Klf8 regulates left-right asymmetric patterning through modulation of Kupffer’s vesicle morphogenesis and spaw expression

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Abstract

Background: Although vertebrates are bilaterally symmetric organisms, their internal organs are distributed asymmetrically along a left-right axis. Disruption of left-right axis asymmetric patterning often occurs in human genetic disorders. In zebrafish embryos, Kupffer’s vesicle, like the mouse node, breaks symmetry by inducing asymmetric expression of the Nodal-related gene, spaw, in the left lateral plate mesoderm (LPM). Spaw then stimulates transcription of itself and downstream genes, including lft1, lft2, and pitx2, specifically in the left side of the diencephalon, heart and LPM. This developmental step is essential to establish subsequent asymmetric organ positioning. In this study, we evaluated the role of krüppel-like factor 8 (klf8) in regulating left-right asymmetric patterning in zebrafish embryos.

Methods: Zebrafish klf8 expression was disrupted by both morpholino antisense oligomer-mediated knockdown and a CRISPR-Cas9 system. Whole-mount in situ hybridization was conducted to evaluate gene expression patterns of Nodal signalling components and the positions of heart and visceral organs. Dorsal forerunner cell number was evaluated in Tg(sox17:gfp) embryos and the length and number of cilia in Kupffer’s vesicle were analyzed by immunocytochemistry using an acetylated tubulin antibody.

Results: Heart jogging, looping and visceral organ positioning were all defective in zebrafish klf8 morphants. At the 18–22 s stages, klf8 morphants showed reduced expression of genes encoding Nodal signalling components (spaw, lft1, lft2, and pitx2) in the left LPM, diencephalon, and heart. Co-injection of klf8 mRNA with klf8 morpholino partially rescued spaw expression. Furthermore, klf8 but not klf8Δzf overexpressing embryos showed dysregulated bilateral expression of Nodal signalling components at late somite stages. At the 10s stage, klf8 morphants exhibited reductions in length and number of cilia in Kupffer’s vesicle, while at 75% epiboly, fewer dorsal forerunner cells were observed. Interestingly, klf8 mutant embryos, generated by a CRISPR-Cas9 system, showed bilateral spaw expression in the LPM at late somite stages. This observation may be partly attributed to compensatory upregulation of klf12b, because klf12b knockdown reduced the percentage of klf8 mutants exhibiting bilateral spaw expression.

Conclusions: Our results demonstrate that zebrafish Klf8 regulates left-right asymmetric patterning by modulating both Kupffer’s vesicle morphogenesis and spaw expression in the left LPM.

Keywords: Zebrafish, Klf8, Spaw, L-R patterning, Kupffer’s vesicle
Background

Despite the outward appearance of bilateral symmetry in vertebrates, internal organs exhibit substantial left-right asymmetry. In humans, genetic disorders that affect left-right asymmetric patterning may result in organ heterotaxy [1], complex congenital heart disease, and asplenia/polydactyly [2]. In order to study the various processes that establish left-right asymmetry in a laboratory setting, several vertebrates, including mice and zebrafish, have been utilized. Largely based on these animal studies, the major developmental processes which establish asymmetry are known to include: symmetry-breaking in the node, the transfer of asymmetric Nodal expression from the node to the left lateral plate mesoderm (LPM), asymmetric expression of Nodal and downstream genes in the left LPM, and the completion of left-right asymmetric organ morphogenesis [3, 4].

Clockwise rotation of nodal cilia creates a directional nodal flow, which is responsible for the preferential activation of Nodal expression on the left side of the embryo [5]. Not surprisingly, mutations in genes involved in ciliogenesis [6] or its regulation [7, 8] have been found to disrupt normal left-right patterning. Leftward nodal flow generates an initial accumulation of NODAL protein on the left side of the embryo. Subsequently, self-enhancement and lateral-inhibition systems involving NODAL, LEFTY1 and LEFTY2 reinforce the asymmetric distribution and restrict Nodal gene expression to the left side of the organism [9].

Nodal signalling is initiated by the binding of NODAL to the ACTIVIN receptor and EGF-CFC co-receptor, which results in the formation of an intracellular regulatory complex. This complex consists of phosphorylated SMAD2, SMAD4 and FoxH1, and directly activates target gene transcription [10]. Left-side specific enhancers (ASEs) with FoxH1 binding motifs are present in the murine Nodal and Lefty2 genes [11]. Thus, NODAL amplifies its own expression in the left LPM via SMADS/FoxH1 interaction with the ASE. Simultaneously, NODAL induces Lefty2 expression, which inhibits low-level NODAL signalling, and thereby restricts Nodal expression to the left LPM. This asymmetric NODAL activation induces expression of Pitx2 in the left LPM, via its ASE. PITX2 is a homeodomain transcription factor implicated in left-right asymmetric organ morphogenesis [12], and loss of Pitx2 expression has been shown to affect the asymmetric distribution of internal organs in several vertebrates [13].

In zebrafish embryos, Kupffer’s vesicle (KV) performs a similar role to the mouse node in initiating left-right asymmetric patterning [14]. KV is derived from dorsal forerunner cells (DFCs), which are formed via a Nodal signalling-dependent ingestion of surface enveloping layer cells from the dorsal blastoderm margin. This ingestion occurs at the blastula stage, when embryonic epiboly initiates [15]. DFCs then migrate toward the vegetal pole and organize into multiple rosette-like, epithelial structures at the end of gastrulation. These epithelial rosettes then merge into a single epithelial rosette and differentiate into the ciliated KV, with the vesicle lumen arising from apical membrane expansion during early somite stages. Tilted cilia are positioned with the basal body at the posterior pole of DFCs, and these motile cilia are asymmetrically distributed along the anterior-posterior axis of KV. Furthermore, cilia asymmetry is established by the Rho kinase, Rock2b, and is essential to generate an anti-clockwise swirling flow that commences asymmetric Nodal signalling [16–18]. In addition to rock2b, deficiencies in several genes involved in the movement, formation or positioning of cilia, such as dnah9, ift88, and vangl2, have been shown to disrupt normal left-right patterning [14, 19, 20].

Three nodal-related genes, namely ndr2 (cyc), ndr1 (sqt), and southpaw (spaw), have been identified in zebrafish [10]. Among these genes, spaw exhibits the earliest expression in the left-side of the LPM, and stimulation of its own transcription during somitogenesis shifts its expression domain from the posterior to the anterior left LPM [21, 22]. Furthermore, morpholino knockdown of spaw decreases expression of genes encoding Nodal signalling components, including spaw, lefty1 (lft1), lefty2 (lft2), and pitx2, in the left LPM [22], affecting the left-right asymmetric distribution of heart, pancreas, and diencephalon. Together, these studies demonstrate the essential role of Spaw, and underscore its relevance as a NODAL homolog in establishing left-right asymmetry of teleosts [22, 23].

Similar to mouse embryos, different repressors of the Nodal signalling pathway have been reported to modulate the induction or maintenance of asymmetric Nodal signalling in teleosts. At the 4–6 s stages, spaw is expressed bilaterally in KV, while charon is expressed in a region adjacent to KV, where it antagonizes Spaw activity and contributes to biased spaw expression in the left LPM [24]. Furthermore, repression of Spaw activity in the right LPM or cardiac field by Lft1 or Lft2 is also essential in the establishment of left-right patterning. Notably, lft1 expression in the notochord is induced via binding of BMP4 with BMP receptor 1aa at the early segmentation stage, while lft2 expression in the left cardiac field is activated by Spaw in the anterior LPM [25–27]. Despite this detailed knowledge about Spaw repressor proteins, it is still unknown whether asymmetric spaw expression in the left LPM can be regulated by transcription factors.

Krüppel-like factor 8 (KLF8) is a member of the KLF family of transcription factors [28, 29], and participates in a broad range of developmental processes. KLF proteins
contain C-terminal zinc finger DNA binding motifs, and distinct N-terminal regulatory elements. KLF8, like KLF3 and KLF12, possesses a regulatory domain that interacts with C-terminal binding protein (CtBP) [30]. Interaction of KLF8 with the co-repressor CtBP inhibits embryonic Gamma-Globin gene expression [31, 32], a role confirmed with C-terminal binding protein (CtBP) [30]. Interaction and KLF12, possesses a regulatory domain that interacts distinct N-terminal regulatory elements. KLF8, like KLF3 contain C-terminal zinc finger DNA binding motifs, and

In this study, we demonstrate that zebrafish Klf8 plays an additional role in regulating left-right asymmetric patterning. Heart jogging, looping and visceral organ positioning were defective in klf8 morphants. At 18–22 s stages, expression levels of spaw, lft1, lft2, and pitx2 were decreased or eliminated in the left LPM, diencephalon, and heart of the majority of klf8 morphants. In contrast, klf8 overexpression resulted in bilateral expression of spaw and its downstream target genes in these tissues. Both dorsal forerunner cell number, and the length and number of cilia in KV were also affected in klf8 morphants. However, klf8 CRISPR-Cas mutant embryos showed bilateral spaw expression in the LPM, which may have been partly due to compensatory upregulation of klf12b.

Methods

Ethics approval
All animal procedures were approved by the Institutional Animal Care and Use Committee of Academia Sinica (Protocol ID: 15–12–918).

Zebrafish maintenance and staging
Adult AB zebrafish, Tg(sox17:gfp)zf470.1 and klf8 mutants (klf8d25, klf8177), generated by a CRISPR-Cas9 system were maintained in high density, self-circulation systems (Aqua Blue), or 20 L aquaria supplied with filtered fresh water and aeration under a photo period of 14 h light and 10 h dark. Embryos were maintained at 28.5 °C, and morphological criteria were defined as described [36].

Plasmid construction, morpholino and mRNA injection
The full-length klf8 coding sequence or klf8 lacking zinc finger domain (klf8−zf) was cloned into the T7TS vector, and used as template to synthesize capped mRNA with the mMESSAGE mMACHINE T7 Kit (Ambion). Previously published morpholinos (MOs) were used [35], including two MOs that prevent Klf8 protein translation: klf8-MO1atg (2.2 ng) and klf8-MO2atg (1.9 ng), two MOs that prevent klf8 mRNA splicing: MOΔO (0.73 ng) and MOΔC (0.73 ng), and one control MO: klf8-4 mm MO1 (2.2 ng). An additional MO to prevent klf8 mRNA splicing: MOΔO2 (0.73 ng; 5′- TGGTGTCACATCATCCTCTCAACGGGGATCATCCTGT-3′; targets the donor site of exon 3) was used. A 1.5-fold greater dosage of P53 MO9 [37] was injected, as compared to the co-injected klf8-MOs. To verify upregulation of klf12b in homozygous klf8d25 mutant F6 embryos, klf12b MO (5 or 10 ng) was used. klf12b MO (5′-ATTCCTGTCATACATACATCTGT-3′), which is complementary to 20 bases of the coding region including the ATG start codon (underlined) and five bases of the 5′ untranslated region, was used. The indicated MO or mRNA was microinjected into one- or two-cell zygotes using a Nanoject II automatic injector (Drummond).

Whole mount in situ hybridization, whole mount immunohistochemistry, and photography
Whole mount in situ hybridization was performed on embryos treated with 0.003% phenylthiocarbamidine, using digoxigenin-antisense RNA probes and alkaline phosphatase-conjugated anti-digoxigenin antibody. Various templates derived from pGEMT or pGEMT-Easy vectors were linearized, and the following antisense RNA probes were generated (restriction site and promoter in parentheses): bmp2b (BamHI/T7), charon (BamHI/Sp6), myl7 (NcoI/SP6), gata5 (SacI/SP6), gata6 (SalII/T7), lft1 (SalII/T7), lft2 (HindIII/SP6), ntl (XhoI/T7), oep (NcoI/SP6), pitx2c (EcoRI/T7), and spaw (SpcI/T7). To produce the dvr1 antisense RNA probe, PCR product that was generated using M13 forward and M13 reverse primers was used as a template and transcribed by T7 RNA polymerase. To detect changes in KV cilia, one- or two-cell zygotes of Tg(sox17:gfp) were microinjected with different klf8-MOs. The 10s stage embryos were fixed in 4% paraformaldehyde for 2 h at room temperature (RT) and dehydrated in methanol at −20 °C. After dehydration, the embryos were permeabilized using acetone at −20 °C and washed by Phosphate-buffered saline with tween 20 (PBST) followed by blocking in 5% serum. The embryos were incubated with anti-acetylated tubulin antibody (1:250, Sigma-Aldrich) for 2 h, at RT, followed by mouse Alexa Fluor 568 for 1 h, at RT (1:250, Invitrogen).

To investigate DFC number alteration, one- or two-cell zygotes of Tg(sox17:gfp) were microinjected with different klf8-MOs. 75% embryos were fixed in 4% paraformaldehyde at 4 °C overnight. After dehydration, the embryos were permeabilized using acetone at −20 °C for 7 min and treated with 0.15 M Tris-HCl, pH 9.0 at 70 °C for 15 min. The embryos were washed with PBST followed by blocking...
in 1% blocking solution (Roche) at RT for 2 h. The embryos were incubated with anti-GFP antibody (1:200) at 4 °C overnight, followed by rabbit Alexa Fluor 488 (1:200, Invitrogen) incubation for 1 h, at RT. Nuclei were then stained with Hoechst 33,341 (1:1000 in PBST, Invitrogen).

Brightfield embryo images were taken using an AxioCam HRC camera mounted on a Zeiss Imager M1 microscope. Fluorescent images were taken using a Leica TCS-SP5-MP confocal microscope.

Generation of klf8 mutants using CRISPR-Cas9 system

klf8 

mutants were generated using a CRISPR-Cas9 system targeting exon 2. sgRNA was designed by ZGENE BIO BIOTECH INC. (Taipei, Taiwan) and DNA template was amplified from pZGB plasmids containing klf8 sgRNA. PCR was conducted using forward (5′-ACA CAGGAAAACGTAGTACCAGTAC-3′) and reverse (5′-GATCCG CACCGACTCGGTGCCACTTT-3′) primers, and klf8 sgRNA were synthesized using the MEGASHortscript T7 Transcription Kit (Ambion, Austin, TX, USA). klf8 sgRNA (86.3 pg) and capped mRNA (34.5 pg, Addgene) [38] were co-injected into one-cell zygotes. Genomic DNA was isolated from pools of 10 embryos at 24 hpf. PCR was conducted using forward (5′- CAGTGGGCAAACACAGAACGGCAG-3′) and reverse (5′-CCACATCGTAGACTCCAAAATGCG-3′) primers and amplified DNA was then digested with T7 endonuclease I to evaluate insertion and deletion efficiency. The rest of the embryos were reared to adult- hood. Injected fish were designated as the F0 generation. To detect the DNA sequence alterations induced by klf8 

sgRNA, genomic DNA was isolated from clipped tail fin of adult F1 fish, and high resolution melt analysis was performed. PCR was conducted in a 20 μl reaction comprising 8–12 ng genomic DNA, 3.5 mM MgCl2, 1× Master Mix containing Taq DNA polymerase, dNTP mix and LightCycler 480 ResoLight dye, and 5 pmol each of forward (5′-ATCTCAGAACTCGGTGCTCATT-3′) and reverse (5′-CCACATCGTAGACTCTCCATT-3′) primers. PCR conditions were 95 °C for 10 min, then 55 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s, followed by a 4 °C pause. Primer pairs for klf8 were forward (5′-TATCCGAATGACATTACCTGTGGG-3′) and reverse (5′-CAGTGGGCAAACACAGAACGGCAG-3′). Primer pairs for klf2 were forward (5′-GAGCGGGTGCTCTTTCTGCACCTG-3′) and reverse (5′-CCACATCGTAGACTCTCCATT-3′). Primer pairs for klf2 were forward (5′-GGCAATCCCTGCTCCATGAGCAG-3′) and reverse (5′-CCACATCGTAGACTCTCCATT-3′).

Quantification of cilia number and length and lumen of Kupffer’s vesicles as well as DFC number

The cilia length and number were quantified using LAS AF and MetaMorph software according to the following steps: (i) merge images with LAS AF for MetaMorph analysis; (ii) “Threshold Image” was set to demarcate cilia and KV cell locations, and the image was converted to grayscale; (iii) from the Arithmetic menu, “Logical AND” was selected, and KV cilia regions were defined; (iv) “Calibrate Distances” was set to define units of length (μm); and (v) the length and number of cilia were quantified by selecting “Integrated Morphometry Analysis” in the Measure menu. The area of KV lumen was quantified using ImageJ software as follows: (i) The merged grayscale images from MetaMorph were loaded into ImageJ; (ii) “Elliptical selections” was selected to demarcate cilia area; (iii) “Set scale” was selected to define units of area (μm²); and (iv) the area was determined by selecting “Measure” in the Analyze menu.

In order to evaluate the DFC number, immunofluorescence confocal images of Tg(sox17:gfp) 75% epiboly embryos were merged using ImageJ software using the following steps: (i) Images were loaded into ImageJ; (ii) “Images to stack” was selected from the “Stacks” item in the Image menu; (iii) “Stacks Focuser” was selected from the “Stacks” item in the Plugins menu. DFC number was then manually counted from images with merged GFP and nuclei.

Reverse transcription PCR (RT-PCR) and reverse transcription quantitative real-time PCR (RT-qPCR)

RT-PCR was used to evaluate the efficacy of sp.-MOs. cDNA from 24 hfp, forward primer (5′- ATCAAGCC GGAGCCAGGAGGT-3′) and reverse primer (5′- GCCGTGGTGAAGTGCCAGGT-3′) were used. RT- qPCR [39] was used to compare expression levels of klfβ, klf12a or klf12b in wild type and homozygous F5 klf8d25 and klf8i17 mutant embryos. cDNA was generated by a GoScript Reverse transcription system (Promega) using total RNA isolated from 10 to 12 s stage wild type or two homozygous klf8d25 and klf8i17 mutant embryos. RT-qPCR was conducted in a 10 μl reaction containing 1× LightCycler 480 SYBR Green I Mix (Roche), respective primer pairs (5 μg) and 1/10 cDNA from wild type or mutant embryos. PCR conditions were 95 °C for 10 min, then 55 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s, followed by a 4 °C pause. Primer pairs for klfβ were forward (5′-TATCCGAATGACATTACCTGTGGG-3′) and reverse (5′-CAGTGGGCAAACACAGAACGGCAG-3′). Primer pairs for klf2 were forward (5′-GAGCGGGTGCTCTTTCTGCACCTG-3′) and reverse (5′-CCACATCGTAGACTCTCCATT-3′).
Statistics
Two-tailed Student’s t-tests with unequal variance were conducted to compare number of cilia, cilia length, lumen area and DFC. To compare the klf8 mRNA rescue effect, Fisher’s Exact Test was used. Statistical tests were performed with Excel software. Differences with p < 0.05 were considered to be statistically significant.

Results
**klf8 morphants display abnormal heart jogging, looping and visceral organ positions**

During a previous study investigating the role of Klf8 in cerebellar development [35], we noted that klf8 morphants often exhibited a no-loop heart at 48 hpf. Because cardiac development is asymmetric, this observation suggested that Klf8 may regulate the general process of left-right patterning. Thus we performed klf8 knockdown experiments with previously validated klf8-MOs and systematically evaluated heart morphogenesis by whole-mount in situ hybridization, using a myl7 antisense RNA probe. A phenotypically normal, L-jog heart tube was readily detected in wild type and klf8-4 mm MO1-injected embryos, while embryos injected with klf8-MO1atg or klf8-MO2atg often showed no-jog (32% for MO1atg, 33% for MO2atg) or right-jog (4% for MO1atg, 3% for MO2atg) heart tubes at 24 hpf (Fig. 1a). Consequently, embryos injected with klf8-MO1atg or klf8-MO2atg frequently developed a no-loop heart (53% for MO1atg, 40% for MO2atg) as compared to the vast majority of wild type or klf8-4 mm MO1-injected embryos showing a D-loop heart at 48 hpf (Fig. 1b). At 72 hpf, a similar percentage of klf8-MO1atg or klf8-MO2atg-injected embryos displayed a no-loop heart (40% for MO1atg, 38% for MO2atg) as compared to wild type or klf8-4 mm MO1-injected embryos, which almost invariably had a D-loop heart (Fig. 1c). Embryos showing a delayed heart cone phenotype were also identified in the klf8-MO1atg (13%) or klf8-MO2atg (27%) groups at 24 hpf (Fig. 1a). However, this delayed phenotype was not observed in the 48 hpf or 72 hpf time points. By these stages, the delayed morphants caught up developmentally and displayed either D-loop, no-loop or L-loop hearts (Fig. 1b&c). We also examined the position of digestive organs in klf8 morphants by whole-mount in situ hybridization, using a gata6 antisense RNA probe. In

![Fig. 1](image-url)

**Fig. 1** Knockdown of zebrafish klf8 caused defects in heart jogging and looping, and visceral organ positions. **a** klf8-MO1atg or klf8-MO2atg-injected embryos stained with myl7 exhibited left (L)-jog, no-jog or right (R)-jog and were compared to stained wild-type or 4 mm-MO1-injected control embryos at 24 hpf. **b** myl7 stained embryos injected with klf8-MO1atg or klf8-MO2atg displayed D-loop, no-loop or L-loop heart and were compared to wild type and control embryos at 48 hpf. **c** myl7 stained embryos injected with klf8-MO1atg or klf8-MO2atg displayed D-loop, no-loop or L-loop heart and were compared to wild type and control embryos at 72 hpf. **d** At 54 hpf, gata6 stained wild type or embryos injected with klf8-4 mm MO1, klf8-MO1atg or klf8-MO2atg exhibited organ positions that were classified as: (Normal) normal positions of liver-left, pancreas-right and intestine with left looping, (Intestine only) only intestine without left looping, (Reverse) reversed position of liver-right, pancreas-left and no looping intestine, or (Bilateral) bilateral extension of liver and pancreas. A, atrium; I, intestine; L, liver; P, pancreas; V, ventricle.
**klf8 deficiency affects the level and pattern of expression for genes in the Nodal signalling pathway**

Genes encoding Nodal signalling components, including *spaw*, *lft1*, *lft2*, and *pitx2*, were asymmetrically expressed in the left side of the diencephalon, heart, or lateral plate mesoderm (LPM) during the 18–22 s stages in zebrafish embryos (Fig. 2a–m). Disruption in the expression of left-side specific Nodal signalling genes results in organ heterotaxy. We observed that *spaw* expression in the left LPM was either decreased (no expression in the anterior LPM and low expression in the posterior LPM, 27%) or absent (46%) in many *klf8* morphants (Fig. 2d–f). A similar effect (32% decreased, 25% absent, 8% bilateral, 5% right) was observed following *klf8-MO2* injection (Fig. 2g–j). Consistent with these results, the expression of genes downstream of *spaw* (*lft1, lft2*, and *pitx2*) was also absent or decreased in the left diencephalon, heart, and LPM of most *klf8-MO1* or *klf8-MO2* injected embryos (Fig. 2h–o). Additionally, we injected three splicing MOs (*MO1atg*, *MOAC*, and *MOsp*) to block splicing of *klf8* mRNA (Additional file 1: Figure S1, A). RT-PCR indicated that splicing of *klf8* mRNA was effectively disrupted in *MOsp*-injected embryos at 24 hpf (Additional file 1: Figure S1, B). Expression of *spaw* in the left LPM was either decreased (8%) or eliminated (63%) in most 18 s stage embryos injected with *MOsp* (Additional file 1: Figure S1, C).

*klf8* was previously shown to repress *p53* expression and induce *met* expression to modulate the development of Purkinje cells and proliferation of granule cells [35]. To confirm that defective left-right patterning did not arise from induction of *p53* due to *klf8* deficiency, we analysed heart looping and gene expression of Nodal signalling components in embryos that were co-injected with *p53-MO* and *klf8-MO1* or *klf8-MO2*.

Of the embryos co-injected with *p53-MO*, together with *klf8-MO1* or *klf8-MO2*, 35–39% exhibited a no-loop heart at 72 hpf, similar to embryos injected with *klf8* MOs alone (Additional file 2: Figure S2, A). Likewise, expression levels of *spaw, lft1, lft2*, and *pitx2* were reduced or eliminated in the left LPM, diencephalon, and heart of high percentages of co-injected embryos during the 18–22 s stages (Additional file 2: Figure S2, B–E). We also found that the expression levels of *gata5* and *oep* (which are known to be expressed in the LPM at the 22 s stage) were unaffected by *klf8* knockdown (Additional file 2: Figure S2, F–M). Together these data clearly indicate that increased *p53* expression and apoptosis are not responsible for the decreased expression of genes involved in Nodal signalling.

To further confirm that the decrease in *spaw* expression is a consequence of *klf8* loss-of-function, we performed rescue experiments by co-injecting embryos with *klf8-MO1* and *klf8* mRNA. Approximately 69% of *klf8-MO1* injected embryos exhibited eliminated or
decreased spaw expression in the left LPM at the 18 s stage (Fig. 3a, e). However, this proportion showed a statistically significant reduction to 44% for embryos co-injected with klf8-MO1\textsuperscript{atg} and klf8 mRNA (Fig. 3b-e). Taken together, these results demonstrate that klf8 loss-of-function causes downregulation of Nodal signalling component genes.

**Overexpression of klf8 mRNA causes bilateral expression of genes involved in Nodal signalling**

Since klf8 knockdown reduced expression of genes involved in Nodal signalling, we hypothesized that klf8 overexpression may have the opposite effect. While the majority of embryos injected with 100 pg of LacZ mRNA expressed spaw exclusively in the left LPM at the 18 s stage, 24% of embryos injected with 50 pg and 51% of embryos injected with 100 pg of klf8 mRNA expressed spaw bilaterally in the LPM (Fig. 4c, e). Thus, a dose-dependent effect of klf8 expression was revealed. Moreover, embryos overexpressing klf8 also frequently exhibited bilateral expression patterns of lft1, lft2, and pitx2 in the diencephalon, heart, and LPM at 19–22 s stages (Fig. 4h–t). On the other hand, ntl expression in the notochord was not altered in 22 s stage embryos overexpressing klf8 as compared to LacZ-overexpressing embryos (Fig. 4u–w). In order to investigate whether the zinc finger DNA binding domain of Klf8 is involved in regulating the expression pattern of spaw or its downstream genes, we injected mRNA for klf8 lacking the zinc finger DNA binding domain (klf8\textsuperscript{Δzf}). We found that injection of 100 pg klf8\textsuperscript{Δzf} only induced a low percentage of embryos to exhibit bilateral expression of spaw (6.3%), lft1 (3.6%), lft2 (1.6%) or pitx2 (11.9%), compared to higher rates of bilateral spaw (48.3%), lft1 (31.6%), lft2 (37.5%) or pitx2 (44.3%) expression in klf8 injected embryos at 18 s or 19–22 s stages (Additional file 3: Figure S3). These results demonstrate that overexpression of klf8 does not affect the midline structure, but induces ectopic expression of spaw and its downstream genes when the Klf8 zinc finger DNA binding domain is intact.

**klf8 deficiency affects morphogenesis of Kupffer’s vesicle and asymmetric charon expression**

Since asymmetric flow, generated by rotation of cilia within KV, is essential to initiate left-right asymmetric patterning, and KV is derived from dorsal forerunner cells (DFCs), we then investigated whether klf8 knockdown affected cilia or DFC number during KV morphogenesis. Individual klf8-MOs or control MO were microinjected into one- or two-cell Tg(sox17:gfp) zygotes, and immunofluorescence was conducted using anti-GFP antibody. We found that the number of DFCs at the dorsal margin was significantly reduced in klf8-MO1\textsuperscript{atg}- (average of 26.5 DFCs) or klf8-MO2\textsuperscript{atg}- (average of 27.6 DFCs) injected Tg(sox17:gfp) embryos as compared to wild type (average of 36.2 DFCs) or klf8-4 mm MO1- (average of 35.7 DFCs) injected embryos at 75% epiboly (Fig. 5a–d, o). Cilia were then detected by immunofluorescence staining of 10s stage embryos using an anti-acetylated tubulin antibody. KV lumen size was smaller, but not significantly so, in Tg(sox17:gfp) embryos injected with klf8-MO1\textsuperscript{atg}, klf8-MO2\textsuperscript{atg}, or MO\textsuperscript{sp}-MOs as compared to wild type or klf8-4 mm MO1-injected control embryos at 10s stage (Fig. 5e–n, p). Significantly reduced number and length of KV cilia were detected in embryos injected with different klf8-MOs as compared to wild type and control embryos (Fig. 5q, r). Since asymmetric charon expression on the right side of the KV was influenced by strength and direction of KV flow [40], we also examined whether klf8 knockdown affected asymmetric charon expression around KV. The majority (61% for MO1\textsuperscript{atg}, 57% for MO2\textsuperscript{atg}) of embryos injected with different klf8-MOs revealed symmetric charon expression with reduced expression area around KV as compared to wild type and control embryos at the 10s stage (Additional file 4: Figure S4). These results indicate that KV morphogenesis, cilia length, cilia number and asymmetric charon expression were affected in klf8 knockdown embryos.

**Generation of klf8 mutant by a CRISPR-Cas9 system**

In order to confirm our morphant results, we generated klf8 mutants using a CRISPR-Cas9 system. Although we designed three klf8 sgRNAs targeting to exon 2 or exon 3, only klf8 sgRNA1, which targets to exon 2, was successful in producing mutants. Administration of sgRNA1 induced efficient deletion or insertion of bases in exon 2 and resulted in two klf8 mutant alleles (klf8\textsuperscript{Δ25} and klf8\textsuperscript{i17}) (Fig. 6a). The klf8\textsuperscript{Δ25} mutant had a 25 bp deletion, which produced a 35 amino acid-long misframed Klf8.
protein, while the \(klf8^{i17}\) mutant had a 17 bp insertion that generated a 49 amino acid-long misframed Klf8 protein (Fig. 6b).

Of note, in 24 hpf homozygous F3 embryos of \(klf8^{d25}\) and \(klf8^{i17}\) mutants, we did not observe smaller eyes and abnormal cerebellar morphology that were detected in \(klf8\) morphant embryos [35]. Next, we investigated whether \(spaw\) expression was affected in \(klf8\) mutant embryos. Bilateral \(spaw\) expression was more frequently observed in embryos from the intercross of respective \(klf8^{d25}\) (27 out of 69, 39.1%) or \(klf8^{i17}\) (9 out of 35, 25.7%) F2 heterozygous mutants as compared to wild type embryos (7%) at 18 s stage (Fig. 6c-g). In order to evaluate the genotype of F3 embryos from \(klf8^{i17}\) and \(klf8^{d25}\) mutants with bilateral \(spaw\) expression, we sequenced seven \(klf8^{i17}\) and 18 \(klf8^{d25}\) embryos with the phenotype. From sequencing data, we obtained two (28.6%) wild type, three (42.9%) heterozygotes and two (28.5%) homozygotes from a total of seven \(klf8^{i17}\) F3 embryos, as well as three (16.7%) wild type, seven (38.9%) heterozygotes and eight (44.4%) homozygotes from a total of 18 \(klf8^{d25}\) embryos. We then deduced that 7.3% of \(klf8^{i17}\) or 17.4% of \(klf8^{d25}\) F3 embryos were homozygous mutant embryos that also had bilateral \(spaw\) expression. This observation was based on the following calculation \([0.285 \times 9 / 35 = 7.3\%\) for \(klf8^{i17}\); \(0.444 	imes 27 / 69 = 17.4\%\) for \(klf8^{d25}\)\]. The other F3 embryos that exhibited bilateral \(spaw\) expression were also deduced to be either heterozygous mutant embryos (11% in \(klf8^{i17}\), 15.2% in \(klf8^{d25}\) or sibling wild type (7.4% in \(klf8^{i17}\), 6.5% in \(klf8^{d25}\) based on similar calculations.

Because human KLF8, KLF3 and KLF12 form a subgroup in phylogenetic tree analysis due to the presence of CtBP-binding sites [41], we wondered whether expression of zebrafish \(klf3\), \(klf12a\), or \(klf12b\) may be upregulated to compensate for \(klf8\) deficiency. We discovered substantial upregulation of \(klf12b\) in \(klf8^{d25}\) F5 homozygous mutant embryos and
downregulation of klf12a in klf8\textsuperscript{\texttt{D25}}. F5 homozygous mutant embryos at 10–12 s stages, while no alteration of klf3 expression was observed in either klf8\textsuperscript{\texttt{D25}} or klf8\textsuperscript{\texttt{I17}} mutant embryos (Fig. 6h-m). Subsequently, we knocked down klf12b in klf8\textsuperscript{\texttt{D25}} F6 homozygous mutant embryos and evaluated the spaw expression pattern at 18 s stage (Fig. 6n). In klf8\textsuperscript{\texttt{D25}} F6 homozygous mutant embryos, bilateral (50.3%) and decreased (6.3%) spaw expression patterns were detected. In homozygous mutant embryos injected with 5 or 10 ng klf12b MO, we found a dose-dependent reduction that did not reach significance in the percentage (42.1% for 5 ng, 29.3% for 10 ng) of embryos with bilateral spaw expression, which was accompanied by the increased occurrence of right (1.6% for 5 ng, 1.7% for 10 ng), decreased (15.9% for 5 ng, 16.4% for 10 ng) or absent (4.0% for 5 ng, 3.4% for 10 ng) spaw expression patterns.

These results indicate that although klf8 mutant embryos display different spaw expression patterns than morphant embryos, this effect may be partly attributed to a compensatory induction of klf12b expression.

**Discussion**

Establishing asymmetric spaw expression in the LPM is essential for left-right patterning in zebrafish embryos. Expression of spaw is first apparent in bilateral cells flanking KV between the 4–6 s stages, while asymmetric spaw expression emerges in the posterior LPM during the 10–12 s stages, and extends to the anterior LPM by the 18 s stage [22]. Knockdown of spaw abolishes spaw expression in the LPM but not in peri-KV domains [21], suggesting that autoregulation of spaw occurs only in the left LPM. We used morpholino antisense oligomers to knockdown klf8, and found that spaw expression in the left LPM was reduced or eliminated in the majority of 18–22 s stage morphants (Fig. 2, Additional file 2: Figure S2). Spaw activates expression of itself, as well as lft1, lft2, and pitx2 in the left LPM during the
segmentation stage [22]. As such, the observed reduction or elimination of lft1, lft2, and pitx2 in the left diencephalon, heart or LPM at 18–22 s stages in the majority of klf8 morphants was not unexpected (Fig. 2, Additional file 2: Figure S2). Overall, defects in the expression of spaw and its downstream genes, and the subsequent defects in internal organ patterning observed in klf8 morphants are consistent with those detected in spaw morphants [22]. Although spaw/sfw mutant embryos more frequently displayed a D-loop heart (68%), compared to klf8 morphants, the difference may be attributed to heart specific actomyosin activity. Furthermore, in sfw mutant embryos, expression of laterality genes including lft1, lft2, and pitx2 were lost, and spaw expression did not propagate toward the anterior of the left LPM [42]. In our study, we saw that overexpression of klf8 but not klf8Δzf resulted in bilateral expression of spaw, lft1, lft2, and pitx2 at 18 s or 19–22 s stages (Fig. 4, Additional file 3: Figure S3), demonstrating a requirement for the Klf8 zinc finger DNA binding domain. Expression of ntl in the notochord was found to be unaltered in klf8-overexpressing embryos, suggesting that the midline structure is in intact. Overall, our results demonstrate that Klf8 may regulate asymmetric spaw expression in the left LPM, which in turn affects left-side specific expression of lft1, lft2, and pitx2 in zebrafish embryos.

Proper morphogenesis of KV is also important for initial asymmetric spaw expression in the posterior LPM. The development of KV involves the formation of DFCs from surface epithelial cells, ingression at the dorsal germ ring margin, DFC migration, formation of rosette-like epithelial structures, coalescence of epithelial rosettes, and differentiation of ciliated KV with interior lumen [15, 43]. Both Tbx16 and Ntl were shown to regulate a mesenchymal to epithelial transition that participates in the formation of rosette-like epithelia [44]. Wnt11- and Prickle1a-mediated planar cell polarity signalling, as well as Cnpy1-mediated FGF signalling, were shown to regulate cell adhesion between adjacent dorsal
forerunner cells to maintain cluster formation [45, 46]. Defects in these signalling events resulted in small KV lumen, with shortened and decreased number of KV cilia. In klf8 morphants, a similar smaller KV lumen, with decreased number and length of KV cilia was frequently observed at the 10s stage. These abnormal structures may result from lower number of DFCs that was observed in 75% epiboly morphants (Fig. 5). Whether Klf8 may participate in Wnt11- and Prickle1a-mediated signalling to modulate DFC cluster formation, remains to be determined.

Zebrafish KV architecture is asymmetric along the anterior-posterior axis, with more ciliated cells in the anterior region. Furthermore, the positioning of the basal body of motile cilia at the posterior end of the epithelial cells may result in cilia tilting [17, 47]. These motile cilia then use a vortical motion to generate swirling fluid flow consisting of a relatively stronger leftward flow across the anterior pole of KV and a weaker rightward flow at the posterior end [16, 18]. Based on experimental tracking of native particles within the KV of wild type, did−/− mutant and dnah7 morphants, and simulated flow by mathematically modelling, it was determined that a threshold of 30 cilia, with dorsal anterior clustering, is essential to generate proper swirling flow in the anti-clockwise direction [40]. In control embryos, with strong left-sided flow across the anterior pole of KV, asymmetric expression is established for charon in the right side of the KV, and spaw in the left LPM. In embryos with non-directional flow, symmetric charon expression and a lack of spaw expression may be found. Embryos without motile cilia, and therefore no KV flow, may exhibit symmetric and slightly weaker charon expression and bilateral spaw expression in the posterior LPM [40]. In klf8 morphant embryos, injected with different klf8 MOs, a significantly reduced number of cilia (< 30), with random distribution was detected (Fig. 5). KV with such a cellular architecture may exhibit a weak and homogenous fluid flow, resulting in the symmetric charon expression around KV that was detected in the majority of klf8 morphants, and leading to drastically reduced spaw expression at late somite stage (Additional file 4: Figure S4, Fig. 2). Overall, our results clearly indicate that Klf8 is required for normal KV morphogenesis, which is known to be critical for initiating asymmetric spaw expression in the left LPM.

In mouse and chick, BMP signalling plays either a positive or negative role in regulating asymmetric Nodal expression. Moreover, the presence of BMP antagonists, such as Noggin, Chordin, or Caronte, can relieve BMP-inhibition to promote asymmetric Nodal expression in the left LPM [48−51]. In zebrafish embryos, heat-activated BMP2b expression inhibits spaw expression, while heat-activated noggin3 induced bilateral spaw expression, indicating that BMP signalling is required to repress spaw expression in the right LPM of early segmentation stage embryos, [25]. In addition, expression of Lft1 in the midline, and Lft2 in the left cardiac field, serve to generate a posterior or anterior barrier to restrict Spaw activity to the left LPM during segmentation stages in zebrafish embryos [27]. Dvr1, a zebrafish Vg1 ortholog, was also shown to facilitate the transfer of spaw expression from the peri-KV region to the left LPM. Thus, reduced or absent expression of spaw and downstream lft1 and lft2 in the LPM, diencephalon, notochord, or heart were detected in dvr1 morphants [52]. In order to investigate whether Klf8 may regulate asymmetric spaw expression via modulation of expressions of bmp2b or dvr1, we then compared expression of these two genes between klf8 morphants and control embryos (Additional file 5: Figure S5). Similar bmp2b expression level around tailbud region was identified in 3 s stage wild type and embryos injected with klf8-MO1$a^g$, klf8-MO2$a^g$ or klf8-4 mm MO1. Likewise, no alteration of dvr1 expression around the tailbud region was detected in wild type and embryos injected with klf8-MO1$a^g$, klf8-MO2$a^g$ or klf8-4 mm MO1 at bud stage. Thus, Klf8 does not act via BMP2b or Dvr1 signalling pathway to regulate asymmetric spaw expression, and the underlying mechanism remains to be determined.

In addition to our studies with klf8 morphants, we generated klf8 mutants by a CRISPR-Cas9 system (Fig. 6). Intriguingly, obvious phenotypic differences were found between morphants and mutants. In 24 hpf homozygous F3 embryos of klf8d25 and klf8i17 mutants, we did not observe smaller eyes and abnormal cerebellar morphology that were detected in klf8 morphant embryos [35]. In addition, bilateral spaw expression was detected in the LPM of klf8d25 and klf8i17 mutants at the 18 s stage (Fig. 6). Discrepant phenotypes between mutants created by TALENs or CRISPR-Cas genome editing systems and antisense morpholino mediated-morphants have been frequently encountered. Previously, differences have been attributed to off-target effects of morpholinos [53], or compensatory effects, which have been described in vasculature development [54], reproduction [55], or neurogenesis [56]. With regard to the two klf8 mutant alleles from our study, more klf8d25 mutant embryos showed bilateral spaw expression in the LPM, compared to klf8i17 mutants (Fig. 6g, n). This difference in outcome may correlate with aberrant upregulation of klf12b in klf8d25 that was further confirmed by klf12b knockdown, but not in klf8i17, mutants (Fig. 6n). Similar compensatory inconsistency was found in stmn4 mutants, which had a low (< 10%) portion of embryos showing similar phenotype to stmn4 morphants. Interestingly, the authors found that stmn1b was upregulated to
compensate in \textit{stmn4–5} but not \textit{stmn4–4} mutants [56]. In our study, we observed that in response to \textit{klf8} deficiency, \textit{klf12b}, a member of a subgroup of KLF family with a CtBP interaction site, was induced to compensate for the loss of \textit{klf8}. However aberrant upregulation of \textit{klf12b} further resulted in bilateral \textit{spaw} expression. On the contrary, downregulation of \textit{klf12a} was detected in \textit{klf8} mutants (Fig. 6). In these mutants, bilateral \textit{spaw} expression was observed to a lesser degree, suggesting that \textit{klf12a} may have undergone functional divergence with \textit{klf12b}, and as such, \textit{klf12a} may play a role in restricting \textit{spaw} expression to the left side of embryos. Overall, \textit{klf8} mutant embryos showed bilateral \textit{spaw} expression, which was quite different from \textit{klf8} morphants that exhibited reduced or eliminated \textit{spaw} expression in the LPM. This dissimilar phenotype may have been partly related to the compensatory induction of \textit{klf12b} expression in the mutant embryos.

Conclusions

In this report, we have demonstrated a novel role for zebrafish \textit{Klf8} in left-right asymmetric patterning. During gastrulation, \textit{Klf8} may regulate DFC cell number to control proper KV morphogenesis, which is essential to initiate asymmetric \textit{spaw} expression in the left LPM. During somitogenesis, \textit{Klf8} may further modulate asymmetric \textit{spaw} expression in the left LPM to ensue asymmetric organ positioning.

Additional files

**Additional file 1: Figure S1.** Knockdown of \textit{klf8} expression by splicing morpholino oligomers resulted in embryos with reduced or absent \textit{spaw} expression in the left LPM. \textbf{A} \textit{klf8} genomic structure showing position of translational morpholino oligomers (\textit{klf8-MO1}\textsuperscript{495}, \textit{klf8-MO2}\textsuperscript{495}) and splicing morpholino oligomers (\textit{klf8-MO1}\textsuperscript{495}, \textit{klf8-MO2}\textsuperscript{495}). Arrows indicate the positions of forward and reverse primers. \textbf{B} RT-PCR showing the efficacy of \textit{klf8} splicing morpholino oligomers. The majority of embryos injected with \textit{klf8} splicing MOs had decreased or absent \textit{spaw} expression in the left LPM. (TIFF 153 kb)

**Additional file 2: Figure S2.** Heart looping and downregulated expression of \textit{spaw} and its downstream genes were not caused by induction of \textit{ps3} expression in \textit{klf8} morphants. Embryos co-injected with \textit{ps3-MO1\textsuperscript{495}} and \textit{klf8-MO1\textsuperscript{495}} or \textit{klf8-MO2\textsuperscript{495}} displayed no-loop or L-loop heart defects at 72 hpf (\textbf{A}). The majority of embryos co-injected with \textit{ps3-MO1\textsuperscript{495}} and \textit{klf8-MO1\textsuperscript{495}} or \textit{klf8-MO2\textsuperscript{495}} exhibited decreased or absent expression of \textit{psaw} (\textbf{B}), \textit{lt1} (\textbf{C}), \textit{lt2} (\textbf{D}), or \textit{pib2} (\textbf{E}) in the LPM, diencephalon or heart at the 18–22 s stages. Expression levels of \textit{gata5} and \textit{aco} which are known to be expressed in the LPM at the 22 s stage were unaffected by \textit{klf8} knockdown (\textbf{F–I}). (TIFF 797 kb)

**Additional file 3: Figure S3.** Overexpression of \textit{klf8} but not \textit{klf8-zf} mRNA induced bilateral \textit{klf8} mRNA which inhibit expression in the LPM. \textit{lt1} in the diencephalon and heart, \textit{lt2} in the heart, and \textit{pib2} in the LPM at 18 s or 19–22 s stages. (TIFF 74 kb)

**Additional file 4: Figure S4.** Symmetric \textit{charon} expression around KV was observed in the majority of \textit{klf8} morphants. Representative images of embryos showing stronger \textit{charon} expression on the right side (\textbf{A}) or left side (\textbf{B}) and symmetric \textit{charon} expression on both sides (\textbf{C}) of KV are shown. Quantification of different \textit{charon} expression patterns in embryos injected with different \textit{klf8} MOs, control MO or wild type embryo is shown (\textbf{D}). Statistical significance was determined by Student’s \textit{t}-test. * \textit{p} < 0.05. Error bars indicate standard deviation. (TIFF 397 kb)

**Additional file 5: Figure S5.** Expression level of \textit{bmp2b} or \textit{drr1} around tailbud region was not affected by \textit{klf8} knockdown. Representative images show similar expression level of \textit{bmp2b} (\textbf{A–D}) or \textit{drr1} (\textbf{E–H}) around the tailbud region in the wild type or embryos injected with \textit{klf8-MO1\textsuperscript{495}}, \textit{klf8-MO2\textsuperscript{495}} or \textit{klf8-4 mm MO1} at 3 s or bud stages. (TIFF 382 kb)

**Abbreviations**

ASE: Left-side specific enhancers; CRISPR-Cas: Clustered regularly interspaced short palindromic repeats- CRISPR-associated system; CtBP: C-terminal binding protein; DFCs: Dorsal forerunner cells; Hpf: Hours post fertilization; Klf8: krüppel-like factor 8; KV: Kupffer’s vesicle; lt1: left1; lt2: left2; LPM: Lateral plate mesoderm; MO: Morpholino oligomer; Ndr: Nodal-related genes; RT: Room temperature; RT-PCR: Reverse transcription PCR; RT-qPCR: Reverse transcription quantitative real-time PCR; S: Somite

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**Availability of data and materials**

All materials are available by the corresponding author.

**Authors’ contributions**

CYL, MYT, YCC, YRL, YHL, HCL, HWL, CHY performed experiments; CYL, MYT, CJH and SPLH analysed data; SPLH conceived the project and wrote the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

All animal procedures were approved by the Institutional Animal Care and Use Committee of Academia Sinica (Protocol ID: 15-12-918).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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