expression in competent HT115 *E. coli* cells with *Cfap206*-dsRNA-L4440 vector (**Figure 5A**). After feeding *Cfap206*-dsRNA-L4440 bacteria, the expression level of *Cfap206* in each group was detected on days 7 and 14. *Cfap206* was markedly suppressed (~70%) in male gonad compared with that of the control group, and no obvious expression change could be detected after L4440-blank feeding (**Figure 5B**). At each check point, the survival rate and body size were roughly equal between different groups, suggesting negligible side effects of this experimental approach. Thereafter, sexually mature individuals from each group were chosen to perform the sperm viability investigation (**Table 6; Supplementary Videos 1–3**). According to the sperm motility, the sperm viability could be rated as A–D four grades, respectively as fast forward movement (A), slow forward movement (B), nonforward movement (C), and extremely slow or inactive movement (D). The results showed that more than 80% of sperms from the control group or L4440-blank group could be rated as grade A/B, while more than 90% of
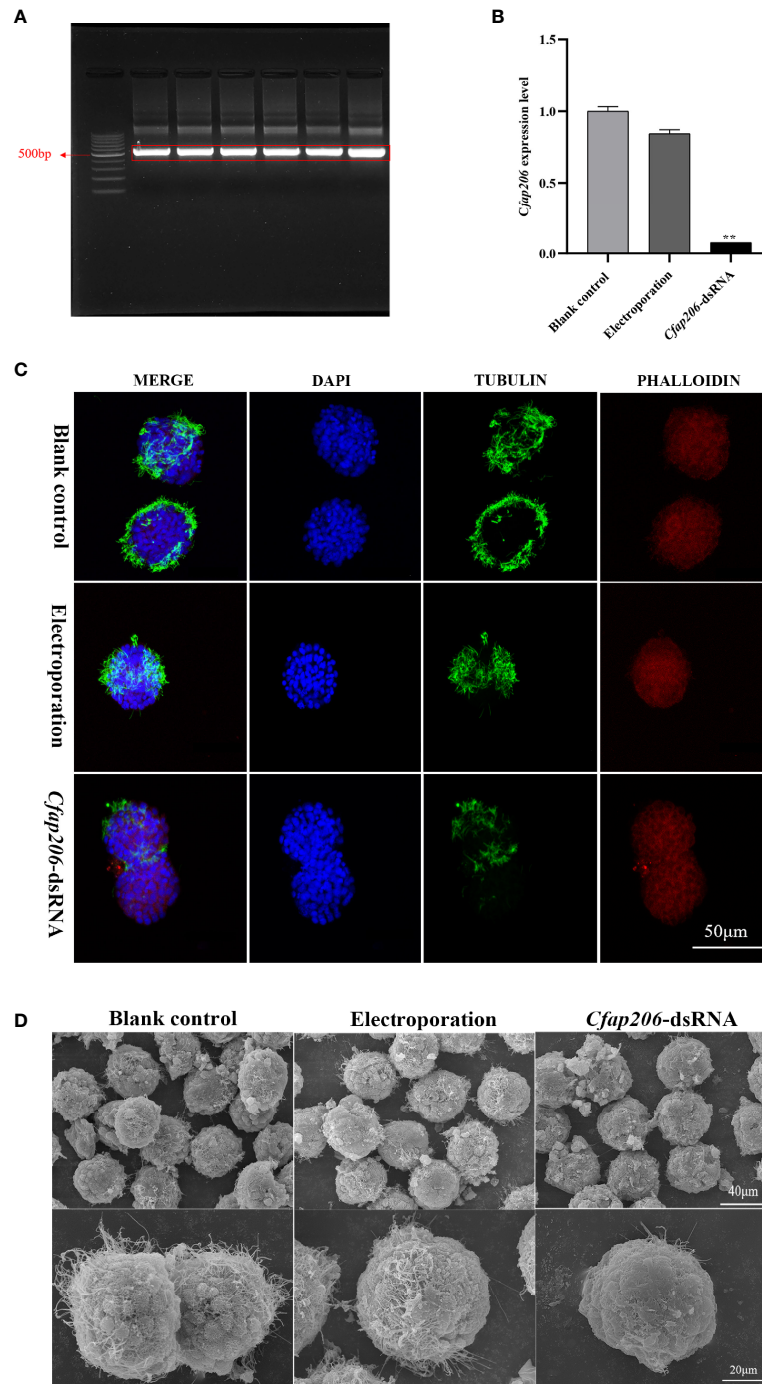
FIGURE 3 | Expression analysis of *Cfap206* and morphological observation of trochophore larvae after RNAi. **(A)** Electrophoresis result of *Cfap206* dsRNA. Lane 1, 100 bp DNA ladder. Lanes 2–7, bands of *Cfap206*-dsRNA (500 bp). **(B)** The expression of *Cfap206* after RNAi (detected by qPCR). **$p < 0.01$. **(C)** Confocal observation of trochophore larvae after RNAi. Cytoskeleton was stained red by phalloidin, nucleus was stained blue by DAPI, and cilia were stained green by tubulin antibody. **(D)** SEM observation of trochophore larvae after RNAi.

sperms from the *Cfap206*-dsRNA-L4440 group was rated as grade D, demonstrating the severe defect of sperm motility after significant suppression of *Cfap206* (**Table 6**). Further morphological observation showed that although the head of

sperm from the *Cfap206*-dsRNA-L4440 group was still normal shaped, the sperm tail appeared to be disintegrated (**Figure 5C**), which could be the obvious cause for their abolished motility. Similarly, it was found that sperm cells with short flagella or
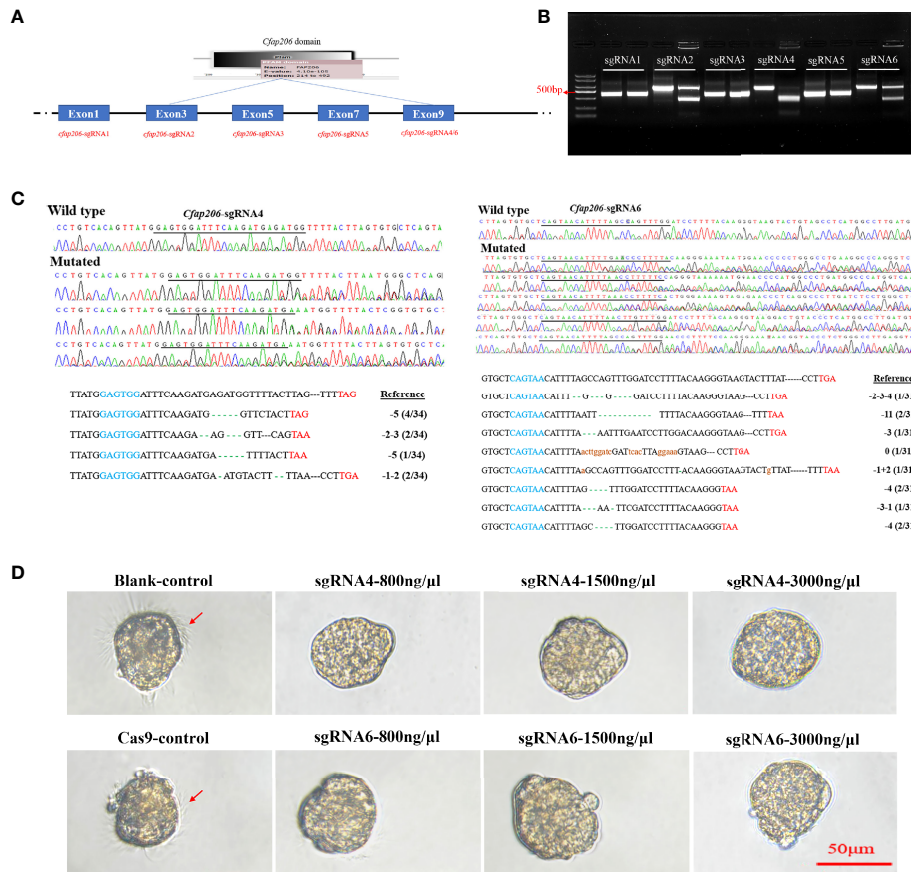
FIGURE 4 | *In vitro* and *in vivo* efficiency detection of *Cfap206*-sgRNAs. **(A)** sgRNA screening for *MlCfap206*. **(B)** *In vitro* cutting efficiency detection of sgRNA1-6. For each sgRNA, the left lane was the control group and the right lane was the experimental group. **(C)** Sanger sequencing of PCR products from injected embryos and the identification of mutations. In the left and right bottom panels, the blue sequence marks the start sequence of *Cfap206*-sgRNA and the red sequence is the stop codon. **(D)** Light microscope observation of trochophore larvae after *Cfap206* knockout.

without flagella were significantly increased in *Cfap206*-KO mice compared with wild-type mice (Shen et al., 2021). Previous studies have shown that the sperm flagellum without *Cfap206* showed severe axoneme disorganization and peri-axonemal defects, resulting in abnormal sperm morphology and decreased motility (Shen et al., 2021). This is because *Cfap206* is closely related to the assembly of the radial spoke RS2 in the axoneme, and the absence of *Cfap206* would result in the radial spoke RS2 either not being assembled or being assembled without the front fork (Shen et al., 2021). *Cfap206* is also a condition for the stable assembly of CSC elements, and CSC connects three major axonemal complexes involved in the dynein regulation of RS2 (Vasudevan et al., 2015). Taken together, our findings showed that *Cfap206* plays critical roles during spermatogenesis, and its participation in the sperm flagella assembly is presented not only in vertebrates but also in marine bivalves. It is worth mentioning that the sexual system of bivalves is diversified, including hermaphrodite, simultaneous hermaphrodite, and sequential hermaphrodite (Collin, 2013). Before the gametes are expelled from the body, hermaphrodite species cannot determine the sex

of their gametes. Considering the important role of *Cfap206* in spermatogenesis, it may be possible to use gene editing technology to knockout *Cfap206* gene to obtain male sterile population, to effectively improve the crossbreeding efficiency of hermaphrodite bivalves.

CONCLUSION

In the present study, we explore the functional role of *Cfap206* during embryonic ciliogenesis as well as during male gametogenesis in a promising bivalve model *M. lateralis*. Through RNAi and CRISPR/Cas9 technologies, *MlCfap206* was effectively knocked down or knocked out in trochophore larvae, and an obvious decreased number of cilia were detected. Through the dsRNA carrier vector feeding, significant suppression of *Cfap206* was observed in the male gonad, together with impaired sperm motility and aberrant sperm tail assembly. Our results suggest that *Cfap206* is critical for the formation and functionalization of both embryonic cilia and
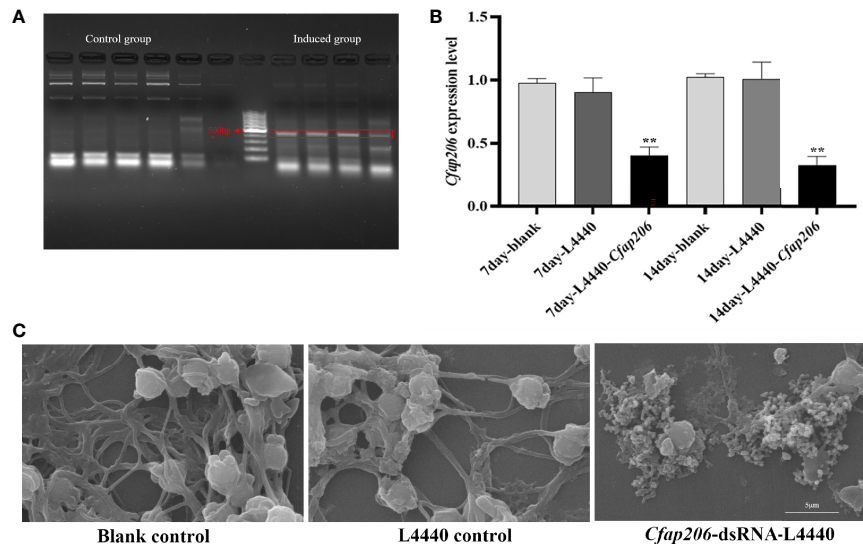
FIGURE 5 | Expression analysis of *Cfap206* and morphological observation of sperm after RNAi. **(A)** Electrophoresis results of dsRNA induced by L4440-*Cfap206*. Lanes 1–5, control group; lanes 8–11, induced group; and lane 7, 100 bp DNA ladder. **(B)** The expression of *Cfap206* on days 7 and 14. **$p < 0.01$. **(C)** Sperm morphology observation using SEM.

TABLE 6 | Sperm motility after feeding *Cfap206*-dsRNA.

Sperm motility	Control	L4440	*Cfap206*-dsRNA-L4440
A	85.5%	78.4%	1%
B	11.6%	8.1%	3%
C	2.9%	10.8%	2%
D	0%	2.7%	94%

sperm flagellum in *M. lateralis*, which may facilitate a better understanding of the ciliogenesis in marine invertebrates. Crossbreeding is an effective way to improve the resistance and other production performance of bivalves in shellfish breeding. However, it is quite challenging to obtain pure gametes for hermaphroditic shellfish, making it difficult to perform crossbreeding. Gene editing technology on target genes (such as *Cfap206* in the present study) would provide a feasible scheme for producing male sterile population to improve the crossbreeding efficiency of hermaphrodite species.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

SL, SW, and ZB conceived and designed the study. YW and XZ performed the experiments. YL and NH participated in data analysis. YW, SL, and XH wrote the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.864037/full#supplementary-material

Supplementary Video 1 | Sperm motility of control group.

Supplementary Video 2 | Sperm motility of L4440-blank group.

Supplementary Video 3 | Sperm motility of *Cfap206*-dsRNA-L4440 group.

REFERENCES

Afzelius, B., and Eliasson, R. (1983). Male and Female Infertility Problems in the Immotile-Cilia Syndrome. *Eur. J. Respir. Dis.* 127, 144–147.

Alten, L., Schuster-Gossler, K., Beckers, A., Groos, S., Ulmer, B., Hegermann, J., et al. (2012). Differential Regulation of Node Formation, Nodal Ciliogenesis and Cilia Positioning by *Noto* and *Foxj1*. *Development* 139, 1276–1284. doi: 10.1242/dev.072728

Andrade, G. R., De Araujo, J. L., Nakamura Filho, A., Guanabens, A. C., Carvalho, M. D., and Cardoso, A. V. (2015). Functional Surface of the Golden Mussel's Foot: Morphology, Structures and the Role of Cilia on Underwater Adhesion. *Mater. Sci. Eng. C Mater. Biol. Appl.* 54, 32–42. doi: 10.1016/j.msec.2015.04.032

Arenas-Mena, C. (2010). Indirect Development, Transdifferentiation and the Macroregulatory Evolution of Metazoans. *Philos. Trans. R Soc. Lond. B Biol. Sci.* 365, 653–669. doi: 10.1098/rstb.2009.0253

Bangs, F., and Anderson, K. V. (2017). Primary Cilia and Mammalian Hedgehog Signaling. *Cold Spring Harb. Perspect. Biol.* 9 (5), a02817. doi: 10.1101/cshperspect.a028175

Beckers, A., Adis, C., Schuster-Gossler, K., Tveriakhina, L., Ott, T., Fuhl, F., et al. (2020). The FOXJ1 Target *Cfap206* Is Required for Sperm Motility, Mucociliary Clearance of the Airways and Brain Development. *Development* 147 (21), dev188052. doi: 10.1242/dev.188052

Calabrese, A. (1970). Reproductive Cycle of the Coot Clam, *Mulinia Lateralis* (Say), in Long Island Sound. *Veliger -Berkeley-* 12, 265–269.

Collin, R. (2013). Phylogenetic Patterns and Phenotypic Plasticity of Molluscan Sexual Systems. *Integr. Comp. Biol.* 53, 723–735. doi: 10.1093/icb/ict076

Danwei, H., Liu, A., Rakeman, A. S., Murcia, N. S., Niswander, L., and Anderson, K. V. (2003). Hedgehog Signalling in the Mouse Requires Intraflagellar Transport Proteins. *Nature* 426, 83–87. doi: 10.1038/nature02061

Dong, F. N., Amiri-Yekta, A., Martinez, G., Saut, A., Tek, J., Stouvenel, L., et al. (2018). Absence of CFAP69 Causes Male Infertility Due to Multiple Morphological Abnormalities of the Flagella in Human and Mouse. *Am. J. Hum. Genet.* 102, 636–648. doi: 10.1016/j.ajhg.2018.03.007

Dubruille, R., Laurencon, A., Vandaele, C., Shishido, E., Coulon-Bublex, M., Swoboda, P., et al. (2002). Drosophila Regulatory Factor X Is Necessary for Ciliated Sensory Neuron Differentiation. *Development* 129, 5487–5498. doi: 10.1242/dev.00148

Elliott, K. H., and Brugmann, S. A. (2019). Sending Mixed Signals: Cilia-Dependent Signaling During Development and Disease. *Dev. Biol.* 447, 28–41. doi: 10.1016/j.ydbio.2018.03.007

Feng, D., Li, Q., and Yu, H. (2019). RNA Interference by Ingested dsRNA-Expressing Bacteria to Study Shell Biosynthesis and Pigmentation in *Crassostrea gigas*. *Mar. Biotechnol. (N. Y.)* 21, 526–536. doi: 10.1007/s10126-019-09900-2

He, X., Liu, C., Yang, X., Lv, M., Ni, X., Li, Q., et al. (2020). Bi-Allelic Loss-Of-Function Variants in CFAP58 Cause Flagellar Axoneme and Mitochondrial Sheath Defects and Asthenoteratozoospermia in Humans and Mice. *Am. J. Hum. Genet.* 107, 514–526. doi: 10.1016/j.ajhg.2020.07.010

Heuser, T., Dymek, E. E., Lin, J., Smith, E. F., and Nicastro, D. (2012). The CSC Connects Three Major Axonemal Complexes Involved in Dynein Regulation. *Mol. Biol. Cell* 23, 3143–3155. doi: 10.1091/mbc.e12-05-0357

Hiruta, C., Kakui, K., Tollefsen, K. E., and Iguchi, T. (2018). Targeted Gene Disruption by Use of CRISPR/Cas9 Ribonucleoprotein Complexes in the Water Flea *Daphnia pulex*. *Genes Cells* 23, 494–502. doi: 10.1111/gtc.12589

Hou, R., Bao, Z., Wang, S., Su, H., Li, Y., Du, H., et al. (2011). Transcriptome Sequencing and *De Novo* Analysis for Yesso Scallop (*Patinopecten yessoensis*) Using 454 GS FLX. *PloS One* 6, e21560. doi: 10.1371/journal.pone.0021560

Hua, K., and Ferland, R. J. (2018). Primary Cilia Proteins: Ciliary and Extraciliary Sites and Functions. *Cell Mol. Life Sci.* 75, 1521–1540. doi: 10.1007/s00018-017-2740-5

Hu, X., Bao, Z., Hu, J., Shao, M., Zhang, L., Bi, K., et al. (2006). Cloning and Characterization of Tryptophan 2,3-Dioxygenase Gene of Zhikong Scallop *Chlamys farreri* (Jones and Preston 1904). *Aquaculture Res.* 37, 1187–1194. doi: 10.1111/j.1365-2109.2006.01546.x

Jakobsen, H. H., Everett, L. M., and Strom, S. L. (2006). Hydromechanical Signaling Between the Ciliate Mesodinium Pulex and Motile Protist Prey. *Aquat. Microb. Ecol.* 44, 197–206. doi: 10.3354/ame044197

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., Mcgettigan, P. A., Mcwilliam, H., et al. (2007). Clustal W and Clustal X Version 2.0. *Bioinformatics* 23, 2947–2948. doi: 10.1093/bioinformatics/btm404

Lin, J., Heuser, T., Carbajal-Gonzalez, B. I., Song, K., and Nicastro, D. (2012). The Structural Heterogeneity of Radial Spokes in Cilia and Flagella Is Conserved. *Cytoskeleton (Hoboken)* 69, 88–100. doi: 10.1002/cm.21000

Lin, C. Y., and Su, Y. H. (2016). Genome Editing in Sea Urchin Embryos by Using a CRISPR/Cas9 System. *Dev. Biol.* 409, 420–428. doi: 10.1016/j.ydbio.2015.11.018

Li, W., Wu, H., Li, F., Tian, S., Kherraf, Z. E., Zhang, J., et al. (2020). Biallelic Mutations in CFAP65 Cause Male Infertility With Multiple Morphological Abnormalities of the Sperm Flagella in Humans and Mice. *J. Med. Genet.* 57, 89–95. doi: 10.1136/jmedgenet-2019-106344

Li, H., Yu, H., Du, S., and Li, Q. (2021). CRISPR/Cas9 Mediated High Efficiency Knockout of Myosin Essential Light Chain Gene in the Pacific Oyster (*Crassostrea gigas*). *Mar. Biotechnol. (N. Y.)* 23, 215–224. doi: 10.1007/s10126-020-10016-1

Lu, J.-K., Chen, T. T., Allent, S. K., Matsubara, T., and Burns, J. C. (1996). Production of Transgenic Dwarf Surfclams, *Mulinia lateralis*, With Pantropic Retroviral Vectors. *Proc. Natl. Acad. Sci. U. S. A.* 93, 3482–3486. doi: 10.1073/pnas.93.8.3482

Lyons, R. A., Saridogan, E., and Djahanbakhch, O. (2006). The Reproductive Significance of Human Fallopian Tube Cilia. *Hum. Reprod. Update* 12, 363–372. doi: 10.1093/humupd/dml012

Nicholas, K. B. (1997). Genedoc: Analysis and Visualization of Genetic Variation, EMBNEW. *Embnew. News* 4, 14. doi: 10.11118/actaun201361041061

Orhon, I., Dupont, N., Pampliega, O., Cuervo, A. M., and Codogno, P. (2015). Autophagy and Regulation of Cilia Function and Assembly. *Cell Death Differ.* 22, 389–397. doi: 10.1038/cdd.2014.171

Paps, J., Xu, F., Zhang, G., and Holland, P. W. (2015). Reinforcing the Egg-Timer: Recruitment of Novel Lophotrochozoa Homeobox Genes to Early and Late Development in the Pacific Oyster. *Genome Biol. Evol.* 7, 677–688. doi: 10.1093/gbe/evv018

Pazour, G. J., and Rosenbaum, J. L. (2002). Intraflagellar Transport and Cilia-Dependent Diseases. *Trends Cell Biol.* 12, 551–555. doi: 10.1016/S0962-8924(02)02410-8

Pazour, G. J., and Witman, G. B. (2003). The Vertebrate Primary Cilium Is a Sensory Organelle. *Curr. Opin. Cell Biol.* 15, 105–110. doi: 10.1016/S0955-0674(02)00012-1

Ross, A. J., May-Simera, H., Eichers, E. R., Kai, M., Hill, J., Jagger, D. J., et al. (2005). Disruption of Bardet-Biedl Syndrome Ciliary Proteins Perturbs Planar Cell Polarity in Vertebrates. *Nat. Genet.* 37, 1135–1140. doi: 10.1038/ng1644

Santos, S. L., and Simon, R. J. L. (1980). Response of Soft-Bottom Benthos to Annual Catastrophic Disturbance in a South Florida Estuary. *Marine Ecol. Prog.* 3, 347–355. doi: 10.3354/meps003347

Satir, P., and Christensen, S. T. (2007). Overview of Structure and Function of Mammalian Cilia. *Annu. Rev. Physiol.* 69, 377–400. doi: 10.1146/annurev.physiol.69.040705.141236

Shen, Q., Martinez, G., Liu, H., Beurois, J., Wu, H., Amiri-Yekta, A., et al. (2021). Bi-Allelic Truncating Variants in CFAP206 Cause Male Infertility in Human and Mouse. *Hum. Genet.* 140, 1367–1377. doi: 10.1007/s00439-021-02313-z

Shimazaki, A., Sakai, A., and Ogasawara, M. (2006). Gene Expression Profiles in *Ciona intestinalis* Stigmatal Cells: Insight Into Formation of the Ascidian Branchial Fissures. *Dev. Dyn.* 235, 562–569. doi: 10.1002/dvdy.20657

Sironen, A., Shoemark, A., Patel, M., Loebinger, M. R., and Mitchison, H. M. (2020). Sperm Defects in Primary Ciliary Dyskinesia and Related Causes of Male Infertility. *Cell Mol. Life Sci.* 77, 2029–2048. doi: 10.1007/s00018-019-03389-7

Stubbs, J. L., Oishi, I., Izpisua Belmonte, J. C., and Kintner, C. (2008). The Forkhead Protein Foxj1 Specifies Node-Like Cilia in *Xenopus* and Zebrafish Embryos. *Nat. Genet.* 40, 1454–1460. doi: 10.1038/ng.267

Tang, S., Wang, X., Li, W., Yang, X., Li, Z., Liu, W., et al. (2017). Biallelic Mutations in CFAP43 and CFAP44 Cause Male Infertility With Multiple Morphological Abnormalities of the Sperm Flagella. *Am. J. Hum. Genet.* 100, 854–864. doi: 10.1016/j.ajhg.2017.04.012

Tao, F., Jiang, T., Tao, H., Cao, H., and Xiang, W. (2020). Primary Cilia: Versatile Regulator in Cartilage Development. *Cell Prolif.* 53, e12765. doi: 10.1111/cpr.12765

Thomas, J., Morle, L., Soulavie, F., Laurencon, A., Sagnol, S., and Durand, B. (2010). Transcriptional Control of Genes Involved in Ciliogenesis: A First Step in Making Cilia. *Biol. Cell* 102, 499–513. doi: 10.1042/BC20100035

Toure, A., Martinez, G., Kherraf, Z. E., Cazin, C., Beurois, J., Arnoult, C., et al. (2021). The Genetic Architecture of Morphological Abnormalities of the Sperm Tail. *Hum. Genet.* 140, 21–42. doi: 10.1007/s00439-020-02113-x

Vasudevan, K. K., Song, K., Alford, L. M., Sale, W. S., Dymek, E. E., Smith, E. F., et al. (2015). FAP206 Is a Microtubule-Docking Adapter for Ciliary Radial Spoke 2 and Dynein C. *Mol. Biol. Cell* 26, 696–710. doi: 10.1091/mbc.E14-11-1506

Verhey, K. J., Dishinger, J., and Kee, H. L. (2011). Kinesin Motors and Primary Cilia. *Biochem. Soc. Trans.* 39, 1120–1125. doi: 10.1042/BST0391120

Vij, S., Rink, J. C., Ho, H. K., Babu, D., Eitel, M., Narasimhan, V., et al. (2012). Evolutionarily Ancient Association of the Foxj1 Transcription Factor With the Motile Ciliogenic Program. *PloS Genet.* 8, e1003019. doi: 10.1371/journal.pgen.1003019

Walker, R. L., and Tenore, K. R. (1984). Growth and Production of the Dwarf Surf Clam *Mulinia lateralis* (Say 1822) in a Georgia Estuary. *Gulf Res. Rep.* 7, 357–363. doi: 10.18785/grr.0704.07

Wang, X., Liu, B., Liu, F., and Huan, P. (2015). A Calaxin Gene in the Pacific Oyster *Crassostrea gigas* and Its Potential Roles in Cilia. *Zoology. Sci.* 32, 419–426. doi: 10.2108/zs150009

Wang, J., Zhang, L., Lian, S., Qin, Z., Zhu, X., Dai, X., et al. (2020). Evolutionary Transcriptomics of Metazoan Biphasic Life Cycle Supports a Single Intercalation Origin of Metazoan Larvae. *Nat. Ecol. Evol.* 4, 725–736. doi: 10.1038/s41559-020-1138-1

Ward, J. E., Macdonald, L. B. A., and Thompson, R. J. (1993). Mechanisms of Suspension Feeding in Bivalves Resolution of Current Controversies by Means of Endoscopy. *Limnol. Oceanography* 38, 466. doi: 10.4319/lo.1993.38.2.0265

Wei, H., Li, W., Liu, T., Li, Y., Liu, L., Shu, Y., et al. (2021). Sexual Development of the Hermaphroditic Scallop *Argopecten irradians* Revealed by Morphological, Endocrine and Molecular Analysis. *Front. Cell Dev. Biol.* 9, 646754. doi: 10.3389/fcell.2021.646754

Xu, F., Domazet-Loso, T., Fan, D., Dunwell, T. L., Li, L., Fang, X., et al. (2016). High Expression of New Genes in Trochophore Enlightening the Ontogeny and Evolution of Trochozoans. *Sci. Rep.* 6, 34664. doi: 10.1038/srep34664

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