A transgenic zebrafish for in vivo visualization of cilia

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.6125214.

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Cilia are organelles for cellular signalling and motility. Mutations affecting ciliary function are also associated with cilia-related disorders (ciliopathies). The identification of cilia markers is critical for studying their function at the cellular level. Due to the lack of a conserved, short ciliary localization motif, the full-length ARL13b or 5HT6 proteins are normally used for cilia labelling. Overexpression of these genes, however, can affect the function of cilia, leading to artefacts in cilia studies. Here, we show that Nephrocystin-3 (Nphp3) is highly conserved among vertebrates and demonstrate that the N-terminal truncated peptide of zebrafish Nphp3 can be used as a gratuitous cilia-specific marker. To visualize the dynamics of cilia in vivo, we generated a stable transgenic zebrafish Tg (β-actin: nphp3N-mCherry) su1001. The cilia in multiple cell types are efficiently labelled by the encoded fusion protein from embryonic stages to adulthood, without any developmental and physiological defects. We show that the line allows live imaging of ciliary dynamics and trafficking of cilia proteins, such as Kif7 and Smo, key regulators of the Hedgehog signalling pathway. Thus, we have generated an effective new tool for in vivo cilia studies that will help shed further light on the roles of these important organelles.

1. Introduction

Cilia are microtubule-based organelles found on the surface of most eukaryotic cells, where they play roles in cell motility, sensory reception and signal transduction. Cilia are classified as motile cilia or non-motile primary cilia (PC), properties reflected in their different microtubule organization [1]. Motile cilia, normally with a ‘9 + 2’ microtubule structure, are found mainly on the surface of migratory cells and some functionally specialized epithelial cells where their synchronized beating facilitates cell movement or fluid clearance, respectively [2]. The PC, by contrast, is a single antenna-like organelle composed of a ‘9 + 0’ microtubule structure and cell membranes attached [3]. PC are important for processes such as sensing mechanical and chemical signals from the extracellular microenvironment. They serve as a hub for the reception and processing of extracellular signals, regulating their integration with receptors and subsequent transduction of the signal inside the cell. Various molecules such as receptors, scaffolds, ion channels, adapter molecules and effector enzymes have been shown to be trafficked in PC, referred to as intraflagellar
trafficking, making this organelle a vital subcellular compartment for cell proliferation, differentiation, metabolism, etc. [4–7]. Several signalling pathways in vertebrates that are critical for cell differentiation and embryonic development, including Hedgehog (Hh) and PDGF, are PC-dependent [8–11]. In humans, mutations in genes required for cilia structure or function led to cilia-associated disorders (ciliopathies), such as ciliary dyskinesia, polycystic kidney disease and craniofacial abnormalities [12–14].

Although the importance of cilia function has long been recognized, progress in their study, especially PC, has been limited by the lack of efficient methods for analysing these tiny subcellular compartments. One important goal is to elucidate the changes in cilia morphology and trafficking of cilia-localized components following the manipulation of genes or cells. To date, this has relied largely on immunohistochemistry (IHC) with fixed materials. Although much information on cilia and cilia-associated cell behaviors has been obtained in this way, the method has its limitations. The real-time dynamic changes inside of cilia in vivo cannot be resolved by IHC. And the approach is time-consuming and labour-intensive when troubleshooting artefacts. Implementing in vivo cilia labelling can in principle address these limitations and enable real-time cilia monitoring. To achieve this, a suitable cilia localization signal is needed to generate specimens in which the cilia are reproducibly labelled. While numerous proteins are localized to cilia, no conserved cilia localization signal has so far been identified. In tissue culture studies, the commonly used proteins for cilia labelling are ARL13b and serotonin receptor 6 (5HT6). ARL13b, a member of the ADP-ribosylation factor-like family, is a GTPase protein that anchors the ciliary membrane and plays an important role in controlling ciliary trafficking [15–19], whereas 5HT6 is a member of the G-protein-coupled receptor (GPCR) family and functions in regulating cilia length, dendritic morphology and cilia protein localization [20–23]. In the absence of short ciliary targeting sequences, C terminal fusions of their full-length forms to fluorescent proteins have been used for cilia labelling. However, as both proteins are essential for cilia structure and function, overexpression of either has effects on cilia, which can affect the processes being analysed [21,24–26]. In addition, the use of the transient expression for labelling purposes leads to inconsistencies among cells with different expression levels. Identification of shorter, more specific, and functionally neutral ciliary peptides could obviate these limitations.

Nephrocystin-3 (NPHP3), identified as a protein associated with nephronphthisis, a genetic ciliopathy, was reported to be localized at the Inv compartment, a distinct proximal segment of the ciliary body [27,28]. Current knowledge of Nphp3 protein shows that it is involved in biogenesis, assembly and disassembly of cilia, as well as transport of certain molecules to cilia, such as myristoylated proteins. Domain analysis indicates that the N terminal region (1–204 amino acid residues [aas]) of mouse Nphp3 is sufficient to drive the localization of the fluorescent protein to the PC in tissue culture without affecting the structure and function of cilia, indicating that it is a good candidate for a ciliary label [29]. Here, we have identified the zebrafish homolog of nphp3 and analysed its spatio-temporal expression during embryogenesis. After confirming its N-terminal ciliary targeting sequence (CTS) peptide, a stable transgenic zebrafish line, named sx1001, has been established by genomic integration of znphp3N-mCherry driven by the β-actin promoter. The cilia of the sx1001 line are uniformly labelled by red fluorescence in both embryonic stages and adult tissues, enabling live in vivo imaging of cilia. We present the characterization of this line and illustrate its use in analysing the processing and mechanism of the cilium during development.

2. Results

2.1. Expression analysis of zebrafish Nphp3

We analysed the temporal expression of zebrafish nphp3 (znphp3), by RT-PCR and quantitative RT-PCR of embryos and larvae from 4 hpf (hours post-fertilization) to 120 hpf. Weak maternally derived znphp3 transcript was detected at 4 hpf, prior to the onset of zygotic transcription. An abundance of nphp3 mRNA increased dramatically from 24 hpf to 72 hpf, then declined slightly, until 120 hpf (figure 1a). The spatial distribution of mRNA was analysed by in situ hybridization (ISH) of embryos and larvae from the 4 hpf to 120 hpf, using a DIG-labelled RNA probe against the 3′ end of the nphp3 coding region (electronic supplementary material, table S2). Transcripts were detectable in all the stages assayed. Moderate signals were observed at sphere stage (figure 1b). At 10 somite stage (ss), the znphp3 mRNA was evenly distributed throughout the embryo, while at 24 hpf, it accumulated ventrally in the retina and the horizontal myoseptum (figure 1c,d). This spatially regulated pattern became much more obvious at 48 hpf (figure 1e). The ISH signal in the retina reduced from 96 hpf to 120 hpf, while the signal at the horizontal myoseptum remained and expanded into the intervening muscle fibres. The transcript was also detected at most of the somite boundaries (figure 1f–i). In addition, cross sections at 120 hpf revealed weak znphp3 mRNA could be detected in the glomerulus of the pronephros, suggesting its conserved function in kidney development (figure 1h).

2.2. Transient expression of zebrafish full-length Nphp3 and its N-segment truncation protein localized to primary cilia

Protein sequence analysis revealed that znphp3 shares 67.04% similarity with mouse Nphp3 and 69.02% with human NPHP3 [27,29] (electronic supplementary material, figure S1A). To test whether the protein also localizes to PC in zebrafish, mRNA encoding znphp3 fused to eGFP at its C terminus was injected into one-cell stage embryos. The znphp3-eGFP fusion protein was robustly expressed in injected embryos and its subcellular localization was assayed by immunofluorescence (IF) staining in 18 hpf embryos. To visualize the PC, injected embryos were incubated with a fluorescently labelled antibody against acetylated tubulin (Ac-tub). Like its mouse orthologue, the tagged znphp3 specifically localized to PC in the otic vesicle, neural tube, and myotome (electronic supplementary material, figure S1B–D).

Previous studies showed that a 204 residue N-terminal region of mouse Nphp3, containing a conserved Gly myristoylation site (G2) and CC domain is the minimum peptide required for cilia localization [29]. We found that the first 185
drive proteins of interest to the cilia in zebrafish embryos. Thus, zNphp3N can be used as a PC-specific peptide to shaft targeting is dependent on the N-terminal myristoylation with only faint labelling of the basal body (electronic supplementary material, figure S2); this indicates that ciliary region during 24–48 hpf; (i) the expression at the horizontal myoseptum remained and spread to the intermediated muscle fibres at 96 hpf; (g–i) znphp3 mRNA was found at the majority of the somatic borders and was slightly expressed at the pronephros glomerulus at 120 hpf (as shown in the dotted line in g). Arrows point to tissues with enriched expression of znphp3. Scale bar, 100 μm.

The αααs of znphp3 are equivalent to the first 204 amino acids of mouse Nphp3 and the first 208 amino acids in human NPHP3. The znphp3N shares 86.16% similarity with that of mouse Nphp3N, as well as the critical myristoylation Gly site and the CC domain, suggesting that the znphp3N may be sufficient for ciliary targeting in zebrafish (figure 2a). As predicted, an N-terminal fusion of eGFP to znphp3N localized to the cilia shaft in various cells of transiently microinjected 18 hpf embryos (figure 2b–c). By contrast, mutant znphp3N (1–185: G2A)-GFP signals were largely restricted to the cytoplasm, with only faint labelling of the basal body (electronic supplementary material, figure S2); this indicates that ciliary shaft targeting is dependent on the N-terminal myristoylation site. Thus, znphp3N can be used as a PC-specific peptide to drive proteins of interest to the cilia in zebrafish embryos.

2.3. Generation of a stable znphp3N-mCherry transgenic line

To generate a stable line in which the cilium is labeled in all cell types, a Tol2 transgenic construct carrying znphp3N-mCherry driven by the β-actin promoter was assembled into pMiniTol2 plasmid by Gibson Assembly method [30] (figure 3a), and introduced into zebrafish embryos by microinjection together with Tol2 transposase mRNA. Stable integrants were identified by fluorescent microscopic screening and six lines were established. The line with the strongest red fluorescence, designated Tg (β-actin: nphp3N-mCherry)sx1001 (sx1001 hereafter), was used for all subsequent analyses in this study. The sx1001 fish developed normally and were fertile. The znphp3N-mCherry transgene is maternally expressed as all the embryos from sx1001 females crossed to wild-type males had strong red fluorescence at 4 hpf. The znphp3N-mCherry protein expression in sx1001 was further confirmed by Western blot analysis as shown in figure 3b. To analyze the PC labelling further, immunostaining against Ac-Tub was performed in 18 hpf transgenic embryos. As illustrated in figure 3c–f, in all cell types analysed, including eye, otic vesicle, neural tube, and myotome, the vast majority of PC labelled by Ac-Tub were also mCherry positive, indicating that the znphp3N-mCherry is a robust marker of the PC. The znphp3N-mCherry was evenly distributed along the whole ciliary shaft with only weak cytoplasmic signals outside of PC. We note that a few cilium-like subcellular structures were labelled by mCherry, but were Ac-Tub negative; this reflects an artefact of the Ac-Tub staining.

2.4. Integration and expression of znphp3N-mCherry in sx1001 has no discernible effect on PC structure or function

Since the abnormal expression or loss of full-length znphp3 leads to cilia abnormalities [31,32], we investigated whether sustained expression of the znphp3N-mCherry fusion protein in embryos impacts the growth and development of transgenic animals, and/or cilia morphology and function. As previously mentioned, no abnormal phenotypes were found among sx1001 transgenic animals at either 24 hpf, 5 dpf or adulthood (n > 5). Nor was significant developmental delay observed (electronic supplementary material, figure S3). No obvious morphological defects were observed in the PC of sx1001 embryos at 18 hpf compared to wild-type in all tissues analysed (figure 4a–d). In addition, the length of PC in the eye, otic vesicle, neural tube, and myotome showed no significant difference from wild-type (figure 4e–h). These results indicate that neither the integration nor expression of the znphp3N-mCherry transgene have deleterious effects, making the transgenic line suitable for investigating structure and function.
Figure 2. Transiently expressed N-terminal peptide of zNphp3 (zNphp3N) fused eGFP localized to PC in zebrafish embryos. (a) Sequence alignment of zNphp3N with human and mouse homologs. The Clustal Omega program was used to align protein sequences from D. rerio (zebrafish), H. sapiens (human) and M. musculus (mouse). High and low amino acid similarities are emphasized in dark and light gray, respectively. (b–e2) At 18 hpf embryos, the transiently expressed fluorescent fusion protein zNphp3N-eGFP was precisely localized in the primary cilia of various cells in the otic vesicle, neural tube, and myotome. (b–e) Ciliary localization of zNphp3N-eGFP protein (in green) expressed transiently in zebrafish embryos. (b1–e1) Cilia labelled by anti-AcTub (red). (b2–e2) Co-localization of transiently expressed zNphp3N-eGFP and cilia labelled by anti-AcTub. Arrows indicate places with obvious co-localization. The frame denotes the enlarged site. Scale bar, 5 µm.
As further confirmation of the gratuitous nature of the tagged peptide, we investigated Hh pathway activity in sx1001 animals. No significant effects on the expression of *ptch1* and *ptch2*, two critical Hh pathway target genes, were detected by qRT-PCR in sx1001 compared to wild-type (electronic supplementary material, figure S4A-B). Additionally, the Hh-dependent specification of muscle cell types was analysed in sx1001 embryos. The superficial slow fibres (SSFs), muscle pioneer cells (MPs) and medial fast fibres (MFF) which are specified by different levels of Hh signalling activity [34], all appeared normal, as revealed by Prox1a+, Eng2a+ and double-positive cells at 24 hpf, respectively (electronic supplementary material, figure S4C–J). Taken together, these data support the conclusion that the transgenic integration and ciliary accumulation of Nphp3N-mCherry in sx1001 has no obvious impact on the structure or functions of PC.

### 2.5. Sx1001 facilitates live imaging of cilia

We next asked whether the sx1001 line is suitable for imaging cilia in live embryos and larvae. Firstly, the PC of sx1001 embryos at 24 hpf were examined. Consistent with the findings in fixed tissue, the PC in the retina, otic vesicle and neural tube in sx1001 clearly expressed red fluorescence (figure 5a–c). Similarly, in live 3 dpf sx1001 larvae, the different ciliary structures were also labelled (figure 5d–i). For example,
zebrafish have both motile and non-motile cilia, and both types of cilia were clearly labelled in sx1001. The kinocilia of the inner ear, which are preserved throughout adulthood and play a role in hearing, were also visible in live sx1001 larvae. In conclusion, sx1001 can precisely and in real-time display the status of different cilia in a variety of cell types in vivo.

2.6. Cilia in adult tissues of sx1001 are uniformly labelled

Having shown that sx1001 efficiently labels various cilia in embryos, we next investigated if this property persists into adulthood. To observe the distribution of labelled cilia in adult fish, we examined various organs including the eye, brain, heart, intestine, liver, kidney and muscle by cryosectioning and immunofluorescence staining. Cilia labelled by zNphp3N-mCherry were observed in all these organs (figure 6a–f), varying in number and length. Although cilia could be seen in all types of vertebrate cells, not all cells are ciliated due to their differing cell cycle status. More and longer cilia were observed in the intestine and retina, while fewer and shorter cilia were found in heart, liver, kidney and somatic muscles (figure 6g). This variation in cilia length and number may reflect functional differences among these tissues.

2.7. Sx1001 facilitates in vivo analysis of vertebrate Hh signalling

Most of the key components of the Hh pathway, including PTCH, SMO, KIF7, SUFU and GLI, are regulated through

Figure 4. Nphp3N-mCherry integration into sx1001 has no influence on the cilium morphology. (a–d) In all tissues examined, no noticeable difference in cilia morphology was detected between sx1001 and wild-type embryos. (a–d), cilia labelled by anti-AcTub in wild-type embryos at 18 hpf (green). (a’–d’) Cilia marked by sx1001 (red); nuclei were labelled by Hoechst (blue). Scale bar, 5 μm. (e–h) Comparative measurements of the length of cilia in the eyes, otic vesicle, neural tube and myotome from wild-type (black bar) and sx1001 (grey bar) using the maximum intensity projection (MIP) method [33]. Each sample has been measured the length of 20 straight cilia (n = 4 for each sample). Unpaired Student’s t-test was used for analysis (n.s., not significant).
translocation to the PC. The sx1001 line provides the possibility of monitoring this translocation in vivo. Fluorescently tagged Kif7-GFP and Smo-GFP fusion proteins were transiently expressed in sx1001 embryos. Consistent with previous studies of fixed specimens, both fusion proteins showed specific ciliary localization [35–37]. The Smo-eGFP was distributed throughout the shaft of the PC, while Kif7-eGFP accumulated at the tip of the PC in otic vesicles, neural tube and myotome (figure 7a–f). These findings illustrate the utility of the sx1001 line for real-time monitoring of Hh-pathway components in a living organism.

3. Discussion

Nphp3 is a ciliary protein that has been reported to function in the maintenance of cilia number and cAMP levels [32,38]. The zebrafish Nphp3 (znphp3) shares 204 aas with mouse Nphp3 and 208 aas with human NPHP3. Although the functions of znphp3 in development at embryonic stages have been studied using morpholino-mediated knockdown, its spatio-temporal expression pattern has not previously been reported. Our qRT-PCR and ISH analyses detected znphp3 transcript at 4 hpf, suggesting a maternal contribution of zNphp3 protein. Subsequently, the expression of znphp3

Figure 5. sx1001 enabled the live imaging of cilia at the tissue level. (a–c) In living sx1001 at 24 hpf, the cilia of the embryo were clearly visible in red fluorescence in the retina, otic vesicle, and spinal canal. Histone2A with C terminal fused eGFP was used to label the nucleoli in live embryos. In live 3 dpf sx1001 larvae, the various ciliary structures have been revealed and identified with a red fluorescent signal from different tissues as indicated (d–i). The frame denotes the enlarged site. Scale bar, 5 µm.
dramatically increased after 24 hpf, implying an important function at these stages. Consistent with this, knockdown of \( \text{znphp3} \) has been shown to result in body curvature, hydrocephalus, positional inversion and pronephric cysts [32]. In line with its function in the kidney, we observed weak expression of \( \text{znphp3} \) in the pronephric glomerulus. Strong expression in the horizontal myoseptum and somite boundaries is consistent with a function in muscle development, potentially in connections between muscle fibres. Expression in muscle is not uniform, as no signal was seen in the first few somites or in the caudal somites.

By contrast to the signal peptides of secreted proteins or the nuclear localization signals of nuclear proteins, ciliary targeting sequences seem quite diverse. Even the ciliary...
targeting peptide (CTP) in the third intracellular loop of the GPR161 and 5HT6 GPCR proteins is not conserved between these two ciliary proteins, despite their belonging to the same family [20,28,39]. Thus, to date, labelling cilia for in vivo monitoring has relied upon the use of whole fluorescently tagged cilia proteins, such as ARL13b and 5HT6. As these fusion proteins are functional, their overexpression is likely to affect the status of cilia. Therefore, we explored the use of the N terminal peptide of zNphp3 fused with eGFP as an alternative gratuitous marker of cilia. Our finding that Hh pathway activity is unaffected by expression of this fusion protein in sx1001 transgenic fish, supports the view that it has no deleterious effects on cilia function. In addition, we have tested both zNphp3N-eGFP and zNphp3N-mCherry fusions, and found that both localize to the whole ciliary shaft. This raises the possibility that zNphp3N could be used to drive any peptide of interest to the cilia structure.

Although no conserved motif for ciliary localization has been identified, there were some clues for the conservation of the ciliary localization signal. The first eight amino acids of Nphp3N are known to be substantially conserved among vertebrates. This sequence matches the recognition sequence for N-myristoyltransferase: M-G-XX-X-S/T-. The GFP signals from the myristoylation site mutant, in which the second glycerine was replaced with alanine, were lost in the ciliary shaft but persisted in the cytoplasm [29]. Conserved with mouse Nphp3N, the G2 of zNphp3N is critical for its ciliary localization. Consistently, in our study, a G to A mutation caused an impairment in zNphp3N ciliary shaft targeting (electronic supplementary material, figure S2). This G2 is supposed to be a myristoylation modification, a type of lipid acylation modification. Furthermore, there are also some other lipid modifications, such as prenylation and palmitoylation that are supposed to be important for proper ciliary localization. For example, the palmitoylation modification at the C-terminus of 5-HT is critical for its ciliary trafficking. Interestingly, the conjugated modification by cholesterol at Asp95 of mouse Smo has been reported to be essential for its localization to the PC, suggesting that lipid modification is a shared requirement for ciliary localization. Further investigation should be focused on the identification of the lipid modification types and sites, potential enzymes and the mechanism of ciliary accessing control.

A growing number of studies have identified critical cellular signalling pathways that act through PC. To our knowledge, this is the first time that the zNphp3-specific cilia localization sequence has been used to generate a cilia-tagged transgenic zebrafish line for live-cell dynamic imaging of cilia. This stably inheritable transgenic line not only enables accurate, real-time monitoring of cilia state in multiple cell types in vivo, but also represents a powerful tool for functional research of receptors and regulatory proteins in cilia at the output of balanced signalling cascades.

4. Experimental procedures

4.1. Zebrafish husbandry

Adult fish, AB strain, imported from China Zebrafish Resource Center, was maintained at 28°C on a 14 h light/10 h dark cycle in Shanxi University zebrafish facility. Embryos were collected from natural crosses and raised in E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl_{2} \cdot 2H_{2}O, 0.33 mM MgSO_{4} \cdot 7H_{2}O), and staged under the standard protocol [40]. Phenylthiourea (PTU, 0.003%) was added to inhibit pigment formation for embryos older than 24 hpf.

4.2. DNA constructs, RNA synthesis, injection and generation of zebrafish transgenic lines

The transient expression constructs of pCS2+_npmp3-eGFP and pCS2+_npmp3N-eGFP, and the transgenic construct of
pMiniTo12-β-actin-nphp3N-mCherry were generated by Gibson assembly. The primers used for cloning were listed in electronic supplementary material, table S2. The transient expression constructs of pCS2+-kif7-eGFP, pCS2+-smo-eGFP and pDB600 were as described [35,41].

For caged mRNA synthesis, the transient expression constructs were linearized by NotI or XbaI for depending on the experiment design. For generating the transgenic lines, the pMiniTo12-β-actin-nphp3N-mCherry was coinjected with Tol2 transposase mRNA into one-cell stage embryos, and the T0 was screened at 24 hpf. The embryos with strong red fluorescence were grown as potential founders. The T0 were out-crossed with wild-type when grown up, and T1 with strong red fluorescence was screened out as the stable transgenic lines.

4.3. RNA isolation, RT-PCR and quantitative RT-PCR
Total RNA was extracted from zebrafish embryos using Trizol at different stages (Invitrogen). 1 mg of RNA was then used to synthesize the cDNA using SuperScript IV reverse transcriptase (Invitrogen) and oligo(dT) primers (Sangon Biotech). The RT-PCR was performed using the 2× EasyTaq PCR SuperMix (Trans). For quantitative RT-PCR, about 0.2 µl of cDNA was used and the qRT-PCR was performed using GoTaq qPCR Master Mix (Promega) on BioRad qRT-PCR system. Normalization was done against β-actin and primers were listed in electronic supplementary material, table S2.

4.4. Whole-mount in situ hybridization
The C terminal of zebrafish npnp3 including parts of its 3’UTR was cloned to pGEM-T easy (Promega) as the RNA probe template. The digoxigenin-labelled npnp3 RNA probe was synthesized by Roche RNA labelling Kit. in situ hybridization was carried out as previously described [42]. The images were taken by Imager M2 microscopy.

4.5. Western blot analysis
The embryo was lysed in lysis buffer (20 mM Tris HCl, pH 7.4; 150 mM NaCl; 1% TritonX-100; 10% Glycerol; 2 mM EDTA; 1 mM PMSF). Samples were microcentrifuge for 20 min at 4°C, loading buffer (37.5 mM Tris HCl, pH 7.4; 3% SDS; 0.01% bromophenol blue; 6.25% glycerol; 100 mM DTT) was added to the supernatant and the equivalent of 30 embryos run on each lane of a 10% acrylamide denaturing gel, and electrophoresis onto Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore). Rabbit anti-mCherry (1:1000; Sigma) or rabbit anti-γ-tubulin (1:3000; Sigma) were diluted in blocking buffer and incubated with membranes at 4°C overnight. Membranes were incubated with HRP conjugated secondary antibody for 1 h at RT. Proteins were detected with an infrared imager (GE Healthcare).

4.6. Cryosection and immunofluorescence
Fresh tissues were dissected from 3 months of zebrafish, then rinsed quickly with cold PBS. After 2 h of fixing in 4% paraformaldehyde, tissues were embedded in 2% low-melting agarose and incubated in 30% sucrose at 4°C overnight. The samples were cryosectioned with the slice thickness of 20 µm, and then proceeded to immunofluorescence. Specific immunostaining methods were performed as described [43]. Hoechst was used to stain the nucleus, see electronic supplementary material, table S1 for exact dilution concentrations.

For antibody immunostaining at embryonic stages, embryos were fixed in 4% paraformaldehyde at room temperature for 2 h and then were permeabilized in acetone at −20°C for 7 min, then quenched. After 1 h of incubation in blocking solution (DPBS, 1% BSA, 0.5% TritonX-100) at room temperature, primary antibodies diluted in blocking solution were added as described in electronic supplementary material, table S1 and the incubation was performed overnight for incubation at 4°C. After subsequent washes in DPBS-Triton, embryos were incubated with Alexa conjugated secondary antibodies (Alexa Fluor 488 anti-mouse and Alexa Fluor 647 anti-rabbit) and Hoechst overnight at 4°C. Again, Embryos were washed several times in DPBS-Triton and soaked in DPBS with 70% glycerol for imaging. All of the antibodies used in this study, see electronic supplementary material, table S1. Fluorescence was visualized on an LSM 710 confocal microscope with 63× oil immersion objective, 40× and 20× objective. Digital images were acquired using ZEN software.

4.7. Statistics
All experiments were replicated at least three times. The experiment results were presented as the mean ± standard deviation. A one-way analysis of variance (ANOVA) and t-test was used to calculate p-values via GraphPad Prism 7.0 software. The value p < 0.05 was considered statistically significant.

Data accessibility. The data are provided in electronic supplementary material [44].

Authors’ contributions. H.Z.: data curation, investigation, methodology, project administration, software, validation, writing—original draft; Z.H.: data curation, investigation, methodology, visualization; L.L.: validation; Y.X.: data curation, investigation; Q.W.: data curation, investigation; F.P.: resources, supervision, validation; L.D.: resources, supervision, validation; C.W.: funding acquisition, resources, supervision; P.W.I.: conceptualization, funding acquisition, resources, supervision, validation, writing—review and editing; Z.Z.: conceptualization, funding acquisition, methodology, project administration, resources, supervision, validation, visualization, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

Funding. This work was supported by National Natural Science Foundation of China (No. 31970738), Social Development Foundation of Shanxi (No. 201903D321019), Youth Foundation of Hundred Talents of Shanxi Province, and Program Fund for the Scientific Activities of Selected Returned Oversea Professionals in Shanxi Province, China.
