

Meteorins regulate the formation of the left-right organizer and the establishment of vertebrate body asymmetry

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eLife Assessment

This study presents **important** insights into the regulation of left-right organ formation. By combining genetic perturbation of all three Meteorin genes in zebrafish and timelapse imaging, the authors identify an essential role for this protein family in the establishment of left-right patterning. They provide **convincing** evidence that Meteorins are required for the morphogenesis of dorsal forerunner cells, the precursors of the left-right organizer (also named Kupffer's vesicle) in zebrafish. In line with this, Meteorins were shown to genetically interact with integrins ItgaV and Itgb1b to regulate dorsal forerunner cell clustering.

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Abstract

Summary

While the exterior of vertebrate bodies appears bilaterally symmetrical, internal organ positioning and morphology frequently exhibit left-right (L-R) asymmetries. In several vertebrates, including human, mouse, frog and zebrafish, left-right symmetry-breaking during embryonic development is initiated by a ciliated organ called the Node or left-right organizer. Within the Node, a leftward flow of extraembryonic fluid named the Nodal flow mediates the asymmetric expressions of Nodal factors. Although downstream Nodal pathway components leading to the establishment of the embryonic left-right axis are well known, less is known about the development and formation of the embryonic Node itself.

Here we reveal a novel role for the Meteorin protein family in the establishment of the left-right axis and in the formation of the Kupffer's vesicle, the Node equivalent structure in zebrafish. We show that the genetic inactivation of each or all three members of the zebrafish Meteorin family (*metrn*, *metrn-like a* and *metrn-like b*) leads to defects in properties of the

Kupffer's vesicle, caused by impaired assembly and migration of the Kupffer's vesicle forming dorsal forerunner cells. In addition, we demonstrate that Meteorins genetically interact with integrins ItgaV and Itgβ1b regulating the dorsal forerunner cell clustering and that *meteorins* loss-of-function results in disturbed Nodal factor expression and consequently in randomized or symmetric heart looping and jogging.

These results identify a new role for the Meteorin protein family in the left-right asymmetry patterning during embryonic vertebrate development.

Introduction

From the outside the vertebrate body plan appears bilaterally symmetric. However, internal organs positioning and morphology often displays left-right (L-R) asymmetries. For instance, in vertebrates the heart generally lies on the left side, the liver and the pancreas are positioned on the right and the gut presents asymmetric rotations.

One major regulator for the establishment of the L-R axis is Nodal, a ligand belonging to the TGFβ protein family [1–6]. Its signaling is activated in the left lateral plate mesoderm (LPM), whereas Nodal remains inactive in the right LPM, creating an embryo-scale left-right asymmetry [3]. In mice, this asymmetry is achieved by an oriented rotation of the cilia of a structure called the Node generating a leftward flow of extraembryonic fluid named the Nodal flow. This results in an asymmetric expression of several Nodal factors and Nodal signaling. Participating in this patterning, Leftys, soluble inhibitors belonging to a subclass of TGF factors, antagonize Nodal signaling [7,8].

The Node and the Nodal flow have been described in numerous vertebrates. For instance, in *Xenopus* the gastrocoel roof plate and in zebrafish the Kupffer's vesicle (KV) [9–14] are key structures of the L-R symmetry breaking during the embryonic development [11,13,15]. Similarly, in humans and other mammals like rabbits and mice, the systematic establishment of left-right body symmetry begins with asymmetric fluid flow driven by rotating cilia [16]. This occurs within the transient primitive node in humans or the embryonic node in mice and rabbits [13,15,17]. However, in other mammalian classes, although left-right symmetry breaking mechanisms are believed to be cilia-independent, they are still poorly understood at present [2,18]. In chick, the symmetry breaking structure known as the Hensen's node, serves as the ciliated organizer responsible for the left-right asymmetry establishment. In this species, the process of symmetry breaking is believed to occur independently of the non-motile cilia present within the Hensen's node. Instead, the breaking of symmetry is primarily attributed to the leftward movement of cells around the node [19–21].

Compared to the relatively flat-shaped mouse node, the zebrafish KV is a fluid-filled sphere with a ciliated epithelium and it is formed by dorsal forerunner cells (DFCs) [9]. DFCs first emerge as cells of the epithelium of the dorsal surface in direct contact with the yolk syncytial layer at 6 hours post-fertilization (hpf) [22]. Around 8 to 9 hpf, following the epibolic movement, 20 to 30 DFCs form a single cluster and migrate towards the vegetal pole in a non-involuting manner at the leading edge [22,23]. During this migration process, polarized DFCs organize into multiple focal points by retaining long-lasting apical contacts within the cluster and remaining delaminated DFCs maintain contact with the cluster by cell-cell contact mechanisms [22,24]. At the final phase of epiboly and after reaching the vegetal pole, the DFC cluster is separating from the dorsal marginal enveloping layer cells by losing the apical contacts [22,24].

The focal points of the cluster are then rearranged by integrating the unpolarized DFCs to form a single focal point that will expand into a monolayer rosette structure containing a lumen to form the KV by 12 hpf [9,22–24]. Along this process, non-motile and motile monocilia are

generated on the apical membrane facing the lumen that create a counterclockwise flow of fluid by the KV, called Nodal flow [22,25]. It was shown that the leftward directed flow within the KV results in asymmetric expression of zebrafish Nodal genes, like *spaw*, *lefty1* and *lefty2* that is fundamental for proper L-R patterning [26,27]. However, the molecular mechanisms involved in correct DFC migration and clustering that will form the KV remain largely elusive. In this context, it has been reported that *nodal*, *lefty* as well as *p-smad2* expression are downregulated in *Meteorin*-null ES-cells. As such, Meteorin (*Metrn*) was hypothesized to be a novel important regulator of Nodal transcription [28].

Meteorin (*Metrn*), a secreted neurotrophic factor highly conserved among vertebrates, is expressed during early mouse development. It is already detected at the blastocyst stage in the inner cell mass and then expands through the extraembryonic ectoderm to the central (CNS) and peripheral nervous system (PNS) during later developmental stages [28]. It was first described to induce glial cell differentiation and to promote axonal extension in dorsal root ganglion (DRG) explants *in vitro* [29]. The disruption of *Metrn* function in Mouse resulted in early embryonic lethality [28] preventing the investigation of *Metrn* proteins function during early embryonic development *in vivo* and in particular their role for L-R patterning. Also conserved among vertebrates, its paralog Meteorin-like (*Metrl*) has been first reported as a downstream target of the Pax2/5/8 signaling pathway during otic vesicle development [30] and was later shown to have neurotrophic properties comparable to the ones of *Metrn* [31].

Here, using the zebrafish larva as a model system, we generated knockout lines for all three-existing zebrafish *metrn* genes (*metrn*, *metrnl* and *metrnlb*). While all three single and compound mutants are viable, *metrns* mutant embryos displayed organ patterning defects notably with randomized or symmetric heart looping at a significantly higher frequency. Like in mice, *metrn* genes are expressed during early development already from the 2-cells stage and transcripts are detected in DFCs as well as in the KV structure. We show that *metrns* loss of function leads to DFC disorganization and Nodal flow formation defects within the KV. Together our study reveals a critical role for *Metrn* proteins in the DFC clustering, KV formation and in the establishment of the L-R axis.

Results

Metrns loss-of-function affects proper heart looping and correct visceral organs positioning

To study the functions of Meteorin proteins during early development, we generated knock out zebrafish lines using the CRISPR/Cas9 technology to target the second exon of each gene that encodes for the signal peptide sequence. The newly generated zebrafish mutant lines carried out-of-frame deletions in the coding sequence of *metrn*, *metrnl* and *metrnlb* (Fig. S1A). In contrast to published embryonic lethal *Metrn* mouse mutants [28], constitutive homozygote zebrafish mutants for all three genes individually (*metrn*^{-/-}; *metrnl*^{-/-}; *metrnlb*^{-/-}), in double (*metrn*^{-/-}; *metrnl*^{-/-}; *metrnlb*^{-/-}; *metrn*^{-/-}; *metrnlb*^{-/-}; *metrnl*^{-/-}; *metrnlb*^{-/-}) and triple (*metrn*^{-/-}; *metrnl*^{-/-}; *metrnlb*^{-/-} or triplMut), were all viable and did not show any obvious morphological defects (Fig. 1A).

We first validated that our mutagenic approach resulted in the generation of null alleles. To do so, we performed *in situ* hybridization on *metrn*, *metrnl* and *metrnlb* from 2-cells stage to 1 day post-fertilization (dpf) on triplMut embryos (Fig. S1B). No expression could be detected for neither of those two genes in the triplMut embryos, indicating the degradation of the transcripts in the *metrn* mutant backgrounds (Fig. S1B). To confirm this observation, we compared the expression level of all three *metrn* genes upon total mRNA extraction from 14 and 48 hpf WT and triplMut embryos by qRT-PCR. *Metrn* and *metrnl* expression was detected in samples of 14 hpf WT embryos and *metrnlb* expression was detected in samples of 48 hpf WT embryos. A significant reduction of the

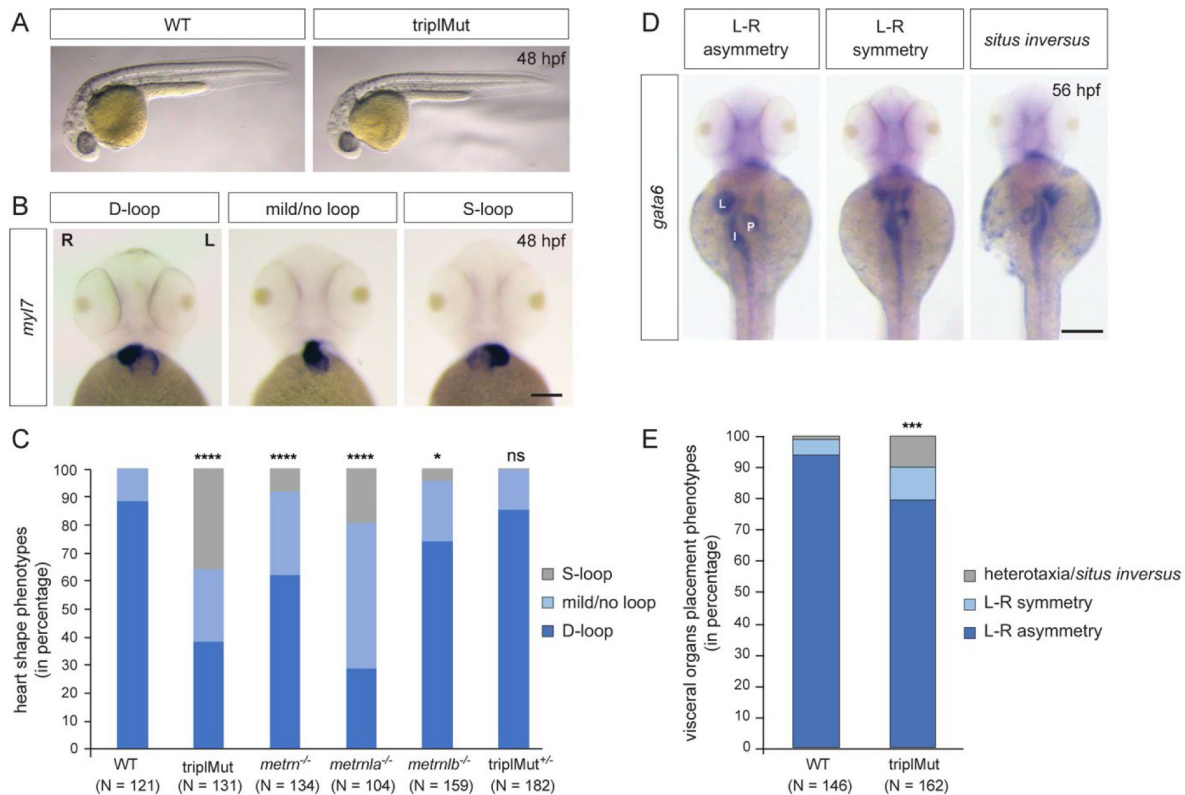


Fig. 1.

Metrn loss-of-function causes heart jogging/looping and visceral organ positioning defects.

(A) 48 hpf triplMut zebrafish display no gross phenotypic defects compared to wild type (WT) embryos. (B) Examples of 48 hpf embryos showing mRNA expression of the heart marker *myl7* and the different heart looping phenotypes (ventral view). (C) Quantification in percentage of heart looping phenotypes at 48 hpf in WT, triplMut, *metrn*^{-/-}, *metrnla*^{-/-}, *metrnlb*^{-/-}, triplMut^{+/-} embryos; displayed *p*-values compared to WT: *****p*-value: <1.0e-5 for triplMut, *****p*-value: <1.0e-5 for *metrn*^{-/-}, *****p*-value: <1.0e-5 for *metrnla*^{-/-}, **p*-value: 0.003 for *metrnlb*^{-/-}, ns *p*-value: 0.49 for triplMut^{+/-}, *p*-value compared to *metrnlb*^{-/-}: **p*-value: 0.034 for triplMut^{+/-} (not displayed). (D) Example of 56 hpf embryos showing *gata6* mRNA expression highlighting the different visceral organ positioning phenotypes (ventral view). (E) Quantification in percentage of visceral organ positioning phenotypes at 56 hpf in WT and triplMut embryos, ****p*-value: 0.00034. In (B) L = left, R = right. In (D) L = liver, P = pancreas, I = intestine. Scale bars in (B): 100 μ m, (D): 500 μ m.

expression of all *metrn* genes could be measured using triplMut samples of the same age. These results suggest that the mRNA produced by the loci that we targeted is degraded by nonsense-mediated decay (**Fig. S1C** [↗](#)).

Despite the absence of gross morphological defects, using *myl7* antisense riboprobe *in situ* hybridization in single mutant embryos, we observed a mis-positioning of the heart in triplMut mutant embryos. Indeed while 88.43% of the wild type control embryos had D-looped shaped hearts, only 38.17% of the triplMut embryos exhibited a WT-like heart looping phenotype (**Table 1** [↗](#) and **Fig. 1B-C** [↗](#)). Similarly, *metrn*^{-/-} and *metrnla*^{-/-} single mutant embryos displayed a high proportion of randomized heart phenotypes, whereas *metrnlb*^{-/-} single mutant embryos showed a small percentage of heart looping phenotypes (**Table 1** [↗](#) and **Fig. 1B-C** [↗](#)). The injection of *metrn* and/or *metrnla* mRNA into one-cell stage triplMut zebrafish embryos could partially rescue the observed phenotypes (**Table 1** [↗](#), **Fig. S1D** [↗](#)). In addition to the heart, morphological L–R asymmetry of the visceral organs was also perturbed in triplMut embryos (using *gata6* expression as a marker). Indeed, 10.49% of the triplMut embryos exhibited symmetrical placement of the gut and pancreas with respect to the midline and 9.88% *situs inversus* or heterotaxy phenotypes compared to wild type embryos (WT: 93.84% L–R asymmetry, 4.79% L–R symmetry and 1.37% heterotaxia) (**Fig. 1D-E** [↗](#)). These results indicate that while *Metrns* are not implicated in the specification of neither the heart nor the visceral organs (*myl7* and *gata6* expression being detected in triplMut embryos and in single mutants for *metrns*), their loss of function reveal that they are implicated in the L–R asymmetry positioning of these organs. The left-jogged and D-looped heart or the right-positioned liver are two of many examples of early L–R asymmetry determination in zebrafish and are well-studied readouts for altered L–R patterning processes. Therefore, our results suggest that *Metrns*, in particular *Metrn* and *Metrnla*, may have a crucial function in establishing L–R asymmetry during the early stages of development. To exclude potential genetic compensation effects among paralogs that were reported in other studies [32 [↗](#)], we focused all subsequent analyses (if not stated otherwise) on the triple mutant line (triplMut).

Metrns are required for proper Nodal factor gene expression

Nodal signaling is one of the major regulatory pathways for the determination of the L–R axis during vertebrate development. In zebrafish, null mutants for several Nodal factors like *spaw* (*Nodal* zebrafish orthologue), *dand5* or *lefty1* (*lft1*) all display symmetric or randomized heart looping and jogging similarly to *Nodal* mutant mice [33 [↗](#)–36 [↗](#)]. We therefore asked whether *Metrns* loss of function could affect the expression of these factors. To do so, we assessed *dand5*, *spaw*, *lft1* and *lft2* expression in our *metrns* mutant embryos. By qRT-PCR, we measured a significant reduction of *dand5* expression at 14 hpf in triplMut embryos compared to wild type controls (**Fig. 2A** [↗](#)). Similarly, the expression of *spaw*, *lft1* as well as *lft2* in the absence of *Metrns* proteins was also downregulated in triplMut embryos compared to wild type embryos (**Fig. 2A** [↗](#)).

In order to assess whether *Metrns* loss of function altered the expression patterns of *spaw*, *lft1* and *lft2*, we performed *in situ* hybridization experiments for all three genes at 16 hpf (**Fig. 2B** [↗](#)). At 16 hpf *spaw* is normally expressed adjacent to the KV and in the left LPM. In *metrn*^{-/-} and *metrnla*^{-/-} single mutants as well as triplMut embryos, we could not detect any *spaw* expression in the left LPM and only faintly around the KV (**Fig. 2B** [↗](#)). The *spaw* inhibitor *lft1* at this stage is expressed along the midline. In all analyzed *metrn* mutants (single *metrn* and *metrnla* and triplMut), its expression was severely disrupted at the midline (**Fig. 2B** [↗](#), middle panel). The same was observed for *lft2* which expression in the left heart field was either absent or randomized in *metrn* mutants, single or triplMut embryos, compared to the wild type embryos (**Fig. 2B** [↗](#), lower panel).

We next characterized *dand5* specific R > L bias expression pattern in triplMut embryos by *in situ* hybridization. In more than 61% triplMut embryos, we could detect particularly reduced *dand5* expression at 14 hpf and 23% displayed an abnormal *dand5* R > L expression bias (**Fig. 2 C-D** [↗](#)). In single *metrn*^{-/-} and *metrnla*^{-/-} mutant embryos, *dand5* expression pattern was also altered (**Fig. S2** [↗](#)). These results are in line with previous findings reporting that *dand5* expression in the KV is

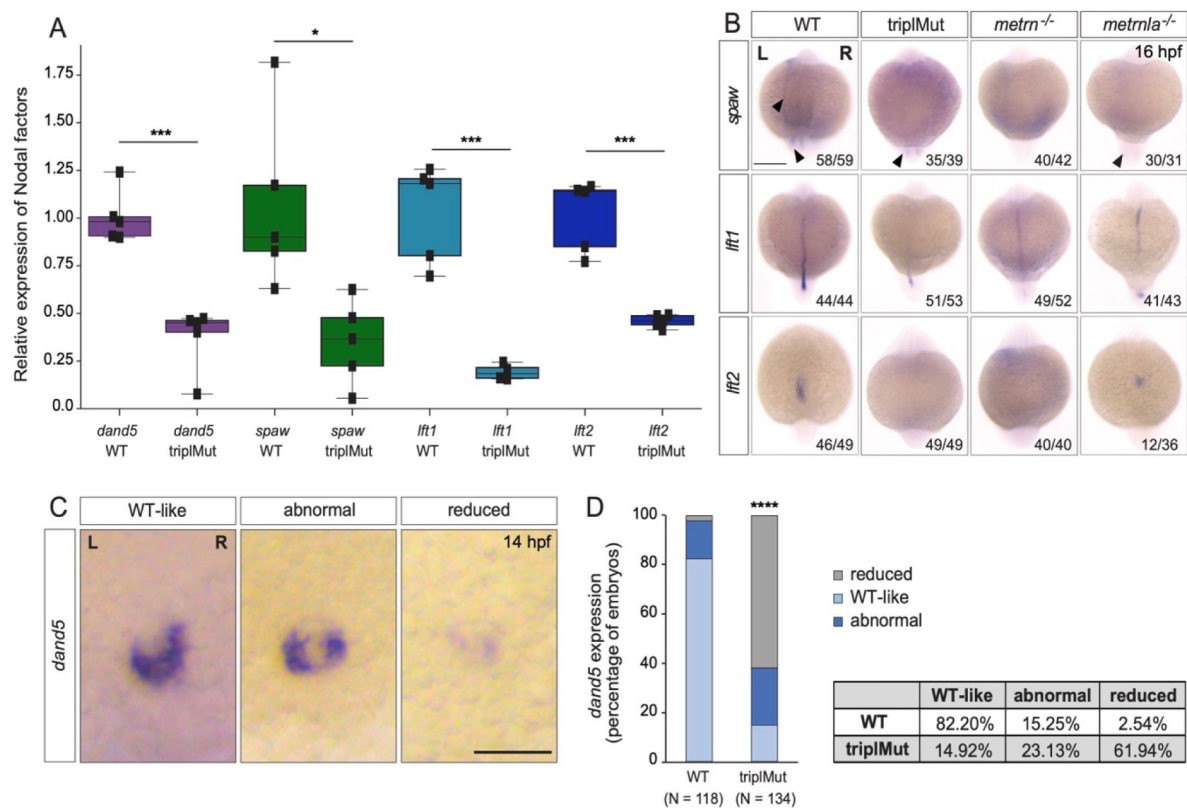


Fig. 2.

Metrn^{ns} are required for proper Nodal factor genes expression.

(A) qRT-PCR analysis for *dand5*, *spaw*, *lft1* and *lft2* expression in 14 hpf wild type (WT) and triplMut embryos (Student t-tests, ****p*-value: 0.00019 for *dand5*; **p*-value: 0.014 for *spaw*; ****p*-value: 0.0004 for *lft1*; ****p*-value: 0.00019 for *lft2*). (B) Dorsal view of 16 hpf triplMut, *metrn*^{-/-} and *metrnla*^{-/-} embryos with reduced expression of *spaw*, *lft1* and *lft2* as revealed by *in situ* hybridization. (C) *Dand5* expression at 14 hpf as revealed by *in situ* hybridization showing different transcripts distribution of *dand5* mRNA. (D) Quantification in percentage of *dand5* expression phenotypes at 14 hpf in WT and triplMut embryos, *****p*-value: <1.0e-5. Scale bar in (B): 250 µm, in (C): 100 µm; L=left, R=right.

only altered if upstream symmetry breaking mechanisms are disrupted [37–39]. These results indicate that *Metrns* are required for the proper expression of Nodal factor genes that are determinants of proper L-R patterning.

***Metrns* are expressed during early zebrafish development**

To investigate how *Metrns* affect the expression of Nodal factors during early development, we analyzed the pattern of expression of all three zebrafish *metrn* genes from 2 cell stage to 48 hpf. We first investigated the expression pattern of *metrn*, *metrnla* and *metrnlb* by *in situ* hybridization and hybridization chain reaction (HCR) experiments. At the 2-cells stage, we detected both *metrn* and *metrnla* maternally expressed transcripts, whereas *metrnlb* expression was not detected (Fig. 3A, Fig. S3A). During gastrulation, the expression of *metrn* and *metrnla* could be detected, whereas *metrnlb* expression was again not observed in our *in situ* hybridization analysis. The earliest time point for zygotic expression of *metrn* and *metrnla* was at 6 hpf (shield stage). At this stage, *metrnla* expression could be detected over the whole animal cap whereas zygotic *metrn* expression onset was observed only around the yolk syncytial layer (YSL) at this developmental stage (Fig. 3A). *Metrn* expression was then restricted to the developing LPM and to the leading edge of the former shield area from 9 hpf (Fig. 3A, Fig. S3B). In comparison, *metrnla* was found expressed throughout the whole enveloping layer and developing midline (Fig. 3A, Fig. S3B). At 12 hpf, the expression of *metrn* was restricted to the KV area while *metrnla* transcripts were mainly found in the midline (Fig. 3A-B). By 14 hpf, *metrn* expression was present in the KV area as well as in the developing brain whereas *metrnla* transcripts were mainly found in the midline and the KV area (Fig. S3B). In contrast, from the 2-cell stage to 14 hpf no *metrnlb* transcripts could be detected (Fig. 3A, Fig. S3A-B). By HCR, we found *metrn* and *metrnla* expression colocalizing in the area of the KV at 12 and 14 hpf (Fig. 3B). By 24 and 48 hpf, both genes were found strongly expressed in the central nervous system (CNS) (Fig. S3C). Finally, *metrnlb* expression onset started from 24 hpf broadly in the embryonic brain (Fig. S3C). At 48 hpf, *metrnlb* transcripts were mostly found in the otic vesicles and at the somite boundaries (Fig. S3C).

We next analyzed the relative expression of *metrn* genes to landmarks of the KV structure like *sox32* a well-studied DFC marker [40,41]. We observed a complete colocalization of *metrn/sox32* and *metrnla/sox32* signals at 10 hpf, indicating that *metrns* genes are expressed in DFCs, the cells that will later form the KV (Fig. 3C-D). To assess whether *metrn* and *metrnla* expression in the KV structure is conserved among vertebrates, we performed *in situ* hybridization for *metrn* and *metrnla* in the chick embryo at early stages. Around stage HH6, we could observe *metrn* and *metrnla* expression at the Hensen's node and around the primitive streak (marked by primitive streak landmark *fgf8*) (Fig. S3D). Remarkably, the chick *metrn* and *metrnla* expression patterns were reminiscent of the ones that we observed in zebrafish, suggesting that *metrn* genes expression at early developmental stages in embryonic Node and midline structures is conserved throughout vertebrates.

***Metrns* loss-of-function leads to DFC disorganization and migration defects**

The specific expression of *metrn* and *metrnla* in DFCs and in the KV area at early developmental stages led us to ask what role they may play for these cells and the KV formation and function. To assess whether the loss of *Metrn* proteins function could affected the migratory and clustering properties of DFCs, we labeled these cells in 9 hpf (80-90% epiboly) gastrulating mutant embryos using the DFC landmarks *sox32* [40,41], *tbxta* [42] or *sox17* [40]. Compared to wild type embryos displaying ovoid DFC clustering, about 65% of the triplMut embryos displayed multiple segregated DFC clusters or abnormal linear cellular distributions as shown using *sox32* marker in 9 and 10 hpf triplMut embryos (Fig. 4A-B, Fig. S4B, Table 2). Similarly, *metrn*^{-/-} as well as *metrnla*^{-/-} single mutant embryos presented clustering defects of *sox32*-expressing DFC compared to wild type embryos (Fig. 4B, Fig. S4A-B, Table 2). In single or triplMut^{+/-} heterozygous

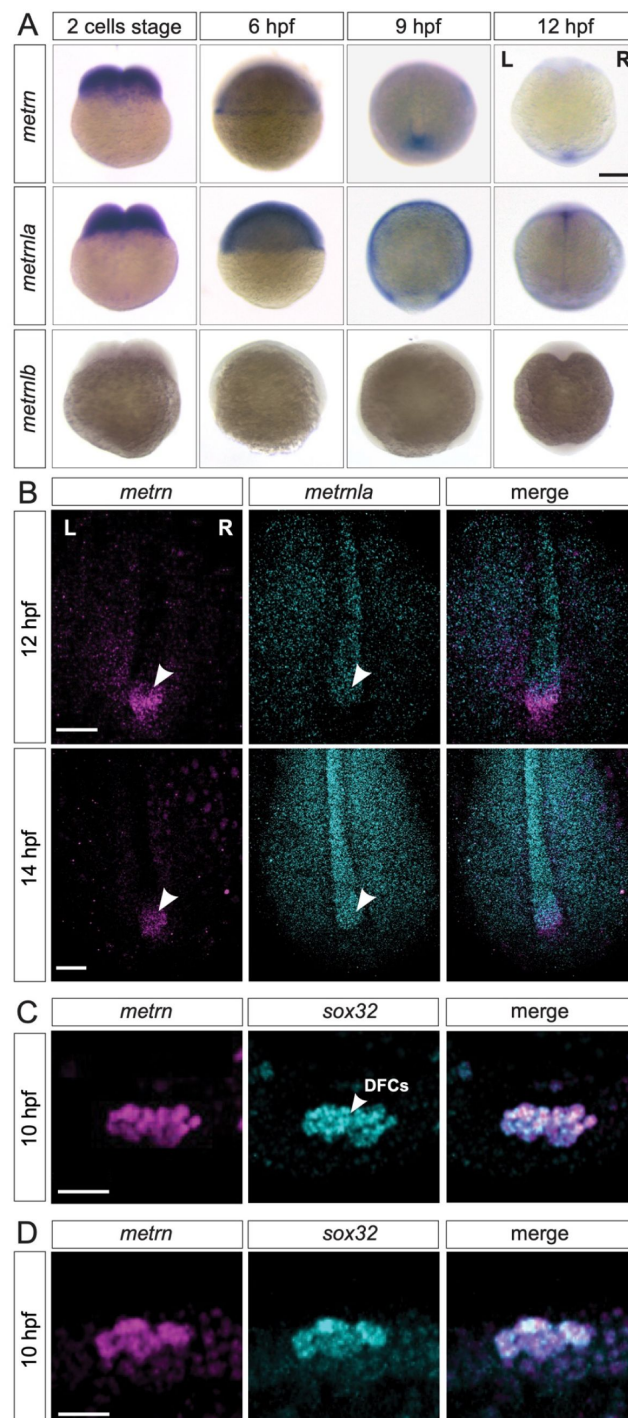


Fig. 3.

***Metrns* are expressed during early zebrafish development.**

(A) Expression patterns of *metrn*, *metrnla* and *metrnlb* during early embryonic development from 2-cell stage to 12 hpf. (9hpf and 12 hpf dorsal view) (B) Confocal cross-section of the midline region of 12 and 14 hpf zebrafish embryo showing mRNA expression of the *metrn* (magenta) and *metrnla* (cyan) and their co-expression in the area of the Kupffer's vesicle (KV, indicated by arrowheads) by HCR. (C) *Metrn* and *sox32* are co-expressed by dorsal forerunner cells (DFCs) as shown by double fluorescence *in situ* hybridization against *metrn* (magenta) and *sox32* (cyan) on 10 hpf wild type embryos. (D) *Metrnla* and *sox32* are co-expressed by DFCs as shown by double fluorescence *in situ* hybridization against *metrnla* (magenta) and *sox32* (cyan) on 10 hpf wild type embryos. Scale bars in (A): 500 μ m, (B) 250 μ m, (C) and (D): 50 μ m. L = left, R = right.

embryos, the DFC clustering was instead not significantly or very mildly affected as revealed (**Fig. S4B**, **Table 2**). The observed defects were also present when using *tbxta* and *sox17* as DFC markers whose expression was detected at 8 and 10 hpf respectively, indicating that although disorganized, the DFCs identity was not altered by the absence of *metrns* expression (**Fig. S4C**). Together, these results indicate that *Metrns* are required for the proper clustering of DFCs.

It was reported that proper adherent cell assembly is crucial for DFCs directional collective cell migration during their animal to vegetal pole (AP to VP) journey [43] (**Fig. 4C**). We therefore evaluated the migration capacity of DFCs during early development in the absence of *Metrns* proteins. To do so, we first labeled *sox32*-expressing DFCs by *in situ* hybridization in wild type and *triplMut* embryos and measured the position of *sox32*-expressing DFCs along the AP-VP axis divided by the total embryo length at 6 hpf, 8 hpf and 10 hpf. Compared to wild type embryos, DFCs in *triplMut* embryos showed migratory delays of about 8% to 13% at all analyzed developmental stages (**Fig. S5B-C**, **Table 3**). By live imaging, we tracked the *in vivo* migration of *sox17*:GFP-labeled DFCs both in *triplMut* and wild type embryos from 50% epiboly to 90% epiboly (from 6 hpf) (**Fig. 4D**, **Fig. S5D**, Video S1-2). In line with the DFC clustering abnormalities in fixed *triplMut* embryos that we observed, the tracking of these cells in *triplMut* embryos *versus* wild type control embryos revealed a diminished convergence rate for *triplMut* DFCs (**Fig. 4E**, upper panel). Furthermore, we measured a significant decrease of their migration speed compared to the wild type DFCs (**Fig. 4E**, lower panel). Intriguingly, individually tracked DFCs exhibited a consistent movement toward the vegetal pole, regardless of the absence of *Metrns* proteins (**Fig. 4D**, **Fig. S5D**, Video S1-2). Taken together, these findings indicate that the absence of *Metrns* proteins does not affect DFC migration directionality but alters instead their migratory abilities including migration speed and convergence rate.

It was shown that during the process of epiboly, initial EVL cells destined to develop into DFCs exhibit an accumulation of ZO-1 along their apical junctions. Subsequently, their apical surface gradually diminishes in size, resulting in the formation of discrete apical domains enriched with ZO-1 [22,24]. DFCs as such originate from dorsal EVL cells through a mechanism involving the delamination of epithelial cells, facilitated by apical constriction. We asked whether the observed DFC organization and migration disruptions in the absence of *Meteorin* proteins might be attributed to perturbations in both ZO-1 accumulation and apical constriction disruption. To explore this hypothesis, we examined the spatial arrangement of these regions enriched with ZO-1 in *triplMut* embryos and WT controls during epiboly at 6 hpf and 8 hpf. As expected, immunohistochemistry against ZO-1 on 6 and 8 hpf *Tg(sox17:GFP)* wild type control embryos where DFCs are labeled with GFP were enriched in ZO-1 at their apical side and apical junctions (**Fig. 4F-G**, **Fig. S5E**). At this same developmental stage, a reduced ZO-1 enrichment at the apical junctions of *triplMut* GFP-positive DFCs could be detected (**Fig. 4F**, **Fig. S5E** left plot). Similarly at 8 hpf, GFP-positive *triplMut* DFCs exhibited diminished ZO-1 at their apical region (**Fig. 4G**, **Fig. S5E** right plot). Furthermore, compared to wild type DFCs, *triplMut* GFP-positive DFCs appear to have lost their apical attachments to the EVL (**Fig. 4F-G**, right panels). Indeed, the distance measured between *triplMut* DFCs and the EVL/YSL at 6 and 8 hpf was significantly higher compared to the one of wild type DFCs (6 hpf: *p*-value: 0.039, N = 4 WT, 30 DFCs measured, *triplMut* N = 6, 46 DFCs measured; 8 hpf: *p*-value: 0.021, N = 7 WT embryos, 47 DFCs measured, N = 7 *triplMut*, 54 DFCs measured; **Fig. S5F**). Together, our results demonstrate that *triplMut* DFCs migrate significantly slower and converge less compared to wild type DFCs. Furthermore, in the absence of *Metrns*, DFCs loose apical ZO1-enrichment and attachment to the EVL/YSL, indicating that *Metrns* proteins are important for proper DFC clustering, migration along the AP-VP axis and their loss affect ZO-1 accumulation for the connection to the EVL/YSL.

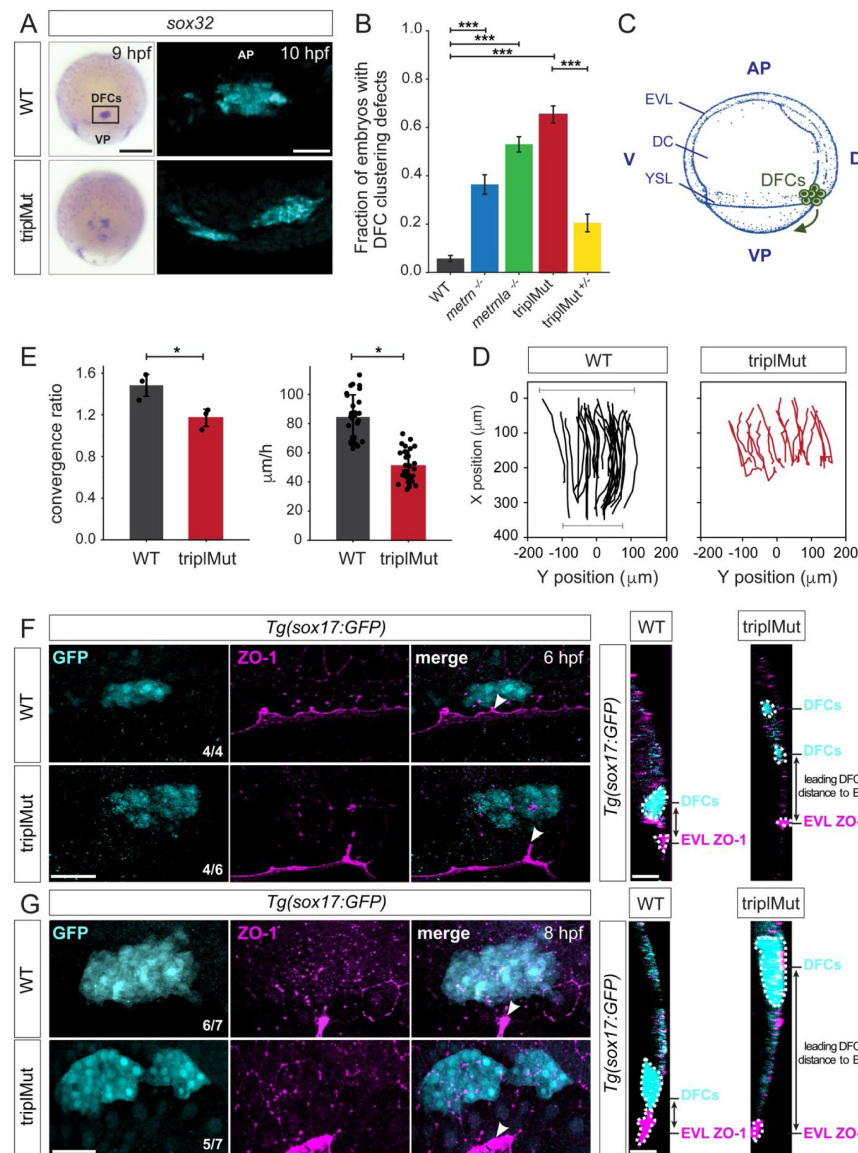


Fig. 4.

***Metrn*s loss-of-function leads to DFC clustering and migration defects.**

(A) Dorsal views of *sox32* expression in DFCs at 9 hpf and 10 hpf (cyan) in wild type (WT) and triplMut embryos reveals DFC misclustering in triplMut embryos. (B) Quantification in fraction of embryos with DFC clustering defects at 9 hpf in WT, *metrn*^{-/-}, *metrnla*^{-/-}, triplMut and triplMut^{+/-} embryos (Fisher exact test, ****p*-value: 1.6e-07 for WT vs. *metrn*^{-/-}; ****p*-value: 3.5e-40 for WT vs. *metrnla*^{-/-}; ****p*-value: 1.5e-49 for WT vs. triplMut and ****p*-value: 7.9e-15 for triplMut vs. triplMut^{+/-}). (C) Lateral view of a schematic representation of an 8 hpf zebrafish embryo visualizing animal pole (AP) to vegetal pole (VP) dorsal forerunner cell (DFC) migration from both poles, adapted from [44]. (D) Tracking plots of combined DFC movement in wild type (left) and triplMut embryos (right) (*n* = 3 embryos per conditions, single embryo traces see Fig. S5D) showing directed DFC movement in both conditions. Bars indicating the analyzed stretch of tracked cells along the y-axis at the beginning and end of each recording. (E) Plots for convergence (upper) and migration speed (lower) analyzed from DFC tracking data show significant decrease in these parameters in triplMut embryos compared to WT controls. (**p*-value convergence ratio: 0.0295; ****p*-value for migration speed 1.217e-04) (F-G) GFP and ZO-1 immunostainings of the dorsal margin and confocal microscopy ZY-planes (right panels) of *Tg(sox17:GFP)* WT and triplMut embryos at (F) 6 hpf and (G) 8 hpf (shield stage and 75% epiboly) showing the apical domains of marginal DFCs with ZO-1 enriched junction points (arrowheads) and revealing the absence of apical ZO-1 enrichment and detachment from the EVL/YSL in triplMut embryos. EVL, enveloping layer; DC, deep cells; YSL, yolk syncytial layer; D, dorsal; V, ventral. Scale bars in (A): 50 μ m, (F-G): 50 μ m. AP = animal pole, VP = vegetal pole.

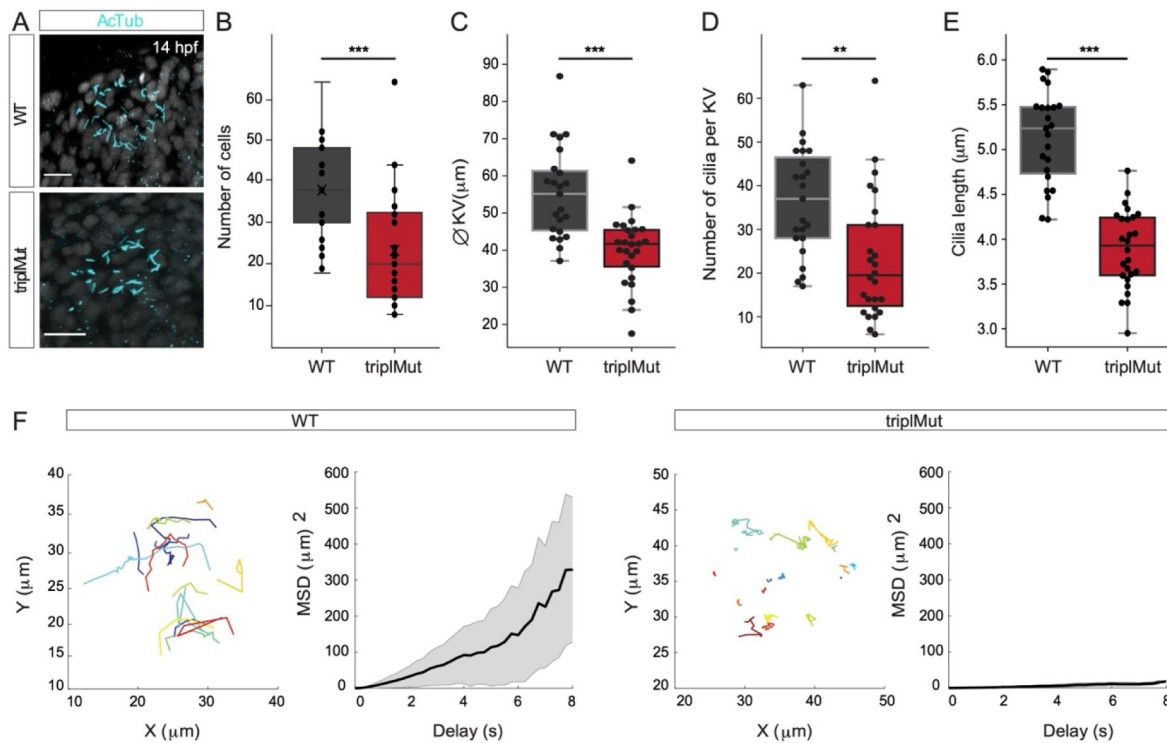


Fig. 5.

***Metrns* loss-of-function impairs the Kupffer's vesicle formation and function.**

(A) Confocal cross-section of 14 hpf wild type (WT) and triplMut counterstained with acetylated tubulin (AcTub) labeling the cilia of the Kupffer's vesicle (KV). (B) The quantification of the cell number per KV in WT and triplMut embryos at 14 hpf shows a significant difference. (C) The KV diameter size measurement, (D) cilia number per KV quantification and (E) individual cilia length measurement in WT and triplMut embryos at 14 hpf are all significantly decreased in triplMut (N WT: 23, N triplMut: 26, Student t-tests, *** p -value: 0.00028 for KV cell number, *** p -value: 9.7e-06 for KV diameter size, ** p -value: 0.0012 for cilia number. For cilia length: average per KV and *** p -value: 5.8e-12). (F) Single microbeads tracking in the KV of 12 hpf –14 hpf WT and triplMut embryos and the mean square displacement (MSD), revealing a directed trajectory in WT samples in contrast to triplMut, displaying short and undirected trajectories. Scale bar in (A): 20 μm .

Metrns loss-of-function impairs the KV formation and function

At the end of gastrulation, DFCs that migrated to deeper layers of the developing zebrafish embryo form a lumen to shape the KV, a ciliated organ transiently present (from around 10.5 hpf to 30 hpf [44]) that is crucial for initiating L-R asymmetric patterning [9,23]. As we observed at 14 hpf, it was reported that *Dand5*, a member of Cerberus/Dan family of secreted factors, is expressed adjacent to the KV with a right – left ($R > L$) bias between 11 and 14 hpf in zebrafish (Fig. 2) [45]. The right-biased expression of *dand5* was shown to be directly influenced by the direction and the strength of the Nodal flow generated by the KV [46]. In return, *Dand5* contributes to the left-biased expression of *spaw* in the LPM by antagonizing *Spaw* activity [45]. The leftward-directed flow generated by the KV was also shown to be important for the asymmetric expression of other Nodal genes, like *spaw*, *lefty1* (*lft1*) and *lefty2* (*lft2*) [26,27]. The observed disturbed expression of these Nodal factors in the absence of *Metrns* led us to hypothesize that *Metrn* proteins might not only be critical for the proper clustering and migration of DFCs but also for the proper formation and function of the KV. To assess this question, we first labeled the cilia present in the KV of wild type and *triplMut* embryos at 14 hpf by anti-acetylated tubulin immunostaining (Fig. 5A). We then measured the number of cells in the KV, the KV diameter and the number of cilia, which were correlating with the number of cells, of both wild type and *triplMut* embryos (Fig. 5B-D). Previous work has shown that the KV diameter and the number of KV cilia cells as well as KV cilia can be highly variable from embryo to embryo [47]. Nevertheless, despite the wide variability, we observed highly significant reduction in all cases (Fig. 5B-D). In addition, compared to the wild type controls, *triplMut* embryos also displayed a significant reduction in the KV cilia length (Fig. 5E).

Furthermore, we investigated both the KV lumen and the process of DFC-to-KV organization. As described recently and observed in ZO-1 stained embryos, by 14 hpf DFCs clustering into a rosette structure lead tight junctions to form the KV lumen [22] (Fig. 56A, left panel). In contrast, ZO-1 immunostained *triplMut* embryos of the same developmental stage displayed an irregular ZO-1 lattice pattern and disrupted KV lumen shape (Fig. 56A, right panel). Additionally, unlike in control embryos, *triplMut* embryos displayed only partially polarized DFCs indicated by absent or irregular aPKC- ζ immunolabeling (Fig. 56B). These results indicate that the loss of *Metrns* function also impacts the proper DFC polarization and subsequent KV formation.

We next evaluated whether the deficient formation of the lumen as well as the reduced number and cilia length in *triplMut* altered the KV function. To do so, we compared the Nodal flow generated by the KV of *triplMut* embryos to the one of wild type embryos. The movement generated by the KV cilia was monitored by injecting fluorescent microspheres (0.5 μm) into the KV at 12-14 hpf. To quantify the flow inside the KV, fluorescent microbeads movement was tracked in WT control ($n = 363$ beads in 4 embryos) and in *triplMut* embryos ($n = 318$ beads in 4 embryos) (Fig. 5F). Beads movement analysis within the KV showed a counter-clockwise rotation of the flow in wild type control (Fig. 5F, Video S3). In contrast, this movement was severely impaired in *triplMut* embryos (Fig. 5F, Video S4). To quantify the properties of the beads displacement, we computed the mean square displacement (MSD), which enables to distinguish between directed and confined motion. This analysis revealed a directed motion of microbeads in the wild type KV (MSD quadratic dependence on Δt) and a confined motion (MSD asymptotic behavior) in *triplMut* [48]. Accordingly, measurement of average bead velocity showed an overall reduction in the beads speed in the *triplMut* mutant embryos compared to the wild types (WT mean = 0.96212 $\mu\text{m}/\text{sec}$; *TriplMut* mean = 0.47996 $\mu\text{m}/\text{sec}$; Fig. 56C). These results demonstrate that the observed defects in the KV formation in the absence of *Metrns* also severely impacts its function and thereby the Nodal flow generation, responsible for proper Nodal factors expression.

Genetic interaction of ItgaV/Itgβ1b and Metrns on the level of the DFCs

In order to further uncover the mechanisms through which Metrns proteins act in the establishment of early L-R patterning, we finally assessed the possible relationship between Metrns and mediators of the extracellular matrix like Integrin αV1 (ItgaV1) and Integrin β1b (Itgβ1b). Integrins are membrane receptors involved in cell adhesion and recognition during embryogenesis amongst others [49]. During gastrulation, *itgaV* as well as *itgβ1b* were shown to be expressed in DFCs and *itgaV* and *itgβ1b* morpholino-injected embryos display DFC phenotypic defects reminiscent to those observed in *metrn* mutants [50].

We therefore asked whether Metrns loss of function could affect the expression of both integrins. To do so, we quantified *itgaV* and *itgβ1b* expression levels in wild type and triplMut embryos at 6, 9 and 24 hpf by qRT-PCR. No significant change in *itgaV* and *itgβ1b* expression levels at either tested developmental stage was measured in triplMut embryos compared to wild type controls (Fig. S7A).

In order to test whether ItgaV1 and Itgβ1b and Metrns genetically interact, we injected insufficient doses of morpholinos for *itgβ1b* (0.5 ng) or *itgaV1* (0.41 ng) into 1 to 4-cell stage in a heterozygous *metrns* mutant background. The injection of the morpholinos at these doses and at sufficient doses (*itgβ1b*: 0.7 ng, *itgaV1*: 1.25 ng) reproduced the previously reported defects on DFCs (Fig. 6A, Table 4) [50]. Instead, the injection of low doses of either *itgβ1b* or *itgaV1* morpholino did not affect DFC clustering, similarly to non-injected triplMut^{+/−} embryos (Fig. 6A, Table 4). However, a significantly higher percentage of embryos with DFC clustering defects could be observed upon the injection of either morpholino at low dose into triplMut^{+/−} (Fig. 6A, Table 4). These results are consistent with the hypothesis that Metrns proteins act together with both Integrins for the proper clustering and migration of DFCs during early embryonic development without directly affecting their expression.

Discussion

Proteins of the Meteorin family were first described as secreted neurotrophic factors highly conserved among vertebrates [28–31]. In mouse *Metrnl*a mutants display phenotypes as impaired angiogenesis after ischemic injury [51] or dysregulated cytokine production [52]. The disruption of *Metrn* resulted in early embryonic lethality [28] preventing to investigate the combined role of Metrns proteins in early embryonic development *in vivo* and in particular its role in L-R patterning. Here, using the zebrafish larva as model system, we generated knockout lines for all three zebrafish *metrn* genes, *metrn*, *metrnl*a and *metrnl*b, which are all viable and fertile, possibly attributed to species specific differences in compensatory mechanisms, allowing for the study of Meteorins functions during early embryonic development.

Our results demonstrate that Metrns are required for symmetry breaking and the establishment of L-R asymmetry, a novel role for Meteorin proteins during vertebrate embryonic development. All three paralogs analyzed, when genetically deleted, had an effect on heart looping formation, with the strongest effect for *Metrnl*a and *Metrn* while *Metrnl*b had a smaller but still significant effect. In our *in situ* analysis, we failed to detect early *metrnl*b expression. We do not exclude that at early stages, *metrnl*b could be expressed at very low level or in a very small number of cells that we cannot detect. However, in the *metrnl*b mutant, the loss of this expression could still be sufficient to induce the low penetrant phenotype. Interestingly, when analyzing the expression of these genes in a single cell transcriptomic atlas at the earliest available time point (10 hpf), in contrast to the robust expression of the two first genes, *metrnl*b expression is indeed found in very few cells [53].

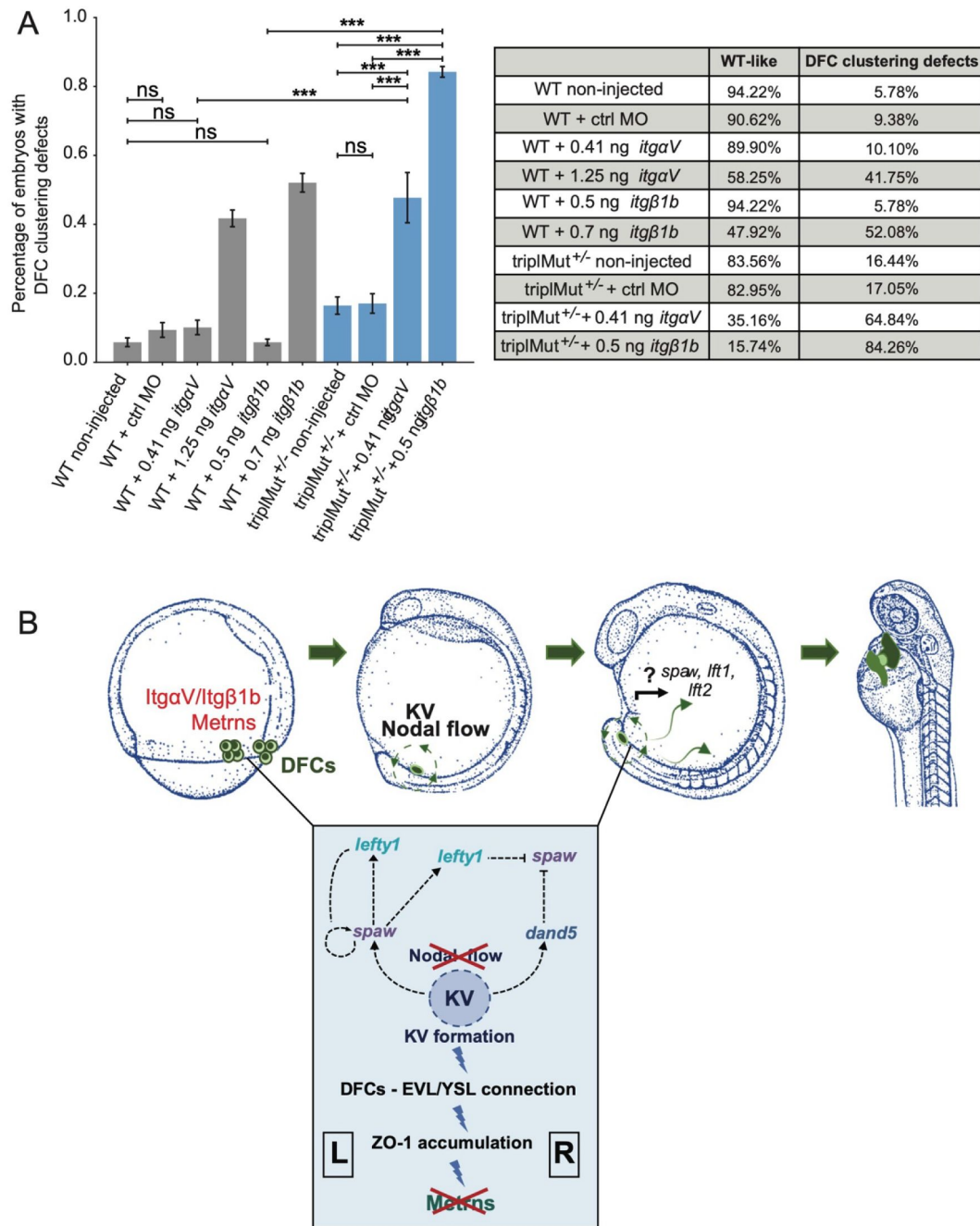


Fig. 6.

Metrns and ItgaV/Itgb1b act together for the proper clustering and migration of DFCs.

(A) Quantification in percentage of embryos with DFC clustering defects at 9 hpf in non-injected wild type (WT), WT + 2.5 ng control morpholino (WT + ctrl MO), WT + insufficient doses of *itgaV/itgb1b* MOs (WT + 0.41 ng *itgaV*; WT + 0.5 ng *itgb1b*), WT + sufficient *itgaV/itgb1b* MO doses (WT + 1.25 ng *itgaV*; WT + 0.7 ng *itgb1b*), triplMut^{+/-} non-injected, triplMut^{+/-} + 2.5ng ctrl MO and triplMut^{+/-} with insufficient doses of *itgaV/itgb1b* MOs (triplMut^{+/-} + 0.41 ng *itgaV*; triplMut^{+/-} + 0.5 ng *itgb1b*). Fisher exact tests indicate **p*-value < 0.01, ***p*-value < 0.001 and *****p*-value < 0.0001 and ns = not significant. (B) Schematic representation modeling the novel role of Metrns for DFCs assembly and migration, for the proper KV formation and subsequent Nodal factors expression distribution at the base of the correct L-R axis establishment during early development (adapted from [44]).

We show that *Metrns* play a crucial role in ensuring this function the genetic interaction with the integrins *ItgaV1* and *Itgb1b* known to facilitate the proper clustering and migration of DFCs based on the sufficient accumulation of ZO-1 and apical constriction, essential to form the KV [22,50]. Furthermore, our results reveal that *Metrns* are essential for several aspects of the KV formation and function including the cilia length, cilia number, KV size and lumen formation. Consequently, *Metrns* are important for the regulation of the proper expression of Nodal flow genes and that this regulation is critical for the proper asymmetric positioning and morphology of vital organs like the heart (Fig. 6B).

Previous work has shown that zebrafish midline mutants display altered *lft1* and *lft2* expression patterns as well as discordance among heart, gut and brain gene expression patterns [54]. From 9 hpf *metrn* expression was visible around the developing LPM close to the midline and the leading edge of the shield. *Metrnla* expression was found throughout the whole enveloping layer and the developing midline which plays an important role in vertebrate L-R development. Despite the expression of both paralog close to or at the midline, we hypothesize that the described body asymmetry defects in *triplMut* embryos do not originate from impaired midline structures but rather impaired DFC clustering and migration. Indeed, *triplMut*^{-/-} embryos display intact *tbxta*-positive notochords (Fig. S4C), as well as randomized visceral organs positioning (Fig. 1D-E), phenotypes that are not characteristic for defects of the anterior midline [54].

DFCs are precursors of the zebrafish KV, organ of laterality, essential for the L-R axis establishment. DFCs develop at the beginning of gastrulation and form the KV by the end of gastrulation [9,23]. Given the early onset of *metrn* and *metrnla* expression and their specific expression within the DFCs, we investigated their role for the clustering and migration capacity of DFCs during these early embryonic developmental steps. In the course of their migration, DFC progenitors intercalate mediolaterally and results in an oval-rosette shape cluster of DFCs by mid-gastrulation that will form the KV [22]. *Metrn*^{-/-}, *metrnla*^{-/-} and *triplMut* zebrafish embryos displayed DFC assembly defects and delayed migration of these cells during gastrulation. Since DFCs in *triplMut* still express DFC specific markers (*sox17*, *sox32*, *tbxta*), we concluded that *Metrns* are not required for the specification of DFCs *per se* but rather that *Metrns* are important for their proper clustering and migration.

It is important to note that the observed DFC clustering and migration abnormalities in *Metrn* mutants primarily affect the apico-ventral axis rather than the mediolateral axis. This is evident from the disrupted connection between DFCs and the YSL/EVL in these mutants, while the migration directionality remains intact. Since a proper connection between DFCs and the EVL facilitates a subset of DFCs to establish apical contacts with the EVL and become pre-polarized, one could speculate that *Metrns* are involved in the polarization and establishment of direct apical contacts of DFCs with the EVL [22–24,55]. This disrupted connection in *metrns* loss-of-function embryos, which usually links DFCs with the vegetal spread of extra-embryonic tissues [24], likely results in the observed DFCs vegetal motion defects.

It has been demonstrated that these apical attachments function as tissue connectors, linking DFCs with the spreading of extra-embryonic tissues towards the vegetal pole [24].

Therefore, the loss of apical attachments in the mutant embryos causes all DFCs to converge less and migrate more slowly than in wild-type controls, as shown by our *in vivo* imaging analysis.

This would imply that a number of DFCs was simply too slow to reach the vegetal pole in time to form a fully functional KV. Given that KV cilia are monocilia, this subsequently results in a diminished count of KV cells (Fig. S5A), along with the observed structural abnormalities in the KV lumen and impairments in its overall functionality.

Additionally it was shown that the formation of the developing KV lumen was directly connected to ciliogenesis as polarized DFCs initiate developing cilia at their membrane domain facing the forming lumen [22]. This indicates that proper DFC polarization is essential for the KV formation and for ciliogenesis. In *metrns* mutants the reduced cilia length and the lack of PKC ζ staining in the developing KV further highlights an impairment of DFC polarization in the absence of *Metrns* consequently leading to KV formation and function defects. Further work would be needed to explore whether *Metrns* directly impact the motility of KV cilia or if the disrupted Nodal flow observed is solely caused by the disturbed KV lumen.

It has been demonstrated that an appropriate asymmetrical Nodal flow plays a crucial role in the asymmetrical expression of Nodal genes such as *dand5*, *spaw*, *lefty1* (*lft1*), and *lefty2* (*lft2*) [26,27,45]. As such, the disrupted formation and function of the KV in *metrns* mutants might contribute to the evident perturbation in Nodal factor expression and may also induce randomization of Nodal factor expression in L-R asymmetry [9,15,47,50]. We do not exclude however that *Metrns* directly impact the expression of *spaw*, *leftys* and *dand5* in addition to their role in DFC cell behaviors and KV formation. One of the earliest functions attributed to Meteorin was as a regulator of mesendoderm development through the enhancement of nodal expression [28]. In this context, several studies have reported that Nodal signaling was shown to regulate DFC specification [56,57].

In line with the impaired KV formation and function that we observed in *metrns* mutants, it was shown that knockdown of Integrin α V as well as Integrin β 1b in zebrafish results in L-R asymmetry defects [50]. This was explained by DFC organization and migration defects and disturbed KV formation. Integrin α V β 1 specific functions have not yet been completely resolved in mammals, mostly due to their broad expression and redundant function with other integrins, as well as the lack of antibodies and inhibitors specific for α V β 1. However, several *in vitro* studies have already connected α V β 1 to developmental processes. For instance, α V β 1 was shown to be implicated in embryonic astrocytes and oligodendrocyte precursors migration in rodents [58] and neural cell adhesion molecule L1 cell binding [59]. Additionally, the fibronectin receptor integrin α 5 β 1 has been found to be crucial for regulating extracellular matrix assembly along tissue boundaries, in coordination with Eph/Ephrin signaling [60].

Our results suggest that one mechanism by which *Metrns* act is through their genetic interaction with Integrin α V β 1b to mediate the proper clustering of DFCs. These interactions could influence Integrin's adhesive functions (or other properties) necessary for DFC clustering. If so, the absence of Meteorins would lead to disturbed adhesion and/or Integrin signaling, resulting in DFC migration defects and disturbed KV development. Interestingly, it was shown that in α V and β 1b morphants DFC-EVL connections remain intact [50], unlike in our *meteorin* mutants. This contrast suggests that Meteorins alone are crucial for DFC polarization and that their interaction with integrins α V β 1b may rather be important for the subsequent steps leading to KV formation. While our genetic interaction studies provide strong evidence of a link between Meteorins and Integrins, further biochemical analyses are required to fully elucidate this interaction.

Despite the recent advancements in uncovering the multiple functions of Meteorins, their receptors during early development are still unknown. It was recently shown that METRNL is promoting heart repair through its binding to the KIT tyrosine kinase receptor, establishing METRNL as a KIT receptor ligand in the context of ischemic tissue repair [51]. In another study, it was reported that *Metrn* is a ligand for the 5-hydroxytryptamine receptor 2b (Htr2b) [61]. Additionally *Metrn* was associated with the regulation of reactive oxygen species levels in hematopoietic stem/progenitor cells via directing phospholipase C signaling [61]. When we analyzed the expression of *kita*, one of the two zebrafish orthologs to KIT tyrosine kinase receptor, at 9 hpf we found that *kita* is solely expressed in the prechordal plate and later (from 11 hpf) in the lateral borders of the anterior neural plate (Fig. S7B, left panel). Its paralog *kitb* is expressed from 11 hpf in the anterior ventral mesoderm (Fig. S7B, right panel), as previously reported

[62]. Additionally, the analysis of *htr2b* expression during zebrafish development revealed that its expression only starts from around 2 dpf in the heart (Fig. S7C). The absence of *kita/b* as well as *htr2b* expression at early embryonic stages in or at proximity of DFCs and in the forming KV suggests that Metrns rather act through other receptor(s) to ensure their L-R patterning functions.

Mechanisms underlying L-R patterning are known to be highly conserved within vertebrates. Hence, we raised the question whether Meteorin protein family is conserved across different species. Genes coding for *metrn* and *metrnl* exist in several vertebrates classes but could not be found in the genomes of invertebrates [63]. *Metrnl* orthologs genes could instead be found in amphioxus and tunicates. Therefore, we propose that Metrns function in the establishment of the L-R axis that we have uncovered here emerged during Chordate evolution (Fig. S7D).

Material and methods

Zebrafish husbandry

Zebrafish (*Danio rerio*) were maintained at 28°C overnight (O/N) at 14 hours light/10 hours dark cycle. Fish were housed in the animal facility of our laboratory which was built according to the respective local animal welfare standards. All animal procedures were performed in accordance with French and European Union animal welfare guidelines with protocols approved by the committee on ethics of animal experimentation of Sorbonne Université (APAFIS#21323-2019062416186982). For all experiments in this study, primarily zebrafish AB strains were used unless stated otherwise. The *Tg(sox17:GFP)* lines were created via injection of a *pTol2-sox17:GFP* plasmid (kindly gifted from Didier Stanier and Stephanie Woo) into one-cell stage zebrafish embryos together with 150 ng/ml of *tol2* mRNA. Injected embryos were grown to adulthood and screened for GFP in their offspring.

Metrn, *metrnl* and *metrnlb* CRISPR-Cas9-mediated mutagenesis

SgRNA guide sequences were cloned into a BsaI-digested DR274 plasmid vector (Addgene 42250). The sgRNAs were synthesized by *in vitro* transcription using (using the Megascript T7 transcription kit, Ambion, AM1334). After transcription, sgRNAs were purified using a RNAeasy Mini Kit (Qiagen). The quality of purified sgRNAs was checked by electrophoresis on a 2 % agarose gel. The target sequences were the following:

	Sequence (5' - 3')
<i>metrn</i>	CCACCACACCCGGCCAGCC
<i>metrnl</i>	GGTGTATCTCCGCTGCGCCC
<i>metrnlb</i>	CCAGAAACAGCGCCCCCTCC

Cas9 mRNA was generated as described in Hwang, Fu et al. 2013 [64]. To induce targeted mutagenesis at the *metrn*, *metrnl* and *metrnlb* loci, 200 ng/ml of sgRNA were injected into one-cell stage zebrafish embryos together with 150 ng/ml of *cas9* mRNA. Injected embryos were grown to adulthood and screened for mutation in their offspring.

DNA extraction and sequencing for analysis of CRISPR/Cas9-mediated mutagenesis

For genomic DNA extraction, pools of 5 dpf embryos were digested for 1 h at 55 °C in 0.5 mL lysis buffer (10 mM Tris, pH 8.0, 10 mM NaCl, 10 mM EDTA, and 2% SDS) with proteinase K (0.17 mg/mL, Roche Diagnostics) and inactivated 10 min at 95 °C. To check for frequency of indel mutations, target genomic loci were PCR amplified using a Phusion High-Fidelity DNA polymerase (Invitrogen). PCR amplicons were subsequently cloned into a *pCR-bluntII-TOPO* vector (Thermo

Fisher). Plasmid DNA was isolated from single colonies and Sanger sequencing was performed by Eurofins. Mutant alleles were identified by comparison with the wild type sequence using Geneious software.

Mutants genotyping and generation of triple mutants

Genotyping of *metrns* mutants was performed as follows: after genomic DNA extraction PCR amplification reactions were conducted in final volumes of 20 µl containing 1x PCR reaction buffer, 1,5mM MgCl₂, 70 ng of gDNA, Taq DNA Polymerase (5U/µl) and 0.5 µM of each primer.

	Sequence (5' - 3')
<i>metrn-fw</i>	CTGTGTTGACTGCTGGCTG
<i>metrn-rv</i>	GTGGTTTAGTGGTGTTCCTTACAATGA
<i>metrnla-fw</i>	TCCCATGCCTGGACCTCATA
<i>metrnla-rv</i>	AGACGGAGAGAAGAGACGCT
<i>metrnlb-fw</i>	TGTTGATCAGCAGTGTGTGCGTAGC
<i>metrnlb-rv</i>	GTCCTCCGCTGATCTACGTG

The DNA amplification was performed with 35 cycles at the annealing temperature of 60°C. *Metrn* and *metrnlb* amplicons were loaded on 2 % agarose gel to discriminate between wild type and mutant alleles (for *metrn*, the expected size of wild type amplicon is 598 bp while the mutant amplicon is 482 bp long using the same primer set; for *metrnlb*, the size of the expected wild type amplicon is 242 bp while the mutant band is 156 bp long using the same primer set). *Metrnla* PCR product was digested at 55°C O/N with the *PasI* restriction enzyme and the resulting digestion was subsequently analyzed on a 2% agarose gel. The wild type amplicon results in two fragments of 107 bp and 94 bp length, respectively. The mutant amplicon band size is instead 201 bp using the same primer set. Triple mutants were obtained first by incrossing homozygous *metrn* and *metrnla* mutants to generate double heterozygous mutants. Double heterozygous mutants were then crossed with homozygous *metrnlb*^{-/-} fish and genotyped to identify triplMut^{+/-} triple heterozygous mutants. triplMut^{+/-} fish were incrossed and genotyped to identify triplMut^{-/-} triple homozygous mutants. In all described experiments of this study, triplMut^{+/-} were obtained by outcrossing female triplMut^{-/-} fish with male wild type fish, if not stated differently to prevent from *metrns* maternal contribution.

Hybridization chain reaction (HCR)

All HCR probes and solutions were purchased from Molecular Instruments®. Dechorionated embryos at the developmental stage(s) of interest were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 2 hours at room temperature, followed by several washes with PBS to stop the fixation. Embryos were then dehydrated with a series of methanol (MeOH) washes and stored at -20°C. The HCR was performed following the manufacturer's instructions. HCR samples were imaged on an inverted confocal microscope Olympus FV-1000, employing a 20x oil immersion objective (NA 0.85). Z-volumes were acquired with a 5 µm resolution and images were processed and analyzed using ImageJ.

***In situ* hybridization in zebrafish and chicken**

The respective cDNA fragments were amplified by PCR from total zebrafish or chicken cDNA of stage 12 hpf – 48 hpf and HH6. *In vitro* transcription of Digoxigenin/Fluorescent-labeled probes was performed using an RNA Labeling Kit (Roche Diagnostics Corporation) according to manufacturer's instructions. Zebrafish and chicken whole-mount *in situ* hybridizations were performed as previously described [65] [66]. Stained embryos were then imaged on a Leica MZ10F stereomicroscope. Images were processed and analyzed using ImageJ software. Color balance, brightness and contrast were applied uniformly. Fluorescently-labeled samples were imaged using an inverted confocal microscope Olympus FV-1000, employing a 40x oil immersion objective (NA 1.30). Z-volumes were acquired with a 0.5 μ m resolution and images were processed and analyzed using ImageJ.

Analysis of DFC phenotype and migration

For the DCF clustering analysis, Bayesian inference was used to recover the maximum-likelihood value for the malformation probability. The outcome of an experiment was considered to be a binary random variable (malformed or wild type phenotype) whose probability depends solely on the experimental condition. The total number of malformed phenotypes per experiment is therefore binomially distributed. The posterior distribution is obtained by inverting this binomial distribution using Bayes theorem, under a uniform prior. Under these hypotheses the maximum-likelihood estimate for the malformation probability is simply given by the empirical malformation frequency. The standard deviation of the posterior distribution was used to provide a confidence interval for the estimate (black error bars). *P*-values were evaluated using Fisher exact test on the number of malformed and wild type phenotypes. AP to VP DFC migration was calculated as the percentage of the total embryo length.

For *in vivo* live DFC imaging, a *pEGFP-sox17* construct (Addgene #31400) and *H2B-RFP* mRNA were co-injected either in wild type or triplMut embryos that were embedded at 50% epiboly (6 hpf) in 1% low melting-point agarose diluted in E3 water. DFC were identified as recently described in [24]. Imaging was performed in a temperature constant environment with a 10x water dipping objective using an upright Yokogawa CSU-X1 spinning disk scan head, mounted on a DM6000 upright Leica microscope and a CCD CoolSnap HQ2 camera at 4 Hz.

Volumetric image stacks were acquired every 6 minutes which was sufficient to manually track individual DFC migration during 3 hours from 50% epiboly. No drift correction was required. The convergence ratio was calculated by comparing how far the tracked cells stretched along the y-axis at the beginning and end of each recording (as visualized in Fig. 4D and described in Pulgar et al., 2021 [67]).

qRT-PCR and analysis

For gene expression analysis, total RNA extraction was prepared from zebrafish embryos with TRIzol reagent (Thermo Fisher) and TURBO DNA-free reagents (Invitrogen™ AM1907M). Total RNA was cleaned up using RNeasy Mini Kit (Qiagen) following the manufacturer's instructions and treated twice with DNase I (1 U/ μ g RNA, Qiagen). The RNA concentration was quantified using the Nanodrop2000 (Thermo Fisher). RNA (1 μ g) was retro-transcribed using random primers and a SuperScript III First-Strand Synthesis system (Invitrogen 18080051). For qRT-PCR, the SYBR Green PCR Master Mix (Thermo Fisher) was used according to the manufacturer's protocol. *Ef1a* was used as reference genes as previously reported [68]. All assays were performed in triplicates and repeated in 3 independent experiments. The mean values of triplicate experiments were calculated according to the delta CT quantification method [69] and a student T-test was applied for the *p*-value calculation.

mRNA synthesis and injection

Zebrafish *metrn* and *metrnla* cDNA fragments were amplified by PCR from zebrafish cDNA of 1 dpf. The fragments were cloned into a *pCS2+* plasmid linearized with Xho1 and Xba1 restriction enzymes using a Quick Ligation Kit (NEB). The *pCS2:h2b-rfp* plasmid readily available in the laboratory was linearized with the NotI restriction enzyme and the mRNA synthesis was performed by *in vitro* transcription using the mMESSAGE mMACHINE Sp6 Ultra kit (AM1340, Ambion) adding 1 μL of GTP as recommended by the manufacturer's instructions and followed by lithium chloride precipitation. 150 $\text{ng}\cdot\mu\text{L}^{-1}$ of the synthesized mRNA was then injected into 1- to 4-cell-stage zebrafish embryos.

Design and injection of antisense morpholino oligonucleotides

The published morpholinos (MO) used in this study for *itgaV* (5'-AGTGTTCGCCATGTTTTGAGTCTC-3'), *itg β 1b1* (5'-GGAGCAGCCTTACGTCCATCTT AAC-3') and for control (ctrl MO) (5'-CCTCTTACCTCAGTTACAATTATA-3') were injected using the same reported concentration of 0.41 ng (*itgaV*) and 0.5 ng (*itg β 1b1*) for insufficient doses, 1.25 ng (*itgaV*) and 0.7 ng (*itg β 1b1*) for full knock down effect and 2.5 ng for the ctrl MO [50]. All MOs were obtained from Gene Tools (Philomath, OR, USA). MOs were injected in 1 to 4 -cells stage zebrafish embryos.

Whole-mount immunohistochemistry

Embryos were fixed in 4% paraformaldehyde in PBS-Tween for 2h at room temperature (RT) and subsequently washed three times in PBS-1% Triton X-100 to promote their permeabilization. They were then incubated for 1 hour at RT in blocking solution (10% Normal Goat Serum, in PBS-Tween) followed by overnight incubation, at 4°C with primary antibodies diluted at 1:200 in 1% blocking solution. The following primary antibodies were used: anti-acetylated tubulin (Sigma T6793); anti-ZO1 (339100, Invitrogen, Carlsbad, CA, USA); anti-GFP (GTX13970, GeneTex); anti-aPKC ζ (sc-216, Santa Cruz Biotechnology Inc., CA, USA). After five washes in 1x PBS-Tween, the embryos were then incubated O/N with respective Alexa Fluor IgG secondary antibody (Life Technologies) diluted 1:200 in 1% blocking solution. The following day embryos were washed five times in 1x PBS-Tween and mounted in 1% low-melting point agarose in glass-bottom cell tissue culture dish (Fluorodish, World Precision Instruments, USA). An inverted confocal microscope Olympus FV-1000 was used for imaging, employing a 20x objective (NA 0.75) or a 40x oil immersion objective (NA 1.30). Z-volumes were acquired with a 0.5 μm resolution and images were processed and analyzed using ImageJ and Imaris. The distances between the DFCs and the EVL at 6 and 8 hpf were measured in fixed whole-mount samples. Using ImageJ, the distances were analyzed from the center of the leading DFCs to the leading edge of the EVL, as marked by ZO-1. The area of ZO-1 enrichments was measured using Fiji in cropped images that included only DFCs and at 6 hpf also the apical part of the EVL.

Fluorescent microspheres injection and analysis

FluoSpheresH fluorescent microspheres injection was conducted as previously described [70]. Embryos were embedded in 1% low melting-point agarose diluted in egg water. Imaging was performed with a 40x water dipping objective using an upright Yokogawa CSU-X1 spinning disk scan head, mounted on a DM6000 upright Leica microscope and a CCD CoolSnap HQ2 camera at 4 Hz using the green channel at 0.25 μm resolution on a single Z-plane. Beads trajectory tracking was performed using an already developed toolbox [71]. The size detection parameter was set to 0.75 – 1.25 μm objects with a brightness superior to the 0.01 percentile of the whole image. Objects that traveled less than 2.5 μm between two consecutive frames were linked into the same trajectory and considered as a single bead. Objects traveling longer distances were analyzed as separate trajectories, even if they originated from the same bead. Analyses were then performed using a custom-made Matlab code. For better visualization, trajectory plots only show 20 tracked beads. MSD analysis was conducted using an already published code (<https://tinevez.github.io>)

/msdanalyzer/ [72]. Average mean square displacement (MSD) plots only show particles tracked between 1 and 8 seconds. Very short tracking durations do not permit to correctly estimate displacement properties and less than 1% of the particles were tracked for more than 8 seconds.

Data analysis

Expression analysis, DFC clustering, and heart and visceral organ positioning experiments were conducted at least three independent times, each with a comparable N number, to ensure reproducibility and account for batch effects. The reported data represent pooled results from these independent experiments. Experiments for heart looping, *dand5* expression and visceral organ phenotypes have multinomial outcomes, and the *p*-values for these distributions were evaluated accordingly. For any given pair of conditions α (control) and β (mutated condition), the number of observed experimental outcomes were counted. The number of outcomes for each condition was denoted as $(n_\alpha, d_\alpha, r_\alpha)$ and $(n_\beta, d_\beta, r_\beta)$, with N_α and N_β denoting the total number of observations. The frequency of each outcome under a particular condition is simply the number of observations for the given outcome divided by the total number of observations. The dissimilarity between two conditions α and β was measured as the sum of absolute differences between their respective frequencies $\delta = |f_{n\alpha} - f_{n\beta}| + |f_{d\alpha} - f_{d\beta}| + |f_{r\alpha} - f_{r\beta}|$. Then the probability of the null hypothesis was estimated, i.e., that the multinomial distributions of outcomes related to the two conditions are the same, as the probability of obtaining a dissimilarity equal or higher to the one observed by simply pooling all the observations together, and again randomly subdivided in two sets of observations of size N_α and N_β . To numerically estimate this probability the procedure of pooling the observations together was repeated for $K = 100'000$ times, randomly separating them again in two sets, and evaluating the new dissimilarity δ' . The probability of the null hypothesis is then given by the numerical frequency with which the dissimilarity $\delta' \geq \delta$ is greater or equal to the one observed in the experiments. [73, 74] Python and Matlab codes used for beads and DFC tracking are available at <https://github.com/jboulanger91/meteorin>. Codes for statistical analysis are available at https://github.com/FannyEggeler/Meteorin_analysis.

Phylogenetic analysis

Protein sequences were obtained from the Ensembl Genome Browser or UniProt. The following sequences were used: *D. rerio* Metrnl (ENSDARP00000041804.5), *D. rerio* Metrnlb (ENSDARP000000131064.1), *D. rerio* Metrnlc (ENSDARP000000126894.1), *G. gallus* Metrnl (ENSGALP00010026416.1), *G. gallus* Metrnl (ENSGALP00010044844.1), *H. sapiens* Metrnl (ENSP00000455068.1), *H. sapiens* Metrnl (ENSP00000315731.6), *M. musculus* Metrnl (ENSMUSP00000127275.2), *M. musculus* Metrnl (ENSMUSP00000038126.8), *X. laevis* Metrnl (ENSXETP00000107425.1), *L. oculatus* Metrnl (ENSLOCP00000003703.1), *L. oculatus* Metrnl (ENSLOCP00000017224.1), *B. lanceolatum* (A0A8K0F3K3), *S. clava* (XP_039261436). *L. oculatus* was chosen as an outgroup, *B. lanceolatum* as an example for Cephalochordata and *S. clava* as a representative for Tunicata. The phylogenetic tree was reconstructed with the phylogeny analysis from www.phylogenie.fr [75–81].

Resource availability

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Filippo Del Bene (filippo.del-bene@inserm.fr).

Materials availability

All unique/stable reagents generated in this study are available from the lead contact with a completed materials transfer agreement.

Data and Code Availability

- All data reported in the paper are available from the lead contact upon request.
- This paper does report original code which is available at <https://github.com/jboulanger91/meteorin> . Codes for statistical analysis are available at https://github.com/FannyEggeler/Meteorin_analysis .
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Supplementary figures and tables

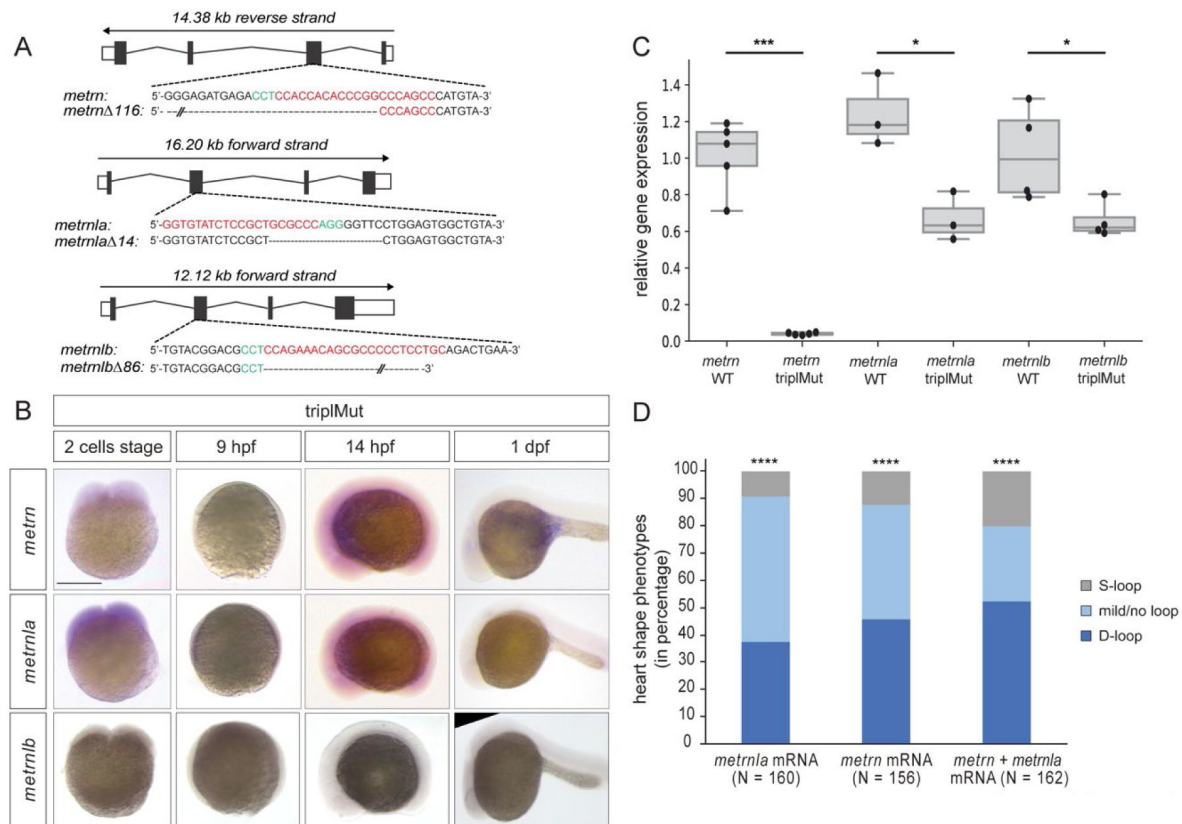


Fig. S1.

(A-C) *Metrn* genes CRISPR/Cas9 mediated knockout generation and validation. (D) *Metrns* loss of function effect on heart morphology.

(A) Targeted *metrns* loci in the zebrafish genome for knockout generation. Red highlights the sgRNA homologous sequence and PAM sequences are highlighted in green. The generated mutation is indicated below by dashed lines and the number of deleted nucleotides is indicated after the Δ sign. **(B)** *metrn*, *metrnla* and *metrnlb* expression in triplMut embryos from 2-cell stage to 1 day post fertilization (dpf) is highly reduced or undetectable by *in situ* hybridization (lateral views). **(C)** *Metrns* expression levels are reduced in triplMut embryos as shown by qRT-PCR analysis of *metrn*, *metrnla*, *metrnlb* expression level at 14 hpf for *metrn* and *metrnla* and at 48 hpf for *metrnlb* in wild type (WT) and triplMut embryos. (Student t-test, ****p*-value: 3.1e-06 for *metrn*; **p*-value: 0.014 for *metrnla*; **p*-value: 0.04 for *metrnlb*). **(D)** Quantification in percentage of the number of embryos with S-looped, D-looped or mild/no looped heart phenotypes at 2 dpf upon injection of *metrnla* mRNA, *metrn* mRNA or both in triplMut embryos. Displayed *p*-values compared to WT: *****p*-value: <1.0e-5 for *metrnla* mRNA, *****p*-value: <1.0e-5 for *metrn* mRNA, *****p*-value: <1.0e-5 for *metrn* + *metrnla* mRNA. Scale bar in (B) 250 μ m.

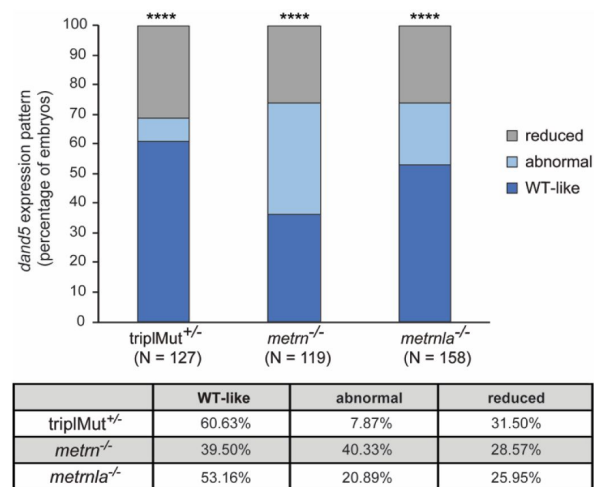


Fig. S2.

Nodal factors gene expression is altered in the absence of Metrns.

Quantification of the number of embryos in percentage with reduced, inverted or wild type (WT) -like *dand5* expression pattern in 14 hpf tripIMut^{+/-}, metrn^{-/-} and metrnla^{-/-} embryos, compared to WT, Permutation test, *****p*-value <1.0e-5 for tripIMut^{+/-}, *****p*-value <1.0e-5 for metrn^{-/-}, *****p*-value <1.0e-5 for metrnla^{-/-}.

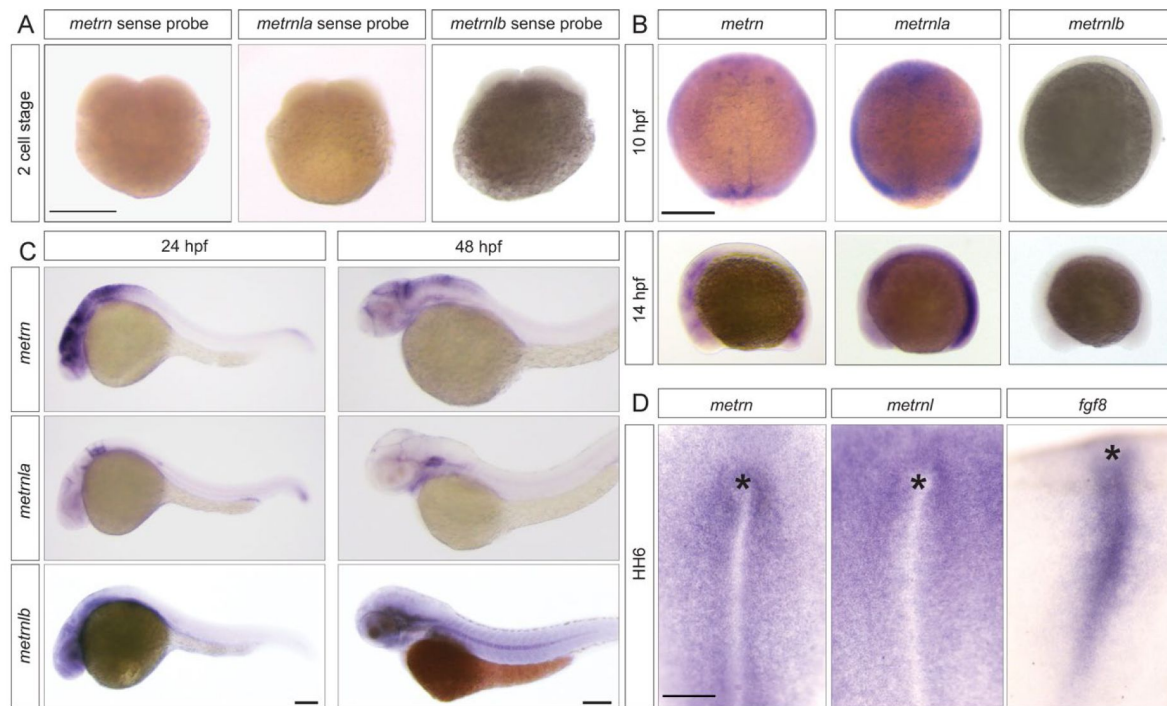


Fig. S3.

***Metrn*s expression during early zebrafish and chick development.**

(A) No expression can be detected for *metrn*, *metrnla* and *metrnlb* upon *in situ* hybridization using sense *metrn*, *metrnla* and *metrnlb* riboprobes on 2-cell stage wild type embryos. (B) *Metrn* expression at 10 hpf can be found in the leading edge of the shield and in the forming Kupffer's vesicle as shown by *in situ* hybridization (dorsal view) and at 14 hpf *metrn* transcripts can be detected in the area of the KV and the developing brain (later view). While *metrnla* at 10 hpf is expressed in the whole enveloping layer and developing midline as revealed here by *in situ* hybridization (dorsal view). At 14 hpf *metrnla* transcripts can be found in the area of the forming KV and midline (later view). No expression can be detected for *metrnlb* at these stages. (C) From 24 hpf, all *metrn* genes are expressed in the developing central nervous system as shown by *in situ* hybridization (lateral views). (D) Dorsal view of *metrn*, *metrnla* and *fgf8* *in situ* hybridization in HH6 chick embryos reveal that *metrn*, *metrnla* are expressed around the Hensen's node (*) and the primitive streak during chick early embryonic development. Scale bars: in (A, B and D) 250 μ m and in (C) 100 μ m.

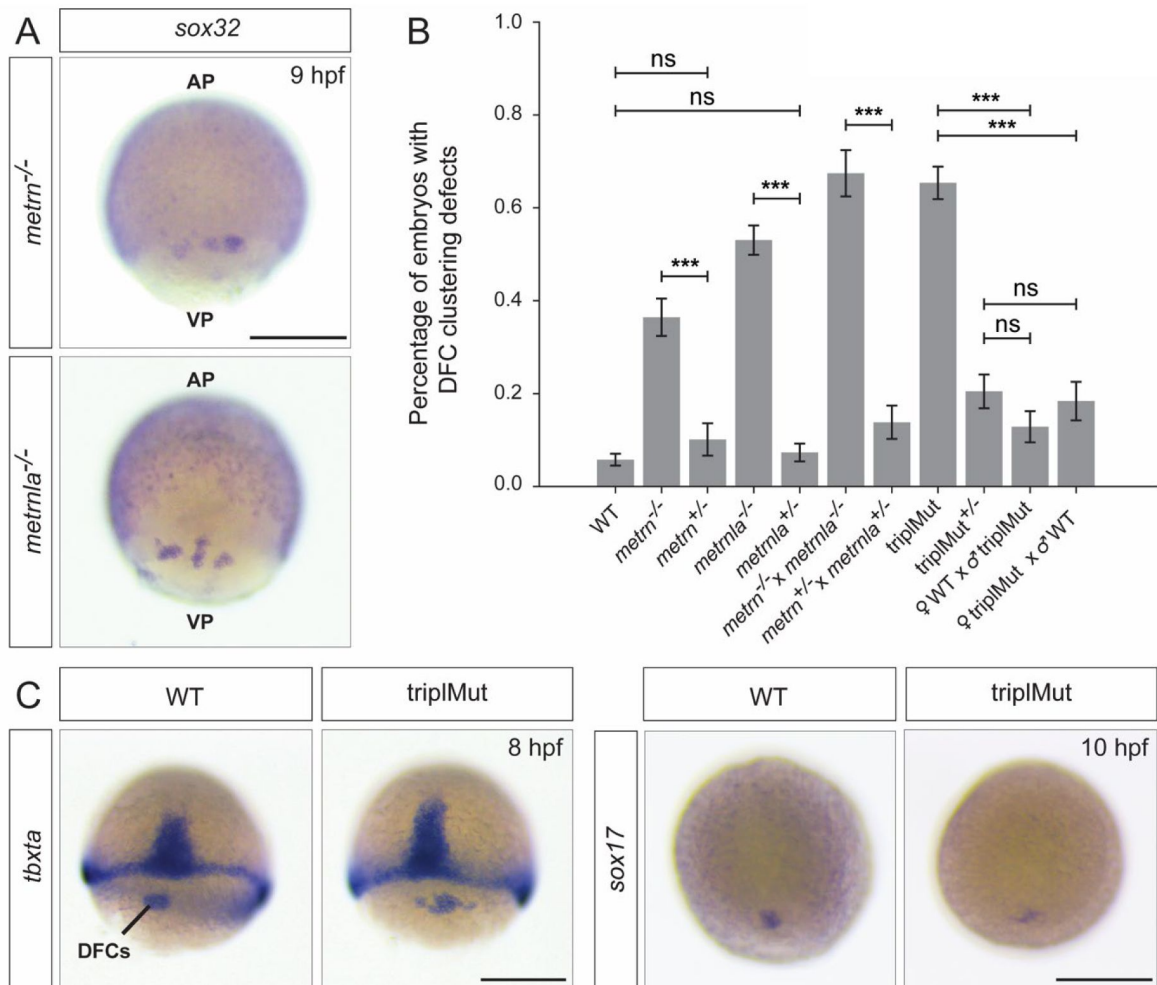


Fig. S4.

***Metrn*s loss-of-function leads to DFC disorganization and migration defects.**

(A) *Sox32* antisense riboprobe *in situ* hybridization revealing DFC misclustering in 9 hpf *metrn*^{-/-} and *metrnla*^{-/-} embryos. (B) Quantification in percentage of the number of embryos with DFC clustering defects at 9 hpf in wild type (WT) and several *metrn*s mutant backgrounds (Fisher exact test, ****p*-value: 1.5e-05 for *metrn*^{-/-} vs. *metrn*^{+/-}; ****p*-value: 2.5e-26 for *metrnla*^{-/-} vs. *metrnla*^{+/-}; ****p*-value: 1.2e-13 for *metrn*^{-/-} x *metrnla*^{-/-} vs. *metrn*^{+/-} x *metrnla*^{+/-}; ****p*-value: 2.2e-18 for tripMut vs. ♀ WT x ♂ tripMut and ****p*-value: 2.1e-13 for tripMut vs. ♀ tripMut x ♂ WT) (C) *tbxta* and *sox17* DFC specification markers are expressed in the absence of *Metrn* proteins as shown by *in situ* hybridization on WT and tripMut at 8 and 10 dpf respectively. Scale bars: in A and C 0.5 mm.

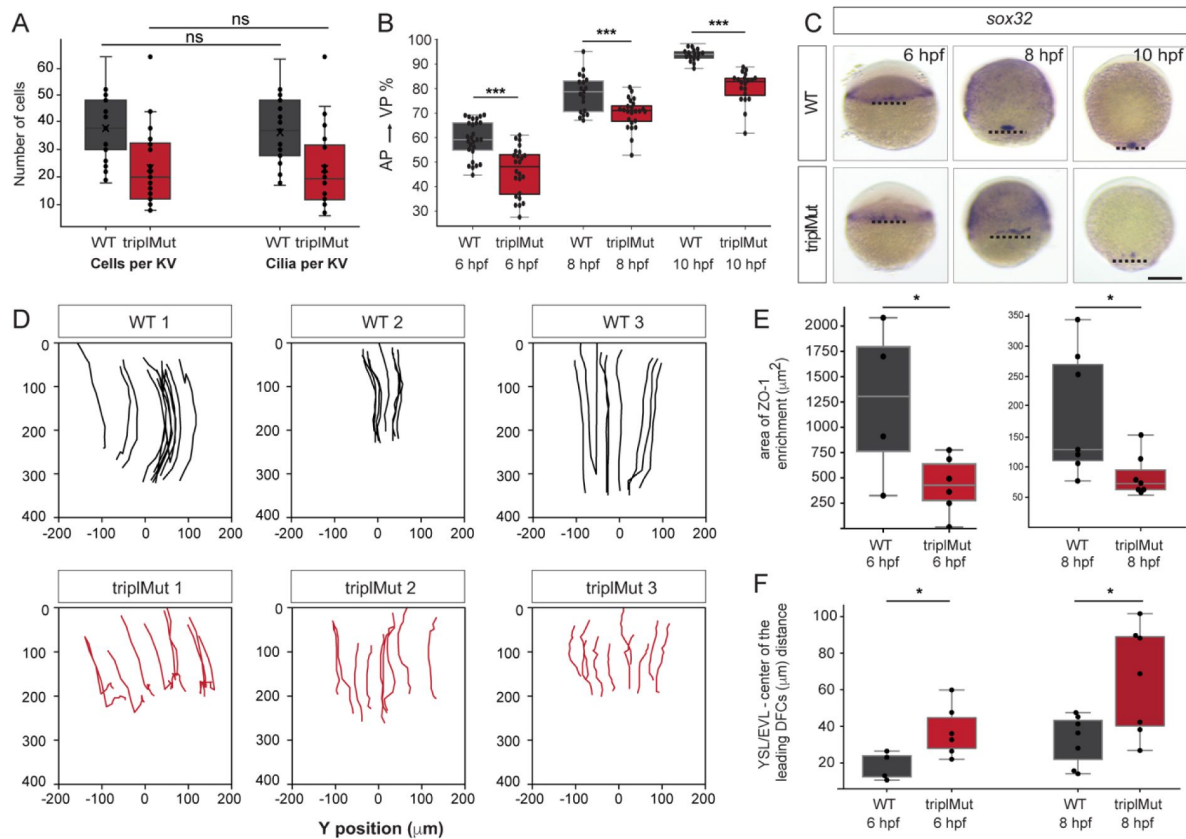


Fig. S5.

***Metrns* loss-of-function leads to DFC disorganization and migration defects.**

(A) The KV cilia number and cells per KV quantification in WT and triplMut embryos at 14 hpf show no significant difference between cilia number and KV cells (N WT: 23, N triplMut: 26, Student T-tests, n.s.: 0.67 for WT KV cells, 0.96 for triplMut KV cells). (B) DFC migration quantification calculated as the percentage of the total embryo length at 6, 8 and 10 hpf in wild type (WT) vs. triplMut embryos. Per condition and group 16 to 27 embryos were analyzed (Fisher exact test, ****p*-value: 2.5e-06 for 6 hpf, ****p*-value: 0.00038 for 8 hpf and ****p*-value: 1.1e-08 for 10 hpf). (C) *Sox32* antisense riboprobe *in situ* hybridization highlighting DFC migration between 6 hpf and 10 hpf in WT and triplMut embryos (point line marks the margin of epiboly). All depicted images are dorsal views. (D) Single traces of *in vivo* tracked DFCs in WT control (upper panel) and triplMut (lower panel) showing the vegetal movement and convergence. (E) Quantification at 6hpf (left plot) and 8 hpf (right plot) of of DFC adjacent ZO-1 enrichments areas reveal a decreased in triplMut embryos in contrast to respective WT controls (T-test; 6hpf **p*-value: 0.043, 8hpf **p*-value: 0.028). (F) The distance of the leading DFCs to the EVL is increased in triplMut compared to WT controls visualized at 6 hpf and 8 hpf (nested T-test, **p*-value: 0.039 N = 4 WT, 30 DFCs measured, triplMut N = 6, 46 DFCs measured; for 6 hpf and **p*-value: 0.021 N = 7 WT embryos, 47 DFCs measured, N = 7 triplMut, 54 DFCs measured for 8 hpf). Scale bars: in (C) 0.25 mm.

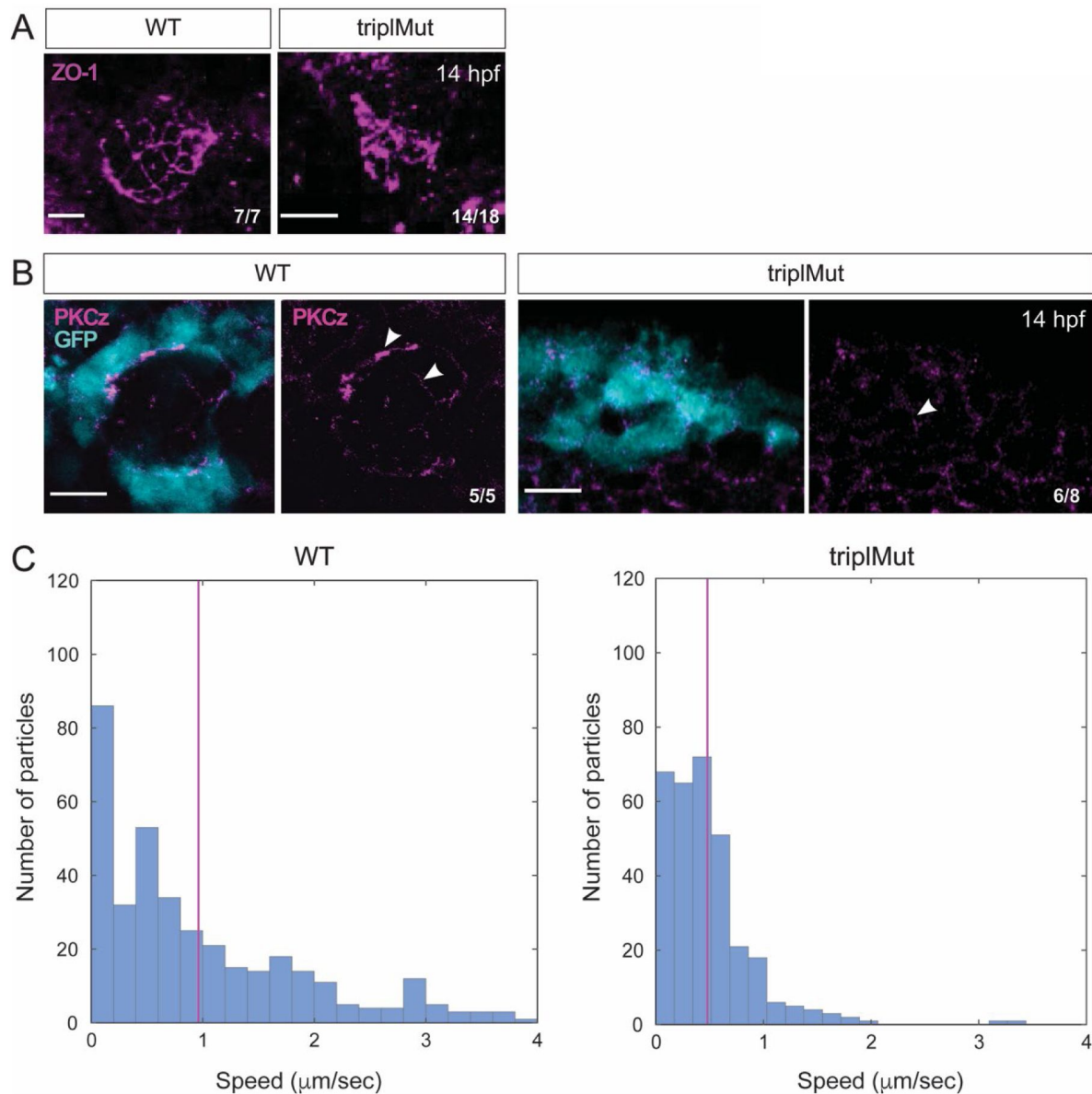


Fig. S6.

***Metrn* loss-of-function impairs the Kupffer's vesicle formation and function.**

(A) Dorsal views of 14 hpf wild type (WT) and triplMut embryos immunostained with ZO-1. Wild type embryos display a large KV lumen with uniform ZO-1 labeled tight junction lattice. TriplMut embryos show disturbed KV lumen and dysmorphic ZO-1 lattice. (B) WT KV cells of 14 hpf *Tg(sox17:GFP)* show proper polarization as visualized with anti- α PKC ζ immunostaining (white arrowhead). 14 hpf TriplMut embryos in contrast exhibit a reduced polarization (white arrowhead). (C) Tracked microbeads velocity analysis in 12-14 hpf WT and triplMut embryos. The purple line marks the mean and the standard deviation (std) for each condition (WT mean= 0.96212; std=0.048143; TriplMut mean=0.47996; std=0.023682). Scale bars: in (A-B) 20 μ m.

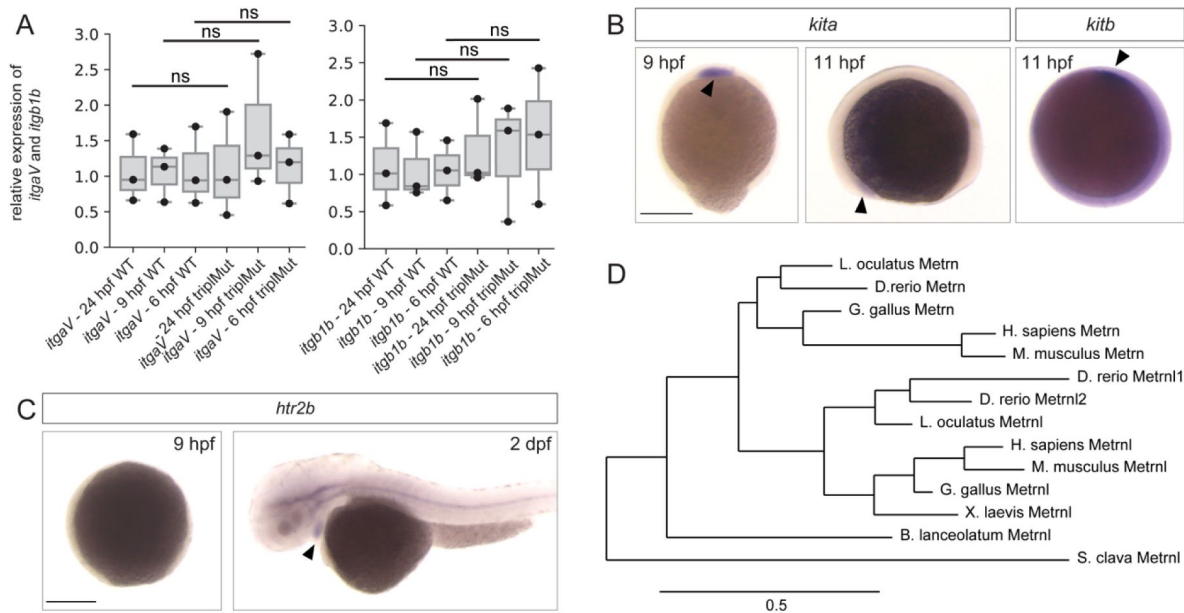


Fig. S7.

Kita, *kitb* and *htr2b* expression patterns during early zebrafish development.

(A) qRT-PCR analysis for *itgaV* and *itgb1b* expression in 6 hpf, 9 hpf and 24 hpf wild type (WT) and triplMut embryos (Fisher exact tests, n.s. = non-significant, *p*-values: 0.95 for 24 hpf *itgaV* WT vs. *itgaV* triplMut, 0.37 for 9 hpf *itgaV* WT vs. *itgaV* triplMut, 0.92 for 6 hpf *itgaV* WT vs. *itgaV* triplMut, 0.64 for 24 hpf *itgb1b* WT vs. *itgb1b* triplMut, 0.7 for 9 hpf *itgb1b* WT vs. *itgb1b* triplMut, 0.46 for 6 hpf *itgb1b* WT vs. *itgb1b* triplMut) (B) At 9 hpf *kita* is expressed in the prechordal plate (black arrowhead, dorsal view) and from 11 hpf in the lateral borders of the anterior neural plate (black arrowhead, lateral view). *Kitb* expression could be detected from 11 hpf in the anterior ventral mesoderm (black arrowhead, lateral view). (C) At 9 hpf, *htr2b* is not yet expressed. From 2 days post-fertilization (dpf), *htr2b* transcripts could be detected in the developing heart (black arrowhead, lateral views). (D) Phylogenetic tree of Metrn proteins. (Scale bar indicates number of amino acids substitutions per site). Scale bars: in (B – C) 250 μ m.

	D-loop	mild/no-loop	S-loop
WT (N = 121)	88.43%	11.57%	0%
<i>metrn</i> ^{-/-} (N = 131)	61.19%	29.10%	8.21%
<i>metrnla</i> ^{-/-} (N = 104)	28.85%	51.92%	19.23%
<i>metrnlb</i> ^{-/-} (N = 104)	74.21%	21.38%	4.41%
triplMut (N = 134)	38.17%	25.95%	35.88%
triplMut ^{+/-} (N = 182)	85.16%	14.29%	0.55%
triplMut + <i>metrnla</i> mRNA (N = 160)	37.5%	53.13	9.37%
triplMut + <i>metrn</i> mRNA (N = 156)	45.51%	42.31%	12.18%
triplMut + <i>metrnla</i> + <i>metrn</i> mRNA (N = 162)	52.47%	27.16%	20.37%

Table 1

Quantification of heart looping phenotypes at 2 dpf using *myl7* riboprobe *in situ* hybridization on wild-type (WT), *metrns* single and triple mutants (triplMut) and *metrn* and/or *metrnla* mRNAs -injected embryos.

'Mild/no loop' designates hearts with no particular L-R orientation. D-loop, dextral loop; mild/no-loop and S-loop, sinistral loop.

Table 2

Quantification in percentage of embryos with DFC clustering defects at 9 hpf in WT, *metrn*^{-/-}, *metrnla*^{-/-}, triplMut and triplMut^{+/-} embryos.

	WT-like (% of embryos)	DFC clustering defects (% of embryos)
WT (N = 202)	93.07	6.93
<i>metrn</i> ^{-/-} (N = 140)	63.57	36.43
<i>metrnla</i> ^{-/-} (N = 245)	46.94	53.06
triplMut (N = 182)	34.62	65.38
triplMut ^{+/-} (N = 140)	79.51	20.49

Table 3

Quantification of the DFC migration calculated as the percentage of the total embryo length at 6, 8 and 10 hpf in wild type (WT) vs. triplMut embryos.

	6 hpf	8 hpf	10 hpf
WT	59.32% (+/- 7.65%, N = 27)	78.18% (+/- 7.94%, N = 19)	93.67% (+/- 2.68%, N = 16)
triplMut	46.26% (+/- 9.83%, N = 24)	69.94% (+/- 6.22%, N = 24)	80.67% (+/- 6.56%, N = 20)

Experiment	Number of embryos
WT non injected	346
WT + ctrl MO	192
WT + 0.41 ng <i>itgaV</i> MO	208
WT + 0.5 ng <i>itgβ1b</i> MO	623
WT + 1.25 ng <i>itgaV</i> MO	412
WT + 0.7 ng <i>itgβ1b</i> MO	336
tripIMut ^{+/-} non injected	219
tripIMut ^{+/-} + ctrl MO	176
tripIMut ^{+/-} + 0.41ng <i>itgaV</i> MO	182
tripIMut ^{+/-} + 0.5 ng <i>itgβ1b</i> MO	559

Table 4

Number of embryos with DFC clustering defects at 9 hpf upon *itgβ1b*, *itgaV* or control (ctrl) morpholino (MO) injection in wild type (WT) or tripIMut^{+/-} embryos.

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Additional information

Authors contribution

F.E. did the experimental work and analyzed the results. L.B. contributed to the experimental work. F.D.S., T.A. and K.D. generated the CRISPR/Cas9 *metrn* mutant lines. J.B.W. performed the KV microbeads tracking analysis and analyzed the cell's tracking experiment. F.E., S.A. and F.D.B. conceived the project and wrote the manuscript. S.A. and F.D.B. co-supervised the study. All the authors reviewed and edited the manuscript.

Additional files

Video S1. *In vivo* imaging of GFP-positive DFCs (single cells labelled with H2B-mRFP mRNA, full stack) in a WT (WT 2 from Fig. S5D) embryo from 50% epiboly for 3 hours showing directed DFC movement and clustering. [🔗](#)

Video S2. *In vivo* imaging of GFP-positive DFCs (single cells labelled with H2B-mRFP mRNA, full stack) in a *triplMut* embryo (*triplMut* 2 from Fig. S5D) from 50% epiboly for 3 hours showing directed DFC movement, but clustering defects. [🔗](#)

Video S3. Microbeads tracking the Kupffer's vesicle of 12-14 hpf wild type embryos showing a distinctive anti-clockwise rotation. [🔗](#)

Video S4. Microbeads tracking the Kupffer's vesicle of 12-14 hpf *triplMut* embryos showing the loss of the distinctive anti-clockwise rotation. [🔗](#)

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Reviewer #1 (Public review):

Summary:

Meteorin proteins were initially described as secreted neurotrophic factors. In this manuscript, Eggeler et al. demonstrate a novel role for Meteorins in establish left-right axis

formation in the zebrafish embryo. The authors generated null mutations in each of the three zebrafish meteorin genes - *metrn*, *metrnla*, and *metrnlab*. Triple mutant embryos displayed phenotypes strongly associated with left-right defects such as heart looping and visceral organ placement, and disrupted expression of Nodal-responsive genes, as did single mutants for *metrn* and *metrnla*. The authors then go on to demonstrate that these defects in left-right asymmetry are likely to due to defects in Kupffer's Vesicle and the progenitor dorsoal forerunner cells including impaired lumen formation and reduced fluid flow, reduced clustering among DFCs, impaired DFC migration, mislocalization of apical proteins ZO-1 and aPKC, and detachment of DFCs from the EVL. Notably, the authors found that expression of marker genes *sox32* and *sox17* were not affected, suggesting Meteorins are required for DFC/KV morphogenesis but not necessarily fate specification. Finally, the authors show genetic interaction between Meteorins and integrin receptors, which were previously implicated in left-right patterning. In a supplemental figure, the manuscript also presents data showing expression of meteorin genes around the chick Hensen's node, suggesting that the left-right patterning functions may be conserved among vertebrates.

Strengths:

Strengths of this study include the generation of a triple mutant line that targets all known zebrafish meteorin family members. The experiments presented in this study were rigorous especially with respect to quantification and statistical analysis.

Weaknesses:

Although the authors convincingly demonstrate a role for Meteorins in zebrafish left-right patterning, data supporting a conserved role in other vertebrates is compelling but limited to one supplemental figure. This aspect would be interesting to follow up in future studies.

Comments on revisions:

I thank the authors for their thoughtful responses to the reviewers. They have adequately addressed all of my concerns.

<https://doi.org/10.7554/eLife.105430.2.sa2>

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<https://doi.org/10.7554/eLife.105430.2.sa1>

Author response:

The following is the authors' response to the original reviews

Public Review:**Reviewer #1 (Public review):***Summary:*

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Weaknesses:

Although the authors convincingly demonstrate a role for Meteorins in zebrafish left-right patterning, data supporting a conserved role in other vertebrates is compelling but limited to one supplemental figure.

We thank the reviewer for their thoughtful summary of our study and for highlighting the strengths of our work, including the generation of the triple mutant line and the rigor of our experimental design and quantitative analyses. We also appreciate the constructive feedback regarding the limited functional data supporting the conservation of Meteorin function in other vertebrates. We agree that this is an important aspect that could be further explored. While functional studies in additional species are beyond the current scope, we will consider such experiments in future work.

We would like to highlight the phylogenetic analysis of Meteorin proteins we have already performed and included in the manuscript (Fig. S7D), which illustrates the evolutionary conservation of this protein family and supports the possibility of a conserved role in left-right patterning.

Additionally, we have expanded the methods and discussion to include: (1) details on zebrafish viability in contrast to reported embryonic lethality in *metrn* mutant mice, (2) the background strains used in our study, (3) observed variability in DFC number and potential batch effects and (4) clarification of our 'convergence ratio' quantification approach.

Reviewer #2 (Public review):

Summary:

In this manuscript the authors describe their study on the role of meteorins in establishing the left-right organizer. The left-right organizer is a transient organ in vertebrate embryos in which rotating cilia cause a fluid flow that breaks the left-right symmetry and coordinates lateralization of internal organs such as gut and heart. In zebrafish, the left-right organizer (also named Kupffer's vesicle) is formed by dorsal forerunner cells, but very little is known about how dorsal forerunner cells coalesce and form this ciliated vesicle in the embryo. The authors mutated the three meteorin-coding genes in zebrafish and observed that mutations in each one of these causes laterality defects with the strongest defects observed in the triple mutant. Loss of meteorins affects nodal gene expression, which play essential roles in establishing organ laterality. Meteorins are widely expressed in developing embryos and expression in lateral plate mesoderm and dorsal forerunner cells was observed. The meteorin triple mutant embryos display defects in the migration and clustering of the dorsal forerunner cells impairing kupffer's vesicle formation and cilia rotation. Finally, the authors show that meteorins genetically interact with integrins.

Strengths:

- These authors went through the lengthy process of generating triple mutants affecting all three meteorin genes. This provides robust genetic evidence on the role of meteorins in establishing organ laterality and circumvented that interpretation of the results would be hard due to redundant functions of meteorins.*
- The use of live imaging on triple mutants is appreciated*
- High-quality imaging of dorsal forerunner to quantify cell migrations and its relation to Kupffer's vesicle formation.*

Weaknesses:

- *Lack of a model how meteorins regulate dorsal forerunner cell migration.*
- *Only genetic data to suggest a link between meteorins and integrins*
- *Besides its role in DFC migration, meteorins may also play a more direct role in regulating Nodal signaling, which is not addressed here.*

We appreciate the recognition of the strengths of our study, particularly the generation of the triple meteorin mutants and the use of high-resolution imaging to quantify DFC behavior and Kupffer's vesicle formation—both of which were central to providing robust evidence for Meteorins' role in left-right patterning.

We also value the reviewer's comments on areas that need further exploration, including the need for a mechanistic model explaining how Meteorins regulate DFC migration, the genetic interaction with integrins, and the potential direct involvement of Meteorins in Nodal signaling.

We agree that deeper mechanistic insights would strengthen the study. While our findings suggest that Meteorins influence DFC migration and clustering through integrin pathways, a detailed mechanistic dissection, particularly regarding the yet unidentified Meteorin receptor, lies beyond the current scope. However, we consider this a key aspect for future research and have discussed it further in the revised discussion section.

In response to the reviewer's suggestions, we have expanded the discussion to address the limitations of the current data linking Meteorins and integrins, including relevant citations to studies that implicate integrins in similar contexts. Additionally, we have added a more detailed discussion of the potential for Meteorins to directly influence Nodal signaling, and we cite a relevant study to support this possibility.

Once again, we thank the reviewer for their insightful and constructive comments. These points raise important directions for future investigation that will further advance our understanding of Meteorin function in left-right axis formation.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

In the Results section (p. 9), the authors state, "...a reduced ZO-1 enrichment at the apical junctions of triplMUT GFP-positive DFCs could be detected." However, in Fig. 4F-G, the areas of ZO-1 enrichment indicated by arrowheads appear quite far from the DFCs themselves, making it unclear if these ZO-1-enriched areas are apical DFC junctions (as stated in the text) or instead are part of the EVL. Is it possible to include an additional cell membrane marker or other landmarks? In addition, the differences in ZO-1 accumulation between mutants and WT appear relatively modest. Is it possible to provide quantification of this effect?

We appreciate the reviewer's request for additional stainings and further clarification and we would like to highlight the requested quantifications of ZO-1 accumulation, including statistical analysis, are already provided in Fig. S5E.

In mouse, loss of Meteorin is embryonic lethal yet the zebrafish triple mutants are viable. Could the authors discuss this discrepancy?

We have expanded the discussion to address this point, suggesting that species-specific differences in compensatory mechanisms may explain the observed differences in viability. We would like to reiterate that while one study has reported embryonic lethality in metrn

mutant mice, this specific mouse line has not been further investigated in any recent publications. Additionally, in collaboration with the lab of Alain Chédotal, we generated independent *metrn* and *metrnl* mutant mouse lines, which did not exhibit the phenotype described in the previously mentioned study.

It has been reported that TL and AB strains exhibit variable numbers of DFCs and thus laterality defects (Moreno-Ayala et al., 2021, Cell Reports 34(2):108606). Would it be possible for the authors to report background strains used in this study and those used to generate the meteorin knock-outs?

We appreciate the comment highlighting the importance of specifying the background strains used in our study. We have now included this information in the methods section, detailing the zebrafish strains utilized throughout our experiments.

For statistical analysis, would be possible for the authors to report the number of clutches examined to control for batch effects (especially given the wide variability in DFC numbers as noted above)?

For further clarification, we have now included additional explanation on number of clutches in the methods section.

In the Methods section (p. 19), the description of how the convergence ratio was computed was somewhat unclear. Could the authors provide a citation or include a diagram/schematic?

We have revised the Methods section to provide a clearer definition of the convergence ratio and have included a schematic (Fig. 4D) to illustrate how it was calculated.

Reviewer #2 (Recommendations for the authors):

- Meteorins are widely expressed in the embryo. Can the authors comment on whether meteorin expression is required in the dorsal forerunner cells (DFCs) or in other cells? This could be addressed by knockdown experiments in DFCs as described by others (PMID: 15716348)

We thank the reviewer for this important comment. In our study, we have shown that Meteorins are not required for the identity of DFCs, as several DFC-specific markers remain expressed in the respective cells within the meteorin mutant background (see Fig. S4).

- In fig1d and 1e the authors use heterotaxy to describe visceral organ placement. The embryo shown in 1d seems to display situs inversus instead of heterotaxy, which is defined as discordance in organ position. The authors should clarify this.

We agree with the reviewer and have revised the figures and figure legends to clarify the distinction between *situs inversus* and heterotaxy.

- In Fig2 the authors show that nodal pathway genes are reduced, suggesting reduced Nodal signaling. How do they explain this as loss of cilia rotation generally leads to randomization of Nodal signaling but not a reduction in signaling.

Following this suggestion we have now added a further discussion on the possibility that Meteorins could directly regulate Nodal signaling in addition to their role in DFC migration and have cited a relevant study.

- *Reduced Nodal signaling in the LPM leads to organ laterality defects. Most anterior tissues like the heart are more sensitive to perturbation in Nodal signaling in the LPM compared to more posterior organs like gut (see also PMID: 25684355). Since in triple mutants the position of the heart is more affected than the position of the visceral organs this suggests that meteorins play an additional role in Nodal signaling in the LPM. As others have shown that meteorins regulate nodal activity (PMID: 24558432), the authors should address this further.*

As described above, we have now added a further discussion on the possibility that Meteorins could directly regulate Nodal signaling in addition to their role in DFC migration and have cited a relevant study. Further investigation into a possible direct role of Meteorins in Nodal signaling will be pursued in future work.

- *The term 'convergence ratio' is not clearly described and confusing as convergence is also used for the movement of LPM cells towards the midline.*

As noted in response to Reviewer #1, we have revised the Methods section and included a schematic in Fig. 4D to better explain this parameter.

We are grateful for the thoughtful critiques from both reviewers, which have been very constructive and improved the clarity of our study. We believe that the revisions we have made address the concerns raised, and we look forward to your evaluation of our revised manuscript.

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