ARTICLE | Article in Press

Hcn4-deficient zebrafish embryos develop a normal left–right axis

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Received: 28 April 2026 / Accepted: 2 September 2026

Abstract

Background Left–right (LR) asymmetry is a well-conserved feature of the Vertebrate body plan and is essential for the correct positioning and morphogenesis of visceral organs. While asymmetric Nodal signaling established by the LR organizer (LRO) is widely accepted as a core mechanism driving LR patterning, additional early processes that act during—or even before—the cleavage stage have been proposed to contribute to LR axis specification, including bioelectrical mechanisms mediated by ion fluxes. On the basis of studies in Xenopus laevis, the hyperpolarization-activated cyclic nucleotide-gated channel HCN4 is a candidate regulator of early LR asymmetry induction. Results To assess evolutionary conservation, we investigated whether Hcn4 contributes to LR patterning in zebrafish. We show that both zebrafish hcn4 ohnologs, namely, hcn4 and hcn4-like (hcn4l), are maternally expressed, with specific mRNAs and proteins detectable from the earliest stages of embryonic development. Despite Hcn4s' maternal synthesis, multiple independent approaches aimed at interfering with their function, including pharmacological and dominant-negative inhibition, as well as the genetic ablation of both genes, have failed to produce defects in cardiac LR patterning. We also performed pharmacological inhibition of Hcn4 in X. laevis without detecting, under our experimental conditions, evidence of alterations in heart or gut laterality, questioning the real role of HCN4 in the establishment of LR asymmetry. Conclusions Together, these results indicate that loss of Hcn4 function does not affect LR patterning in zebrafish and argue against a conserved role for Hcn4 as a molecular mediator of early bioelectrical contributions to Vertebrate laterality. Our findings do not exclude the existence of an ion flux-based mechanism that acts during early development but reveal that such a process is not mediated by Hcn4 function.

Keywords Left-right axis · Left-right organizer (LRO) · *situs inversus* · Heterotaxy · HCN4 · Zebrafish

https://doi.org/10.1038/s41598-026-70409-5

Published online: 14 September 2026

scientific reports

Introduction

Asymmetry is a fundamental characteristic of life, with the left–right (LR) difference being one of its most intriguing and essential manifestations. Although bilaterian organisms appear externally quite symmetrical with respect to the anteroposterior and dorsoventral axes, a deeper investigation revealed that internal organs exhibit distinct and consistent asymmetry in terms of position and function along the LR axis. This asymmetry (termed *situs solitus*) is clearly visible, for example, in cardiac looping, which heavily influences the heart pumping function[1], as well as in the positioning of the liver and pancreas[2], and even in the morphology of the brain, whose lateralization is also evolutionarily conserved across Vertebrates and is responsible for distinct functional divisions among different cerebral areas[3]. Consequently, deviations from such asymmetry, ranging from the inversion of one or more organs (heterotaxy, or *situs ambiguus*) to the complete inversion of the LR axis (*situs inversus*), often lead to severe congenital disorders[4].

The concept of LR asymmetry dates to ancient times, when early anatomists observed the asymmetrical placement of organs in the human body. However, scientists began to unravel the molecular and genetic mechanisms behind this phenomenon only in the 20th century. Seminal discoveries in Vertebrate model organisms have laid the foundation for understanding how symmetry is broken during embryogenesis and persists in adult animals[5,6]. The main embryonic structure where symmetry breaking occurs is the LR organizer (LRO). Although the LRO basically characterizes all Vertebrates, the mechanisms responsible for the LR asymmetry might differ among them. For example, mouse, zebrafish and *Xenopus laevis* rely on the unidirectional fluid flow generated by motile cilia at the LRO, whereas Reptiles and birds LRO does not host motile cilia; thus, the unidirectional fluid flow is absent, and the breaking of LR symmetry is related to the asymmetric positioning of cells with nonequivalent signaling activity. Nonetheless, in both cases, the crucial event triggering symmetry breaking and consequent laterality of the body plan is the LR asymmetric activity of the specific intercellular Nodal signaling—higher on the left side—first at the LRO and then transferred to the lateral plate mesoderm (LPM)[7–9]. The Nodal cascade consists of Nodal, its repressor Lefty, and the transcription factor Pitx2[10–12]. The activation of this cascade is essential and adequate for promoting the proper asymmetric arrangement of organs[13,14]. Historically, the ciliated LRO in zebrafish, the Kupffer's vesicle (KV), is established during early somitogenesis[15], and is similar to the node in mouse and the gastrocoel roof plate in *X. laevis*[16]. During zebrafish gastrulation, a cluster of approximately 20–30 cells, called dorsal forerunner cells (DFCs), migrates at the forefront of the embryonic shield (the zebrafish organizer), where the DFCs proliferate and then differentiate into the KV, an epithelial sac containing rotating cilia[15]. Between the 4- and 6-somite stages, the KV initiates the cilia-powered flow that converts the initial bilateral accumulation of Spaw, a Nodal-related protein, into an asymmetric left-sided distribution[15,17,18].

Although the asymmetric Nodal signaling cascade that takes place at the LRO during gastrulation has been elucidated, the existence of a second mechanism that acts very early during development, within a time frame encompassing the zygote-cleavage stages, has been suggested so that the Nodal cascade is the result of the activation of such an upstream mechanism rather than the actual trigger of symmetry breakage. This mechanism would link the LR asymmetry to blastomere membrane potential in Vertebrates and invertebrates[19–21]. In zebrafish, the early determination of the LR asymmetric body plan seems to rely on H+/K+-ATPase[22] and Na, K-ATPase 2 and Ncx4a[23] action. In evolutionary accordance with this perspective, *X. laevis* maternal ion pumps (ATP4 and ATP6), initially evenly distributed in the zygote, are reallocated asymmetrically during the first cleavage divisions[19,24], thus generating an intracellular voltage and pH gradient that leads to the accumulation of determinants, such as the maternal monoamine serotonin[25], in the blastomeres on the right side of early-stage embryos. Later, in an unknown fashion, these determinants would block Nodal on the right side, hence triggering the asymmetric activation of the cascade at the LRO[7]. This second mechanism, known as the ion-flux model, has been criticized for several aspects (i.e., serotonin asymmetric distribution) by Blum and coworkers in their 2014 elegant paper, where the mechanism itself and the approaches employed to demonstrate its actual existence have been questioned[7]. Nevertheless, further publications supporting this last model also added to the picture of the main role

of the cationic channel Hcn4, which is well known for its importance in heart rate regulation[26,27], in defining the positioning of the heart[28] and the general LR asymmetry in Amphibians[29]. Strikingly, as Pai and colleagues stated in their manuscript[29], the regulation of *X. laevis* LR patterning by Hcn4 is completely independent of the asymmetric expression of the Nodal cascade, whereas Pitcairn and colleagues, from the same research group, state the opposite, at least with respect to heart modeling[28].

In such a scenario, we employed zebrafish to investigate whether Hcn4 might play a role in the early definition of the LR axis in Vertebrates via both chemical (Hcn4 inhibitors) and genetic (dominant-negative Hcn4, and *hcn4*$^{-/-}$/*hcn4like*$^{-/-}$ - the 2 zebrafish ohnologs - double mutant line) approaches to block the contribution of the channel to embryonic development since the zygotic phase.

Although we provide the first evidence of the expression of the zebrafish *hcn4* genes at very early developmental stages, thus revealing its maternal origin, all our attempts to show signs of heart LR asymmetry defects following Hcn4 misregulation failed, questioning whether this phenomenon would be evolutionarily conserved between *X. laevis* and zebrafish. To deepen our analysis, we conducted, in parallel, chemical inactivation of the Hcn4 channel in *X. laevis*, and surprisingly, we were not able to replicate the results obtained by Pai and colleagues[29].

Based on the results achieved in both biosystems, we conclude that Hcn4, although maternally expressed, does not play any early role in the determination of the LR axis in zebrafish and, probably, in *X. laevis*. These findings strongly suggest that Hcn4 is involved in neither flow-based nor ion-flux mechanisms to break LR symmetry in Vertebrate embryos.

Methods

Fish husbandry and lines used

The fish were maintained in the facility of the University of Milan, Via Celoria 26, 20,133 Milan, on a 14 h light/10 h dark cycle. The temperature of the water in the aquarium was between 28 °C and 28.5 °C, with the pH ranging between 6.8 and 7.5. The fish in the aquarium were fed three times per day with granular food of different dimensions according to growth phase. Our facility strictly complies with the relevant European (EU Directive 2010/63/EU for animal experiments) and Italian (Legislative Decree No. 26/2014) laws, rules, and regulations (Auth. Min. 21/2015-UT and 0269080/25), as also confirmed by the authorization issued by the municipality of Milan (PG 198,283/2019). All animal procedures were performed in accordance with European (Directive 2010/63/EU) and Italian (D.Lgs. 26/2014) guidelines and regulations. The study was approved by the Institutional Animal Care and Use Committee (IACUC)/OPBA of the University of Milan (Approval No. OPBA 58/2026). However, since the embryos used in this study were not capable of independent feeding, these procedures do not fall under the scope of the EU Directive 2010/63/EU and the Italian Legislative Decree No. 26/2014 on the protection of animals used for scientific purposes. Nevertheless, all efforts were made to ameliorate animal welfare and to follow the ARRIVE guidelines. For the experiments involving drugs exposure and microinjections, we used the wild-type ABTL strain, while the *hcn4/hcn4l* homozygous mutant strain *(hcn4*^{sa11188}; *hcn4l*^{sa32539}*)* derived from the *hcn4*^{sa11188} × *hcn4l*^{sa32539} (KIT - European Zebrafish Resource Center) crosses. Embryos were collected by means of natural spawning and staged in embryo water-containing Petri dishes for downstream analysis, according to Kimmel and co-workers[30]. To avoid pigmentation, embryos used in whole mount in-situ hybridization (WISH) were raised in embryo water supplemented with 0.003% 1-phenyl-2 thiourea (PTU) until the desired developmental stage was reached. For WISH experiments, embryos were fixed overnight at −4 °C in 4% paraformaldehyde (PFA), pH 7.2, in 1X PBS. For the immunofluorescence experiments, the embryos were typically fixed for 2 h at room temperature in 4% PFA, pH 7.2, in 1X PBS to avoid extensive background fluorescence. Following fixation, they were dehydrated with increasing concentrations (30%, 50%, 70%, 100% two times) of methanol (MetOH) in PBT (1X PBS, 0.1% Tween 20®) for 5 min. Dehydrated embryos were stored in absolute methanol at −20 °C for at least 1 h or until needed.

RNA extraction and cDNA synthesis

Total RNA was extracted from pools of at least 40 embryos at the desired developmental stage via the ReliaPrep™ RNA Tissue Miniprep Kit (Promega). cDNA synthesis was carried out with the M-MLV Reverse Transcriptase Kit (GeneSpin) starting from 2 µg of total RNA; negative controls (no enzyme) were also included.

Expression analysis

For *hcn4* and *hcn4l*, we used XM_680986.9 and XM_679638.10 NCBI sequences, respectively. A first round of PCR was carried out on cDNAs with the following primers: hcn4_f (5'-TTACACCCAAACTGGATTCG-3'), hcn4_r (5'-CCCGCTTGATAATACCTCTCGT-3'), hcn4l_f (5'-CAGCCAGCGTCGCCTCTCA-3'), hcn4l_r (5'-GTACATCCTCTCATCGTCC-3'). Seminested PCRs were then performed using the previous PCR products as templates and the following primers: hcn4_f1 (5'-ATTGAGTTGATCGCGCACC-3'), hcn4_r, hcn4l_f1 (5'-GTTCTGTTGAGGTGGTCGA-3'), hcn4l_r. RT-PCR expression analysis at the 1- to 16-cell stage was also performed with a second primer set as follows: new_hcn4_f (5'-CGTAAAGGAGGACAACGCAG-3'), new_hcn4_r (5'-AGTAATCGTGGATCCGCTGT-3'), new_hcn4_f1 (5'-CGTGGTGGACTTCATCTCCT-3'), new_hcn4l_f (5'- ACATGGGGTCAGCAGTACTC-3'), new_hcn4l_r (5'-CTTTCCTATGCGGTCAAGGC-3'), new_hcn4_f1 (5'-CGCAGACAGTACCAGGAGAA-3'). For *hcn1*, *hcn2b*, *hcn3* and *hcn5*, we used XM_017352974.4, XM_680322.10, XM_684105.7, and NM_001431346.1 NCBI sequences, respectively, to design gene-specific primers. The same seminested PCR approach (2 forward and 1 reverse primers) was used for each cDNA: hcn1_f (5'-CCGCTTGGACCGCATCGGGA-3'), hcn1_r1 (5'-GCTGAATGGAAGCGAGTGAT-3'), hcn1_f1 (5'-TCAAGAGAACGAGCTGCTGA-3'), hcn2b_f (5'-TGGCACTCGCATGCCCGCGT-3'), hcn2b_r1 (5'-GTTCCTGCTCCTTCTCCACT-3'), hcn2b_f1 (5'-CGTAAAATCTGGCTGCGTCA-3'), hcn3_f (5'-ATCTCATAACCGACGCGTGA-3'), hcn3_r1 (5'-AGGTTTCCCATCATCAGGCA-3'), hcn3_f1 (5'- TGACAACAAGACGAGGAGCT-3'), hcn5_f (5'-AAATTACTCTGCGTCCGTGC-3'), hcn5_r (5'-GCAGACGGACAAGGCTAATG-3'), hcn5_f1 (5'-TTTGTCTACGGCAGTGAGGT-3'). *rpl8* was amplified as a control. For each amplification, all negative controls were always performed, including those with no reverse transcriptase, to check for potential false positives due to genomic contamination. The PCR products were loaded onto a 1% agarose gel for electrophoresis and visualized with a Vilber Lourmat UV transilluminator equipped with a Ubitec CCD camera. The images, visualized on the screen equipment, were acquired using a Narzo 30 5G (RealMe) camera phone.

Whole-mount in-situ hybridization (WISH) For a detailed visualization of the zebrafish heart *situs*, the heart-specific Dig-tagged *cmlc2* antisense riboprobe was synthesized with T7 RNA polymerase (Roche) from a pre-existing template. In-situ hybridizations were carried out via standard procedures on embryos rehydrated with decreasing concentrations (75%, 50%, 25%) of MetOH/PBT for 5 min each and then washed twice with PBT.

Whole-mount immunofluorescence

Dehydrated embryos stored in absolute MetOH at −20°C were rehydrated with decreasing concentrations (75%, 50%, 25%) of MetOH/PBT for 5 min each, washed twice with PBT and incubated for 2 h at room temperature in a blocking solution consisting of 5% bovine serum albumin (Sigma–Aldrich) in PBS-TD (0.5% DMSO in PBT). The embryos were incubated overnight at 4 °C with primary antibody in blocking solution. The primary antibody used to label Hcn4 was a rabbit polyclonal anti-HCN4 antibody (Alomone labs APC-052) at a ratio of 1:100; a negative control was also included (no primary antibody). The next day, several washes with PBS-TD were performed, and the embryos were subsequently incubated overnight at 4 °C in PBS-TD with the Alexa Fluor 555 F(ab')2-Goat anti-rabbit IgG (H + L) secondary antibody (Thermo Fisher Scientific) at a ratio of 1:200. The last day, several washes in 1X PBS were performed.

Pronase, drug, and temperature treatments

When needed, the chorion of the embryos was enzymatically removed with a 2 min incubation under gentle agitation in 1 mg/mL pronase (Merck), followed by several washes in embryo water as previously described[31]. The embryos were then incubated with the HCN inhibitors Ivabradine (Merck) and ZD7288 (Merck) at concentrations of 500 µM and 1 mM, respectively. Following washout at 20 hpf, the embryos were allowed to grow in PTU until 48 hpf. As a positive control for LR axis alteration, mild cold stress (22 °C) was applied, as previously described[32].This embryo treatment is well known for being able to induce LR axis inversion in a certain percentage of embryos.

Microinjection of *hcn4* dominant-negative mRNA

The zebrafish *hcn4* dominant-negative (DNHcn4) mRNA was transcribed in vitro from a pregenerated plasmid[33] with the T7 mMessage mMachine (Thermo Fisher). To generate DNHcn4, the GYG motif (positions 441–443) within the selectivity filter was replaced with an AAA triplet. This substitution completely abolishes normal channel activity, resulting in a lack of functional currents, consistent with reports on other HCN subtypes [33 and reference therein]. mRNAs were injected at the one-cell stage at concentrations of 400 and 800 pg/embryo. GFP-mRNA-injected embryos were used as experimental controls.

Genotyping of *hcn4sa11188; hcn4lsa32539*

Genomic DNA was extracted with an in-house adaptation of the zebrafish HotShot genomic DNA extraction protocol[34]. To minimize animal distress, adult $hcn4^{sa11188}$; $hcn4l^{sa32539}$ adult fish were non-invasively sampled. Epithelial cells and superficial mucus were gently collected by swabbing the lateral body side with sterile cotton buds. The procedure was approved by the Institution IACUC/OPBA (Approval No. OPBA 58/2026). The buds were immediately placed in 1.5 mL Eppendorf tubes containing ~ 400 µL of 50 mM NaOH and agitated several times. After the buds were removed, the tubes were heated at 95 °C for 30 minutes and then neutralized with a 1/10 volume of 1 M Tris-HCl, pH 8. The DNA-containing solutions were then pelleted to remove debris, and the supernatants were used as templates for PCR. For each animal, the genomic regions containing *hcn4* and *hcn4l* mutations were amplified with the following primer pairs: hcn4_f1_gen (5'-GTGTACTGACCTCCTCC CAC-3'), hcn4_r1_gen (5'-TGGACCCTCAGCAGATCAAG-3'), hcn4l_f1_gen (5'-ACACATGGACTGGCTT GTAA-3'), and hcn4l_r1_gen (5'-AGTTGGCGAATCGTTGCTAT-3'). The PCR products were purified with the Wizard sv gel and PCR clean-up system (Promega) and sequenced by Eurofins Genomics with the following sequencing primers: hcn4_f2_seq (5'-GATGTATCGTATGAGGCGCG-3') and hcn4l_f3_seq (5'-TTGGCCAC CCAGCAGTCTGA-3'). Sequencing chromatograms were visualized with FinchTV (Geospiza). Gene-specific primers for genotyping were designed based on the following NCBI reference sequences: *hcn4* (XM_680986.9) and *hcn4l* (XM_679638.10).

Heart beat measurement

Heart rate (expressed in bpm) was counted by eye under an MZFLIII fluorescence stereomicroscope (Leica), after acclimatization of the larvae to the new temperature and light exposition.

Imaging

Embryos subjected to WISH were mounted ventrally on a Petri dish with 80% glycerol, and the heart *situs* was observed and imaged with a DFC310FX digital camera (Leica) and Leica Application Suite V4.7 software on an MZFLIII fluorescence stereomicroscope (Leica). Confocal images were obtained by maintaining the same

acquisition parameters on a Nikon A1 laser-scanning confocal microscope (Nikon Instruments Inc.) with a Plan Apo 10x/0.95 lens and NIS-Elements C software. Optical sections (Z-stacks) were merged into one maximum projection with Fiji. The final panels of images were set up and, when needed, adjusted in postproduction for both contrast and brightness via Adobe Photoshop CS6 (Adobe Systems Inc.).

Xenopus laevis experiments

Adult *X. laevis* (Nasco, USA) were housed at the facility of the University of Milan, Via Celoria 26, 20,133 Milan, in an automated breeding system (TecnoPlus, Techniplast, Italy) under standardized environmental conditions (20 ± 2 °C; pH 7.5 ± 0.5; conductivity 1000 ± 100 µS). The animals were maintained on a 12 h light/12 h dark photoperiod and fed a semisynthetic diet twice weekly (XE40, Mucedola). In compliance with the refinement principle of the 3Rs and in contrast to the classic FETAX methodology (ASTM Standard Guide E1439), embryos were obtained by overnight natural mating without the use of human chorionic gonadotropin injections. Breeding was performed in a controlled mating system with standardized humidity and air/water temperature. The collected embryos were cleaned by gentle swirling in a 2.25% L-cysteine solution adjusted to pH 8.0, followed by several rinses in FETAX solution (625 mg/L NaCl, 96 mg/L $NaHCO_3$, 30 mg/L KCl, 15 mg/L $CaCl_2$, 60 mg/L $CaSO_4 \cdot 2H_2O$, and 70 mg/L $MgSO_4$)[35]. Ivabradine was dissolved in FETAX medium at a final concentration of 200 µM, and pre-10-stage embryos were incubated for at least 24 h at 23 °C (IACUC/OPBA Approval No. OPBA 58/2026). Heart and gut laterality were evaluated under an MZFLIII stereomicroscope (Leica) in tadpoles at stage 45. Stadiation was performed according to Nieuwkoop and Faber[36]. As the larvae used were not yet capable of independent feeding, these experiments did not require approval under Directives 2010/63/EU or 26/2014. Nonetheless, high-level welfare standards, including optimized environmental monitoring and authorized facility protocols, were maintained, consistent with those detailed for the zebrafish experiments.

Statistical analysis and data visualization

Statistical analyses were performed in R. Differences were considered statistically significant at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****). Each experiment was performed at least in duplicate, and the data were pooled and analyzed via a χ^2 test. For the heart rate experiments, the number of embryos analyzed are specified in the dedicated figure captions and statistical significance was assessed by applying a t-test. Graphs were generated with GraphPad Prism, with data presented as the percentage of each outcome per condition.

Results

Expression analysis of *hcn4*-encoding genes

Through RT–PCR, we first analyzed the developmental expression of *hcn4* and *hcn4like* (*hcn4l*), the two zebrafish ohnologs of human *HCN4.* We started the analysis by extracting and reverse-transcribing the total RNA at different time points, from 1-cell-stage embryos to 72-h postfertilization (hpf) larvae. The cDNAs were used for a first round of PCR, which we employed as a template in a subsequent seminested PCR to increase specificity and yield. Both genes were expressed starting from the 1/16-cell stage (Fig. 1). For both *hcn4* and *hcn4l*, we also performed a second experiment employing a different set of primers targeting a completely different region, with the same positive outcome (Additional Fig. 1). Taken together, these data indicate that both *hcn4* and *hcn4like* are maternally expressed, thus supporting a potential role during the very early stages of zebrafish embryonic development. We next sought to confirm the mRNA expression data via whole-mount immunofluorescence. By using a polyclonal anti-HCN4 antibody[37], we detected a homogeneous signal in the blastomeres of 1-, 2-, 4- and 8-cell stage embryos (Fig. 2A-D), corroborating the gene expression data and confirming the maternal nature of the two

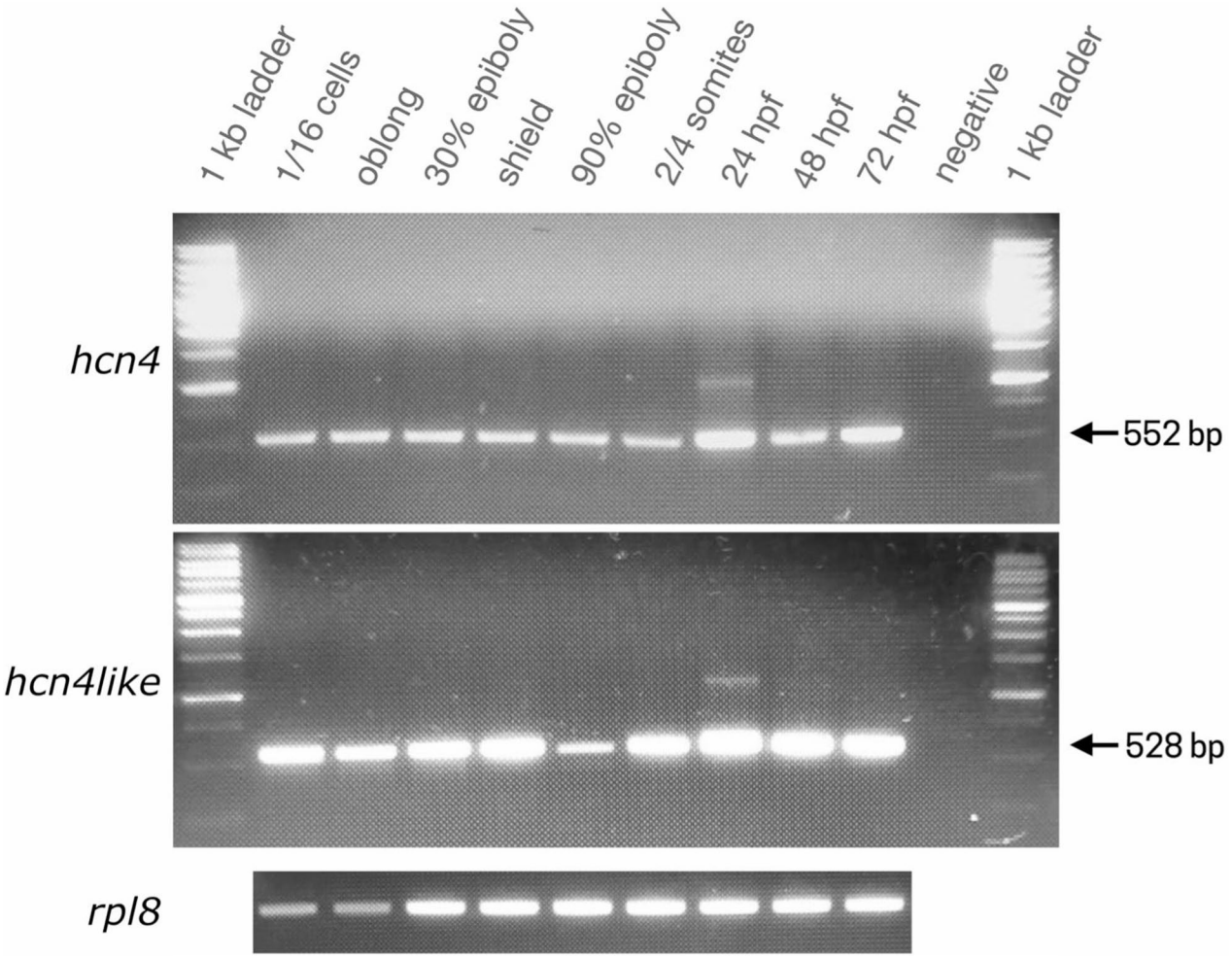


Fig. 1 RT–PCR expression analysis of the hcn4 and hcn4l genes during zebrafish development. Ethidium bromide-stained gels of the seminested RT–PCRs (see Materials and Methods for details). The corresponding developmental stage is reported for each lane. The genes are expressed from the 1- to 16-cell stage and up to 72 hpf. RT–PCR was also performed with *rpl8*-specific primers for RNA integrity and cDNA synthesis quality control. The arrows indicate the length of the gene-specific amplicons (552 bp for *hcn4* and 528 bp for *hcn4l*).

proteins, Hcn4 and Hcn4l, which share 69.21% and 75.70% identity and similarity, respectively. Moreover, the epitope used to generate the antibody is rather conserved between HCN4 and Hcn4/Hcn4l; thus, it is possible to hypothesize that the signal observed might be derived from both proteins.

We also considered the activity of other Hcn channels to compensate for the loss of Hcn4 function in LR axis establishment. Therefore, we analyzed the early expression of all the other *hcn* channel-encoding genes in zebrafish (*hcn1*, *hcn2b*, *hcn3*, and *hcn5*) at the 1- to 16-cell stage. Since *hcn2b*, *hcn3*, and *hcn5* are maternally expressed (Additional Fig. 2), we used concentrations of both Ivabradine and ZD7288 (see below) to ensure the blockage of all Hcn channels.

Heart laterality analysis

Previous work performed in *X. laevis* as a model system supported the role of Hcn4 in the determination of the LR asymmetry of visceral organs, including the heart and gut[28,29]. The key aim of the present work was to investigate whether the role of Hcn4 in this seminal developmental process might be evolutionarily conserved in Vertebrates

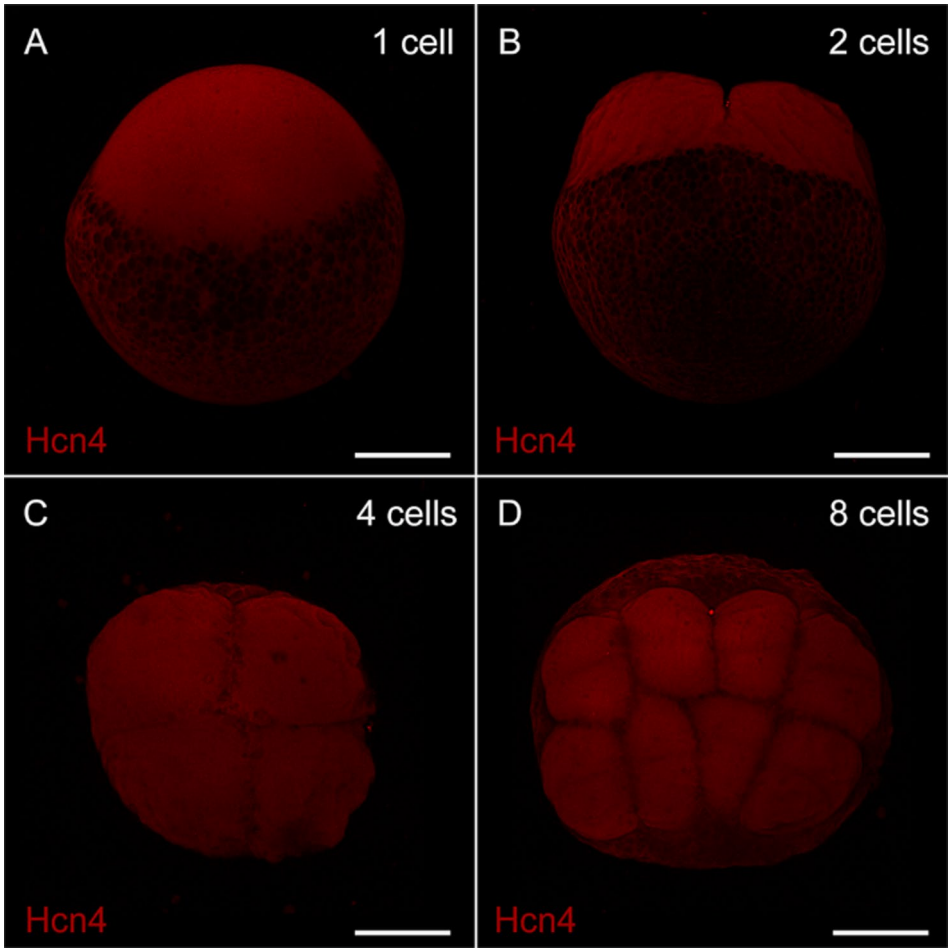


Fig. 2 Immunofluorescence expression analysis of Hcn4 and Hcn4l proteins during the early developmental stages of zebrafish. Hcn4 uniformly localizes in the blastomeres of (**A**) 1-, (**B**) 2-, (**C**) 4-, and (**D**) 8-cell stage embryos. A and B, lateral view; C and D, animal pole view. Scale bars indicate 200 µm.

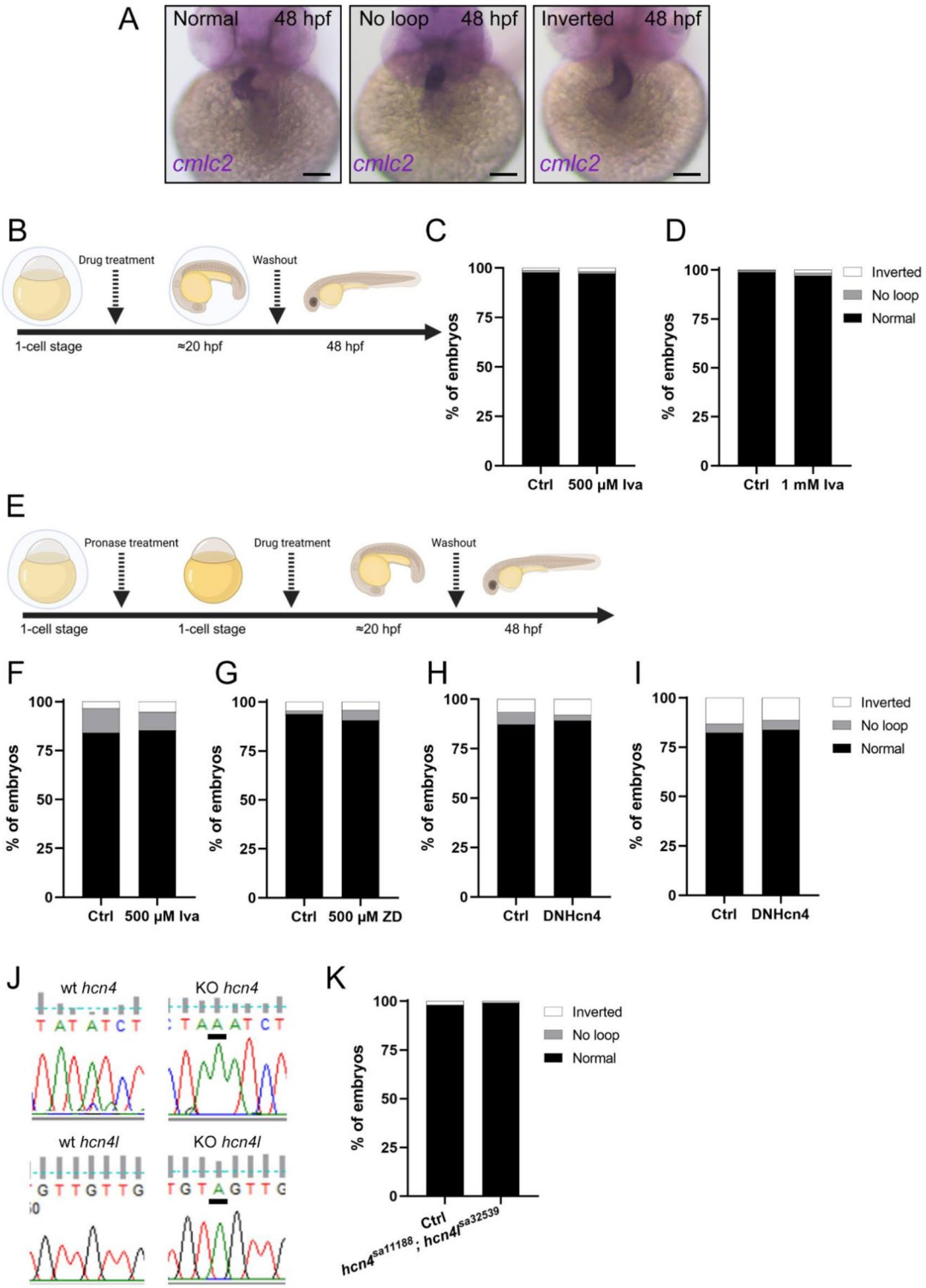
via the use of zebrafish as a model. Therefore, a variety of approaches to block the Hcn4 channel during zebrafish early development have been employed, such as pharmacological inhibition, ablation of protein function via a dominant-negative version of the zebrafish Hcn4 protein, and embryos of the *hcn4*^{sa11188}; *hcn4l*^{sa32539} double knockout line. For all the experiments, we focused on the shape of the heart as a fast readout and categorized the cardiac laterality into 3 different classes, namely, "normal", "no loop" and "inverted" (Fig. 3A). We started the analysis by incubating chorion-bearing 1-cell stage embryos in the HCN inhibitor Ivabradine at concentrations of 500 µM and 1 mM, which were previously reported to be effective in reducing the heart rate in zebrafish embryos[33]. After 20 h of incubation, the drug was removed, and the embryos were allowed to grow until 48 hpf (Fig. 3B). No significant percentage of embryos with a reversed heart loop at either concentration were observed (Fig. 3C, D and additional tables - Tables 1 and 2). This first null result led us to hypothesize a possible event of Ivabradine sequestration by the zebrafish chorion via an interplay of charges. In such a scenario, we decided to perform the same experiment by enzymatically removing the chorion with pronase at the 1-cell stage and then immediately washing and incubating the embryos with the inhibitor at a developmental time point not beyond the 1-cell stage (Fig. 3E). In this case, we used a lower Ivabradine concentration not only to avoid potential toxicity and off-target effects but also because of the absence of the sponge effect, putatively exerted by the chorion, which previously could has led to the underestimation of the amount of inhibitor in the fish medium. Once again, the percentage of heart loop inversion caused by the pharmacological inhibition of Hcn4 via 500 µM Ivabradine was comparable to that obtained without pronase treatment (Fig. 3F, G and additional tables - Table 3). To further increase our confidence in the data, we inhibited Hcn4 by incubating the embryos with a second HCN4 inhibitor named ZD7288, previously used in zebrafish[33], at a concentration of 500 µM without observing any evidence

of laterality defects (Fig. 3G and additional tables - Table 4). These prolonged exposures, combined with high concentrations, ensured adequate intracellular uptake and sustained bioavailability of the HCN4 inhibitors. Consistently, pronounced and persistent bradycardia—a hallmark effect of HCN4 inhibition—was observed at 24 and approximately 40 hpf following Ivabradine washout (additional Fig. 3A, B). Concurrently, a comparable reduction in heart rate was induced by incubating embryos in 200 and 500 µM ZD7288 from the one-cell stage to 48 hpf, without causing cardiac laterality defects. These findings confirm the efficacy of both pharmacological treatments (additional Fig. 3C, D). Following these unexpected results obtained through chemical inhibition, we decided to block channel function via zebrafish DNHcn4, a dominant-negative form of the protein[33] whose synthetic mRNA was microinjected into the zygote at two different concentrations (400 and 800 pg/embryo). No statistically significant differences in heart *situs* were detected between DNHcn4-injected embryos and control embryos (Fig. 3H, I and additional tables - Tables 5 and 6). Nevertheless, embryos injected with 800 pg of DNHcn4 mRNA exhibited a reduced heart rate, indicating that the dominant-negative construct was functionally active despite the lack of an effect on cardiac laterality (additional Fig. 4). Furthermore, to rule out any potential incomplete drug effects or insufficient activity of the dominant-negative Hcn4-encoding mRNA, we employed an *hcn4-/-; hcn4l-/-* double knockout zebrafish line carrying premature stop codons in both genes ($hcn4^{sa11188}$; $hcn4l^{sa32539}$) (Fig. 3J). Also in this case, we did not observe any cardiac *situs* phenotype (Fig. 3K and additional tables - Table 7). As a positive control, embryos incubated under mild cold stress[32] robustly displayed LR cardiac laterality defects compared with embryos incubated under standard conditions (28 °C), confirming the sensitivity of our experimental setup and scoring pipeline (additional Fig. 5 and additional tables - Table 8). These results strongly suggest that the events following the blockage of Hcn4 that lead to *situs inversus* in *X. laevis*[29] do not take place in zebrafish. Given this wealth of unexpected results, we sought to replicate previous data obtained in *X. laevis*, where the functional misregulation of Hcn4 resulted in the inversion of visceral organs[29]. Surprisingly, by treating the embryos before Nieuwkoop and Faber stage 10, as previously described by Pai and colleagues[29], we did not obtain any evidence of heart or gut laterality defects (additional Fig. 6A-C and additional tables - Table 9). Notably, we were forced to downscale the concentration of Ivabradine used to inhibit Hcn4 to 200 µM, as the 400 µM concentration previously used by Pai and colleagues[29] resulted in a massive rate of death, potentially due to toxicity, thus making it impossible to analyze any outcome.

Discussion

Among the four hyperpolarization-activated channels (HCNs), the *HCN4* gene has the most abundant transcript in the adult heart sinoatrial node in the species analyzed thus far[38]. HCN4 is also the most highly expressed HCN channel in the mouse embryonic heart at the very early stages of development[38,39] and dynamically marks heart conduction system precursors[40]. HCN4 is a well-known driver of the funny current *(I_f)* in cardiac pacemaker regions, and most of the studies presented in the literature have focused their attention on its well-established function in regulating cardiac pacemaker currents and rhythmic activity[41].

Hcn4 was recently reported to be maternally expressed in *X. laevis*, with crucial functions during early development in defining the LR axis of the organism through bioelectric signaling tuning[29]. In zebrafish, the only functional study presented in the literature highlights the importance of the two orthologs of human *HCN4*, namely, *hcn4* and *hcn4like (hcn4l)*, in regulating heart rate[33]. However, their maternal derivation and potential functions during the early phases of embryonic development have never been investigated. In the present work, RT–PCR analyses revealed the presence of the two transcripts starting from the zygote stage, indicating that the *hcn4* genes are maternally expressed, as shown in *X. laevis*[29] and in rat oocytes[42]. Nevertheless, such results were unexpected, as early expression of *hcn4* and *hcn4l* has not been reported in common scRNA-seq databases such as DanioCell[43,44]. In addition to the great difference in sensitivity between the two techniques in favor of RT–PCR, a reason for this apparent discrepancy might be that these repositories present gene expression data starting from 3.3 hpf, a time point in development at which the clearance of maternal RNAs has already started, thus possibly



contributing to the decrease/disappearance of *hcn4/hcn4l* RNAs at such early stages[45]. Moreover, immunohistochemistry experiments with an anti-human HCN4 antibody already tested in zebrafish[37] confirmed the presence of the protein in all the developmental phases analyzed (from the 1- to 8-cell stage). As for *X. laevis*[29], the signal appeared uniformly distributed within the blastomere, lacking the membrane-specific localization typical of ion channels. This finding challenges the ion-flux hypothesis, which posits that transmembrane ionic exchanges drive

Fig. 3 Hcn4 inhibition does not lead to heart laterality defects in zebrafish. (**A**) In-situ hybridization with the *cmlc2* riboprobe at 48 hpf (ventral view), showing the three possible outcomes of heart laterality: normal, no loop, and inverted. (**B**) Schematics of the drug treatment performed on chorion-bearing embryos. The embryos were incubated with the Hcn4 inhibitor at the 1-cell stage up to 20 hpf and analyzed at 48 hpf via WISH with the cardiac-specific *cmlc2* riboprobe. (**C**, **D**) Percentages of the three heart laterality outcomes in 48 hpf embryos compared with those in untreated controls following incubation with (C) 500 µM or (D) 1 mM Ivabradine. (**E**) Schematic of the drug treatment performed on embryos following chorion removal. One-cell-stage embryos were enzymatically deprived of the chorion, immediately incubated with the Hcn4 inhibitor up to 20 hpf, and then analyzed at 48 hpf via WISH with the heart-specific *cmlc2* riboprobe. (F, G) Percentages of the three types of heart laterality outcomes in 48 hpf embryos compared with those in untreated controls following incubation with (**F**) 500 µM Ivabradine or (**G**) 500 µM ZD7288. (**H**, **I**) Percentages of the three heart laterality outcomes in 48 hpf embryos, compared with those in controls, following the expression of a dominant-negative Hcn4 protein-encoding mRNA, which was injected at concentrations of (H) 400 pg/embryo and (I) 800 pg/embryo. (**J**) Sequencing chromatograms showing the results of the genotyping of adult animals from the homozygous *hcn4*^{sa11188}; *hcn4l*^{sa32539} line. Each mutation-bearing locus was amplified and sequenced; the mutated nucleotides for *hcn4* (upper panel, wt *hcn4* vs. KO *hcn4*) and *hcn4l* (lower panel, wt *hcn4l* vs. KO *hcn4l*) are underlined. Primers utilized for the identification of *hcn4*^{sa11188} and *hcn4l*^{sa32539} genotypes were derived from NCBI sequences XM_680986.9 and XM_679638.10, respectively. (**K**) Percentages of the three heart laterality outcomes in 48 hpf embryos of the *hcn4*^{sa11188}; *hcn4l*^{sa32539} line compared with wild-type controls. Iva and ZD stand for Ivabradine and ZD7288, respectively.

the electrical signaling essential for LR asymmetry. Indeed, Pai and colleagues reported that Hcn4 is important for *X. laevis* LR patterning of internal organs, as its inhibition from the earliest stages of development leads to errors in the placement of the heart, gut, and gallbladder. Surprisingly, the role of the channel was found to be independent of LRO-*nodal/lefty* asymmetric gene expression[29]. Similarly, ion-flux models based on maternal transcripts, which are initiated before the start of zygotic transcription, have also been proposed for zebrafish[22,23]. In these latter cases, the LR asymmetric body plan depends on H^+/K^+-ATPase[22] and the action of Na, K-ATPase 2 and Ncx4a[23], which, similar to HCN4 in *X. laevis*, takes place in early developmental stages before the activation of the cilia-dependent fluid flow mechanism in Kupffer's vesicle (KV). Nevertheless, unlike those in *X. laevis*, these early mechanisms are not LRO independent and indeed heavily influence the establishment of the asymmetric expression of the Nodal gene *spaw* and its downstream target *pitx2* in the lateral plate mesoderm (LPM), leading to impaired LR axis specification before KV formation[46]. On the basis of these data and given the high similarity among Vertebrates in terms of the molecular processes regulating LR axis establishment[47–49], we sought to replicate the Hcn4-linked heterotaxy phenotype reported in *X. laevis* in various zebrafish models of Hcn4 deficiency, employing both pharmacological and genetic approaches. Notably, for all the zebrafish experiments, we used heart LR asymmetry as a fast readout for abnormal *situs* screening. We started incubating the embryos from 0 to 20 hpf in Ivabradine, a well-known open HCN4 channel inhibitor[50] that was previously reported to be effective in reducing the heart rate of zebrafish [33, and data from our laboratory]. Using previously published concentrations, we could not observe any sign of a reversed loop in the heart or general toxicity. The increase in the concentration to higher values did not result in a different response to the compound, strongly suggesting that, at least in zebrafish, this ion channel did not exert any function during the early developmental stages concerning the definition of the LR axis. However, Ivabradine reportedly has a positive charge at pH 7.4[51]. This might lead to sequestering of the drug by the negatively charged zebrafish chorion, which might act as a physical trap for the compound molecules[52,53]. To overcome the putative limitations in drug absorption, we repeated the experiments by depriving the embryos of their envelope. Also in this case, we were unable to replicate the heterotaxic phenotype observed in *X. laevis*[29]. A second pharmacological block was performed with ZD7288, a less specific and structurally different HCN4 inhibitor[33,54]. Again, neither heterotaxia nor toxicity was observed. Our pharmacological treatments covered the entire developmental window of the LR axis definition, encompassing presumed ion-flux establishment, dorsal forerunner cell (DFC) specification, KV formation, cilia-driven fluid flow generation, and subsequent asymmetric gene expression in the LPM. Therefore, under these experimental conditions, failure to observe cardiac laterality defects is unlikely due to insufficient dosage or timing. Notably, both HCN4 inhibitors are able, at the high concentrations we used, to block the other members of the family of channels[33,50,55,56], thus allowing us to exclude the possibility that the lack of phenotype was a consequence of a

redundancy effect of other maternally expressed *hcn*$_s$. Our results strongly suggest that the pharmacological inhibition of Hcn4 and Hcn4l does not interfere with LR axis establishment in zebrafish, regardless of the mechanism involved. To independently corroborate our results with a genetic approach, we microinjected embryos at the 1-cell stage with an mRNA encoding a dominant-negative form of zebrafish Hcn4 (DNHcn4), kindly provided by Liu and colleagues, which was designed to affect both Hcn4 and Hcn4l channel function by interfering with the assembly of the wild-type tetrameric structure[33]. As for the Hcn4-pharmacological inhibition, no sign of defects in cardiac looping or laterality was detected. In parallel, we injected the mouse version of the Hcn4 dominant-negative, kindly provided by Pai and coworkers[29], but once again, we did not detect any alterations in the LR axis (data not shown).

Finally, we analyzed the zebrafish larvae of a double-knockout *hcn4*$^{-/-}$; *hcn4l*$^{-/-}$ strain. The double mutant did not exhibit any evident cardiac laterality defects, thus supporting all previous evidence. Finally, in all the zebrafish experiments proposed in this work, we did not observe the typical curled or "whirlpool-shaped" body axis phenotype, typically associated with defective cilia function causing the disruption of the LR signaling in zebrafish embryos[57–59], thus strongly supporting the notion that Hcn4 deficiency does not affect the induction and/or construction of the KV. Given the relatively highly conserved nature not only of Hcn4 but also of the molecular signals governing LR specification across Vertebrates[8,60], the absence of laterality defects across all experimental strategies employed in this study appears quite surprising and unexpected in comparison to what was previously published in Amphibians[29]. The findings proposed in this study raise doubts about the real applicability and reproducibility of an Hcn4-linked ion-flux model proposed by Pai and colleagues[29] to explain laterality mechanisms in early Vertebrate development before the morphological onset of LRO. In such a scenario, we sought to replicate the findings of Pai and colleagues in *X. laevis* embryos, where Hcn4 was reported to regulate, in a *nodal* and *lefty* asymmetric expression-independent manner, the early events underlying LR patterning[29]. We closely followed the reported experimental conditions but implemented minor adjustments to optimize not only embryo survival but also consistency. Indeed, we used a lower concentration of Ivabradine (200 µM instead of 400 µM), as the higher dose resulted, under our conditions, in significant toxicity and embryo lethality even though laterality defects did not occur (data not shown). The high lethality of the 400 µM concentration was unaltered even when the larval density increased. Indeed, it has been previously proposed that *X. laevis* embryos 'assist' each other in terms of morphogenesis and, collectively, can resist chemical insults better than single or few individuals through the elicitation of the so-called cross-embryo morphogenetic assistance (CEMA) effect[61]. Evidently, the CEMA phenomenon was not protective against Ivabradine.

Conclusions

The ion-flux model initially proposed by Levin and colleagues[19,62] mostly relies on the asymmetric expression (LR) of both ATP4 and its downstream effector serotonin[24,25]. Conversely, subsequent studies revealed opposite results concerning the distribution of these molecules—with the symmetric expression of ATP4 and serotonin—and their involvement in classic ciliated LRO morphogenesis[8,63,64], thus weakening the veracity of their contribution to the early ion-flux model. In a subsequent study, Pai and colleagues pinpointed Hcn4 as a crucial player in generating the flux of ions necessary to establish LR asymmetry during the early stages of *X. laevis* development[29]. Also strikingly, the authors stated that the involvement of Hcn4 in the model would result independently from the Nodal cascade, which is well conserved among Vertebrates in dictating the establishment of the body *situs solitus*. However, our results tell a different story, showing the absence of any role for Hcn4 in LR axis generation in zebrafish and, very likely, in *X. laevis*, thus providing, from an evolutionary point of view, a more reasonable picture that questions the tendency of *X. laevis*, as previously proposed by Pai and colleagues[29], to follow a path so different from that chosen by the other Vertebrates investigated. In such an evolutionary scenario, it is worth noting that the lack of Hcn4 in mice, even though it is embryonic lethal, has never been reported to cause

heart laterality defects (i.e., the heart of the embryo is perfectly formed and functional, and only a reduction in the cardiac rate has been described for the isolated organ)[38].

In conclusion, our work does not rule against the existence of an earlier mechanism representing the foundations on which the induction and/or the assembly of the latter LRO could be based[22,23], but it presents strong evidence that, whether the mechanism(s) might involve ion fluxes, these fluxes are definitively not mediated by the intervention of the Hcn4 channel.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-026-70409-5.

Author contributions L.D.G. and A.D. designed the study and supervised the project. A.D., A.M. and L.D.G produced the zebrafish data. A.F. and C.J. obtained the double KO zebrafish line. E.M. and F.D.R produced the Xenopus data. All the authors contributed to data analysis. A.D., A.M. and L.D.G wrote the manuscript. All the authors read and approved the final draft.

Funding This work is supported by PNRR M4.C2.1.1. P202272M73_001 fund from the Italian Ministry of University and Research (MUR).

Data availability The reference sequences for hcn4 and hcn4like used for comparative analysis with the mutants in this study are publicly available in the NCBI database under accession numbers XM_680986.9 and XM_679638.10, respectively. All other data generated or analyzed during the current study are included within this article and its supplementary information files.

Declarations

Competing interests The authors declare no competing interests.

Ethics All animal care and experimental procedures were carried out in compliance with applicable European and Italian regulations, including EU Directive 2010/63/EU and Legislative Decree No. 26/2014. The experimental facility operates under ministerial authorizations Auth. Min. 21/2015-UT and 0269080/25, with confirmation from the Municipality of Milan (PG 198,283/2019). The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC)/OPBA of the University of Milan (Approval No. OPBA 58/2026). While the embryos utilized in this study were not yet capable of independent feeding, and therefore technically fall outside the formal regulatory scope of the aforementioned EU and Italian decrees regarding the protection of animals used for scientific purposes, all efforts were nevertheless made to uphold the highest standards of animal welfare and research integrity, aligning with the ARRIVE guidelines. With the objective of minimizing animal distress, all sampling procedures for adult fish were strictly non-invasive. Epithelial cells and superficial mucus were gently retrieved by swabbing the lateral aspect of the body using sterile cotton buds. These specific sampling protocols were also reviewed and approved by the Institutional IACUC/OPBA of the University of Milan (Project Approval No. OPBA 58/2026).

References

1. Putotto, C. et al. Laterality, heterotaxy, and isolated congenital heart defects: The genetic basis of the segmental nature of the heart. *Genome Med.* **16**, 100 (2024).

2. Womble, M., Amin, N. M. & Nascone-Yoder, N. The left-right asymmetry of liver lobation is generated by Pitx2c-mediated asymmetries in the hepatic diverticulum. *Dev. Biol.* **439**, 80–91 (2018).
3. Corballis, M. C. The evolution of lateralized brain circuits. *Front. Psychol.* **8**, 1021 (2017).
4. Forrest, K., Barricella, A. C., Pohar, S. A., Hinman, A. M. & Amack, J. D. Understanding laterality disorders and the left-right organizer: Insights from zebrafish. *Front. Cell Dev. Biol.* https://doi.org/10.3389/fcell.2022.1035513 (2022).
5. Levin, M., Johnson, R. L., Stern, C. D. & Kuehn, M. Tabin, C. A molecular pathway determining left-right asymmetry in chick embryogenesis. *Cell* **82**, 803–814 (1995).
6. Hirokawa, N., Noda, Y., Tanaka, Y. & Niwa, S. Kinesin superfamily motor proteins and intracellular transport. *Nat. Rev. Mol. Cell Biol.* **10**, 682–696 (2009).
7. Blum, M., Schweickert, A., Vick, P., Wright, C. V. E. & Danilchik, M. V. Symmetry breakage in the vertebrate embryo: When does it happen and how does it work?. *Dev. Biol.* **393**, 109–123 (2014).
8. Blum, M., Feistel, K., Thumberger, T. & Schweickert, A. The evolution and conservation of left-right patterning mechanisms. *Development* **141**, 1603–1613 (2014).
9. Kajikawa, E. et al. Nodal paralogues underlie distinct mechanisms for visceral left-right asymmetry in reptiles and mammals. *Nat. Ecol. Evol.* **4**, 261–269 (2020).
10. Shen, M. M. Nodal signaling: developmental roles and regulation. *Dev. Camb. Engl.* **134**, 1023–1034 (2007).
11. Schier, A. F. Nodal morphogens. *Cold Spring Harb. Perspect. Biol.* **1**, a003459 (2009).
12. Marjoram, L. & Wright, C. Rapid differential transport of Nodal and Lefty on sulfated proteoglycan-rich extracellular matrix regulates left-right asymmetry in Xenopus. *Development* **138**, 475–485 (2011).
13. Hamada, H., Meno, C., Watanabe, D. & Saijoh, Y. Establishment of vertebrate left-right asymmetry. *Nat. Rev. Genet.* **3**, 103–113 (2002).
14. Hirokawa, N., Tanaka, Y. & Okada, Y. Cilia, KIF3 molecular motor and nodal flow. *Curr. Opin. Cell Biol.* **24**, 31–39 (2012).
15. Essner, J. J., Amack, J. D., Nyholm, M. K., Harris, E. B. & Yost, H. J. Kupffer's vesicle is a ciliated organ of asymmetry in the zebrafish embryo that initiates left-right development of the brain, heart and gut. *Dev. Camb. Engl.* **132**, 1247–1260 (2005).
16. Smith, K. A. & Uribe, V. Getting to the heart of left–right asymmetry: Contributions from the zebrafish model. *J. Cardiovasc. Dev. Dis.* **8**, 64 (2021).
17. Long, S., Ahmad, N. & Rebagliati, M. The zebrafish nodal-related gene southpaw is required for visceral and diencephalic left-right asymmetry. *Dev. Camb. Engl.* **130**, 2303–2316 (2003).
18. Schneider, I. et al. Zebrafish Nkd1 promotes Dvl degradation and is required for left-right patterning. *Dev. Biol.* **348**, 22–33 (2010).
19. Levin, M., Thorlin, T., Robinson, K. R., Nogi, T. & Mercola, M. Asymmetries in H+/K+-ATPase and cell membrane potentials comprise a very early step in left-right patterning. *Cell* **111**, 77–89 (2002).
20. Raya, A. et al. Notch activity acts as a sensor for extracellular calcium during vertebrate left-right determination. *Nature* **427**, 121–128 (2004).
21. Shimeld, S. M. & Levin, M. Evidence for the regulation of left-right asymmetry in Ciona intestinalis by ion flux. *Dev. Dyn. Off Publ Am. Assoc. Anat.* **235**, 1543–1553 (2006).
22. Kawakami, Y., Raya, A., Raya, R. M., Rodríguez-Esteban, C. & Izpisúa Belmonte, J. C. Retinoic acid signalling links left-right asymmetric patterning and bilaterally symmetric somitogenesis in the zebrafish embryo. *Nature* **435**, 165–171 (2005).
23. Shu, X. et al. Na,K-ATPase α2 and Ncx4a regulate zebrafish left-right patterning. *Development* **134**, 1921–1930 (2007).
24. Adams, D. S. et al. Early, H+-V-ATPase-dependent proton flux is necessary for consistent left-right patterning of non-mammalian vertebrates. *Development* **133**, 1657–1671 (2006).
25. Fukumoto, T., Kema, I. P. & Levin, M. Serotonin signaling is a very early step in patterning of the left-right axis in chick and frog embryos. *Curr. Biol.* **15**, 794–803 (2005).
26. Baruscotti, M., Barbuti, A. & Bucchi, A. The cardiac pacemaker current. *J. Mol. Cell. Cardiol.* **48**, 55–64 (2010).
27. Baruscotti, M. et al. Deep bradycardia and heart block caused by inducible cardiac-specific knockout of the pacemaker channel gene Hcn4. *Proc. Natl. Acad. Sci. U S A.* **108**, 1705–1710 (2011).
28. Pitcairn, E. et al. Coordinating heart morphogenesis: A novel role for hyperpolarization-activated cyclic nucleotide-gated (HCN) channels during cardiogenesis in *Xenopus laevis*. *Commun. Integr. Biol.* **10**, e1309488 (2017).
29. Pai, V. P. et al. HCN4 ion channel function is required for early events that regulate anatomical left-right patterning in a nodal and lefty asymmetric gene expression-independent manner. *Biol. Open* **6**, 1445–1457 (2017).
30. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Schilling, T. F. Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253–310 (1995).
31. Bragato, C., Persico, A., Ferreres, G., Tzanov, T. & Mantecca, P. Exploring the Effects of Lignin Nanoparticles in Different Zebrafish Inflammatory Models. *Int. J. Nanomed.* **19**, 7731–7750 (2024).
32. Liu, M. et al. Mild cold stress specifically disturbs clustering movement of DFCs and sequential organ left-right patterning in zebrafish. *Front. Cell. Dev. Biol.* **10**, 952844 (2022).

33. Liu, J., Kasuya, G., Zempo, B. & Nakajo, K. Two HCN4 channels play functional roles in the zebrafish heart. *Front. Physiol.* https://doi.org/10.3389/fphys.2022.901571 (2022).
34. Meeker, N. D., Hutchinson, S. A., Ho, L. & Trede, N. S. Method for isolation of PCR-ready genomic DNA from zebrafish tissues. *BioTechniques* **43**, 610–614 (2007).
35. Battistoni, M. et al. Modified *Xenopus laevis* approach (R-FETAX) as an alternative test for the evaluation of foetal valproate spectrum disorder. *Reprod. Toxicol.* **107**, 140–149 (2022).
36. *Normal Table of Xenopus Laevis (Daudin): A Systematical & Chronological Survey of the Development from the Fertilized Egg till the End of Metamorphosis* (Garland Science, 2020). https://doi.org/10.1201/9781003064565.
37. Stoyek, M. R., Quinn, T. A., Croll, R. P. & Smith, F. M. Zebrafish heart as a model to study the integrative autonomic control of pacemaker function. *Am. J. Physiol. Heart Circ. Physiol.* **311**, H676–H688 (2016).
38. Stieber, J. et al. The hyperpolarization-activated channel HCN4 is required for the generation of pacemaker action potentials in the embryonic heart. *Proc. Natl. Acad. Sci. U S A*. **100**, 15235–15240 (2003).
39. Yasui, K. et al. I(f) current and spontaneous activity in mouse embryonic ventricular myocytes. *Circ. Res.* **88**, 536–542 (2001).
40. Liang, X. et al. HCN4 Dynamically Marks the First Heart Field and Conduction System Precursors. *Circ. Res.* **113**, 399–407 (2013).
41. Copier, J. S., Verkerk, A. O. & Lodder, E. M. HCN4 in the atrioventricular node. *Heart Rhythm* https://doi.org/10.1016/j.hrthm.2025.02.030 (2025).
42. Yeh, J. et al. The expression of hyperpolarization activated cyclic nucleotide gated (HCN) channels in the rat ovary are dependent on the type of cell and the reproductive age of the animal: A laboratory investigation. *Reprod. Biol. Endocrinol.* **6**, 35 (2008).
43. Farrell, J. A. et al. Single-cell reconstruction of developmental trajectories during zebrafish embryogenesis. *Science* **360**, eaar3131 (2018).
44. Sur, A. et al. Single-cell analysis of shared signatures and transcriptional diversity during zebrafish development. *Dev. Cell.* **58**, 3028–3047e12 (2023).
45. Zhao, B. S. et al. m6A-dependent maternal mRNA clearance facilitates zebrafish maternal-to-zygotic transition. *Nature* **542**, 475–478 (2017).
46. Barth, K. A. et al. Fsi zebrafish show concordant reversal of laterality of viscera, neuroanatomy, and a subset of behavioral responses. *Curr. Biol.* **15**, 844–850 (2005).
47. Yost, H. J. Left-right development in *Xenopus* and zebrafish. *Semin. Cell Dev. Biol.* **9**, 61–66 (1998).
48. Levin, M. & Mercola, M. Evolutionary conservation of mechanisms upstream of asymmetric Nodal expression: Reconciling chick and Xenopus. *Dev. Genet.* **23**, 185–193 (1998).
49. Matsui, T. & Bessho, Y. Left–right asymmetry in zebrafish. *Cell. Mol. Life Sci. CMLS.* **69**, 3069–3077 (2012).
50. Bucchi, A., Tognati, A., Milanesi, R., Baruscotti, M. & DiFrancesco, D. Properties of ivabradine-induced block of HCN1 and HCN4 pacemaker channels. *J. Physiol.* **572**, 335–346 (2006).
51. Saponaro, A. et al. Structural determinants of ivabradine block of the open pore of HCN4. *Proc. Natl. Acad. Sci. U. S. A.* **121**, e2402259121 (2024).
52. Hart, N. H. & Donovan, M. Fine structure of the chorion and site of sperm entry in the egg of Brachydanio. *J. Exp. Zool.* **227**, 277–296 (1983).
53. Bonsignorio, D., Perego, L., Del Giacco, L. & Cotelli, F. Structure and macromolecular composition of the zebrafish egg chorion. *Zygote* **4**, 101–108 (1996).
54. Nakashima, K., Nakao, K. & Matsui, H. Discovery of Novel HCN4 Blockers with Unique Blocking Kinetics and Binding Properties. *Slas Discov*. **26**, 896–908 (2021).
55. Wu, X. et al. Is ZD7288 a selective blocker of hyperpolarization-activated cyclic nucleotide-gated channel currents? *Channels Austin Tex.* **6**, 438–442 (2012).
56. Haechl, N., Ebner, J., Hilber, K., Todt, H. & Koenig, X. Pharmacological Profile of the Bradycardic Agent Ivabradine on Human Cardiac Ion Channels. *Cell. Physiol. Biochem. Int. J. Exp. Cell. Physiol. Biochem. Pharmacol.* **53**, 36–48 (2019).
57. Hjeij, R. et al. CCDC151 mutations cause primary ciliary dyskinesia by disruption of the outer dynein arm docking complex formation. *Am. J. Hum. Genet.* **95**, 257–274 (2014).
58. Song, Z., Zhang, X., Jia, S., Yelick, P. C. & Zhao, C. Zebrafish as a Model for Human Ciliopathies. *J. Genet. Genomics Yi Chuan Xue Bao*. **43**, 107–120 (2016).
59. Ghilardi, A. et al. Inner ear and muscle developmental defects in Smpx-deficient zebrafish embryos. *Int. J. Mol. Sci.* **22**, 6497 (2021).
60. Hamada, H. & Tam, P. Diversity of left-right symmetry breaking strategy in animals. *F1000Res.* **9**, 123. (2020).
61. Tung, A. et al. Embryos assist morphogenesis of others through calcium and ATP signaling mechanisms in collective teratogen resistance. *Nat. Commun.* **15**, 535 (2024).
62. Vandenberg, L. N. & Levin, M. A unified model for left–right asymmetry? Comparison and synthesis of molecular models of embryonic laterality. *Dev. Biol.* **379**, 1–15 (2013).

63. Walentek, P., Beyer, T., Thumberger, T., Schweickert, A. & Blum, M. ATP4a Is Required for Wnt-Dependent Foxj1 Expression and Leftward Flow in Xenopus Left-Right Development. *Cell. Rep.* **1**, 516–527 (2012).
64. Beyer, T. et al. Serotonin Signaling Is Required for Wnt-Dependent GRP Specification and Leftward Flow in *Xenopus*. *Curr. Biol.* **22**, 33–39 (2012).

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