Autotaxin/Lpar3 signaling regulates Kupffer’s vesicle formation and left-right asymmetry in zebrafish

Shih-Lei Lai¹, Wan-Ling Yao¹, Ku-Chi Tsao¹, Anna J. S. Houben², Harald M. H. Albers², Huib Ovaa², Wouter H. Moolenaar³ and Shyh-Jye Lee¹,²,³,⁴,⁵,⁶, *

SUMMARY
Left-right (L-R) patterning is essential for proper organ morphogenesis and function. Calcium fluxes in dorsal forerunner cells (DFCs) are known to regulate the formation of Kupffer’s vesicle (KV), a central organ for establishing L-R asymmetry in zebrafish. Here, we identify the lipid mediator lysophosphatidic acid (LPA) as a regulator of L-R asymmetry in zebrafish embryos. LPA is produced by Autotaxin (Atx), a secreted lysophospholipase D, and triggers various cellular responses through activation of specific G protein-coupled receptors (Lpar1-6). Knockdown of Atx or LPA receptor 3 (Lpar3) by morpholino oligonucleotides perturbed asymmetric gene expression in lateral plate mesoderm and disrupted organ L-R asymmetries, whereas overexpression of lpar3 partially rescued those defects in both atx and lpar3 morphants. Similar defects were observed in embryos treated with the Atx inhibitor HA130 and the Lpar1-3 inhibitor Ki16425. Knockdown of either Atx or Lpar3 impaired calcium fluxes in DFCs during mid-epiboly stage and compromised DFC cohesive migration, KV formation and ciliogenesis. Application of LPA to DFCs rescued the calcium signal and laterality defects in atx morphants. This LPA-dependent L-R asymmetry is mediated via Wnt signaling, as shown by the accumulation of β-catenin in nuclei at the dorsal side of both atx and lpar3 morphants. Our results suggest a major role for the Atx/Lpar3 signaling axis in regulating KV formation, ciliogenesis and L-R asymmetry via a Wnt-dependent pathway.

KEY WORDS: Autotaxin, Lysophosphatidic acid, Calcium, Left-right asymmetry, Zebrafish

INTRODUCTION
Left-right (L-R) patterning is a fundamental process in vertebrate embryogenesis. Establishment of L-R asymmetry in vertebrates has been studied extensively and two embryonic structures are essential for proper L-R patterning: the node (or Kupffer’s vesicle, KV, in zebrafish) and the midline, mainly consisting of the floor plate and notochord (Capdevila et al., 2000; Raya and Izpisúa Belmonte, 2006). An intact midline serves as a barrier and, along with lefty1 expression at the midline, prevents the left-side signals from leaking to the right side (Spéder et al., 2007). KV is a ciliated organ originating from dorsal forerunner cells (DFCs), a group of non-involuting cells at the leading edge of the embryonic shield (dorsal organizer) (Essner et al., 2005; Hirokawa et al., 2006; Raya and Izpisúa Belmonte, 2008). The architecture of KV cells and asymmetric KV cilia along the anterior-posterior (AP) axis generate a counter-clockwise nodal flow. The nodal flow then leads to the asymmetrical expression of early laterality genes, such as nodal-related southpaw (spaw) and its downstream targets pitx2 and lefty in the left lateral plate mesoderm (LPM) (Yost, 1999; Capdevila et al., 2000; Long et al., 2003; Hirokawa et al., 2006; Raya and Izpisúa Belmonte, 2006; Spéder et al., 2007; Wang et al., 2011). During the mid-epiboly stage, elevated calcium levels at the shield/DFCs region are essential for the cohesive migration of DFCs to form KV in the tail bud during later stages. Loss of calcium signaling perturbs KV formation and L-R patterning (Schneider et al., 2008). However, the signal transduction pathway leading to the calcium rise remains unknown.

Lysophosphatidic acid (LPA) is a multifunctional lipid mediator that is produced in many tissues and in blood (Moolenaar et al., 2004). LPA is mainly produced from lysosphatidylcholine by a secreted lysophospholipase D (lysPLD), named Autotaxin (Atx; Enpp2 – Zebrafish Information Network), originally identified as an autocrine motility factor for tumor cells (Stracke et al., 1992; for a review, see van Meeteren and Moolenaar, 2007). LPA acts on six cognate G protein-coupled receptors, Lpar1-6, in mammals (Choi et al., 2010). By coupling to multiple G proteins, LPA receptors convey various downstream signals, including activation of Ras and Rho GTPases, phosphoinositide-3-kinase and phospholipase C and mobilization of calcium (Moolenaar et al., 2004; Choi et al., 2010). These signals are then translated into altered gene transcription (Stortelers et al., 2008) and a wide range of cellular responses, including stimulation of cell proliferation, migration and survival as well as cytoskeletal reorganization and morphological changes (Fukushima et al., 1998; Moolenaar et al., 2004; Choi et al., 2010). In mice, Atx and LPA signaling are critically involved in vascular development (Tanaka et al., 2006; van Meeteren et al., 2006b), nervous system function (Fukushima et al., 2002; Yuan et al., 2003; Matas-Rico et al., 2008), lymphocyte homing (Kanda et al., 2008) and tumor progression (Mills and Moolenaar, 2003; Houben and Moolenaar, 2011). Knockout of the genes encoding the three major and closely related LPA receptors (Lpar1-3) in mice resulted in relatively mild phenotypes (Choi et al., 2008). Lpar1–/– mice show perinatal lethality due to defective suckling (Contos et al., 2000). In addition, defects in neural development have also been observed (Matas-Rico et al., 2008). Lpar2+/– mice show no obvious abnormalities, whereas Lpar1–/–/Lpar2+/– double-null mutants have an exacerbation of the frontal hematomas present in the Lpar1–/– mutants (Contos et al., 2002). Lpar3–/– mice are viable and grossly normal, but pregnant

¹Institute of Zoology, ²Department of Life Science, ³Center for Systems Biology, ⁴Center for Biotechnology, Research Center for Developmental Biology and Regenerative Medicine, National Taiwan University, 1 Roosevelt Road, Sec. 4, Taipei 10617, Taiwan, Republic of China. ⁵Division of Cell Biology, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands.

*Author for correspondence (jefflee@ntu.edu.tw)

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female nulls show delayed implantation, altered embryo spacing and reduced litter size (Ye et al., 2005).

Atx is encoded by Enpp2. Enpp2<sup>−/−</sup> knockout mice die at E9.5 with severe vascular defects (Tanaka et al., 2006; van Meeteren et al., 2006b). Similarly, Lpar4<sup>−/−</sup> mice display hemorrhages and/or edema and some embryos die during gestation (Sumida et al., 2010), but the phenotype is much less severe than that of the Enpp2 knockout. We previously showed that Lpar1 is essential for lymphatic vessel development in zebrafish (Lee et al., 2008). A recent study showed that Atx regulates vascular development via multiple LPA receptors in zebrafish (Yukiura et al., 2011). However, it remains unknown to what extent LPA signaling might affect early embryonic development, including L-R patterning.

In this study, we describe a novel role for Atx and Lpar3 in L-R patterning in zebrafish embryos. We find that Atx is highly expressed in the shield during mid-epiboly, and knockdown of either Atx or Lpar3 abolishes calcium fluxes in DFCs at the same embryonic stage. This, in turn, disrupts later KV formation and ciliogenesis, leading to disrupted asymmetric gene expression and organ morphogenesis. Similar effects on L-R asymmetry were observed in embryos treated with inhibitors and antagonists of Atx and Lpar1-3, respectively, and by knockdown of Atx and Lpar3. Taken together, our results reveal a novel role for Atx/LPA signaling in L-R patterning during early zebrafish development.

**MATERIALS AND METHODS**

**Zebrafish**

Wild-type AB zebrafish (*Danio rerio*), Tg(sox17:dsRed) and Tg(sox17:GFP) fish (obtained from Zebrafish International Resource Center, ZIRC) were maintained at 28.5°C on a 14-hour light/10-hour dark cycle. Embryos collected from natural mating were cultured and staged according to Kimmel et al. (Kimmel et al., 1995).

**Embryo microinjections**

Antisense morpholinos designed against atx (MO: CTGGTG-GCTCTCTTCCACTGACAC; sMO: GGAGAATACCTGGGTCGAGACACCG) and lpar3 (MO1: TTGGAACCAATCCCTAGGACAGTGA; MO2: TAGCAGATGTTGTGCCTGGCCAT) and a standard control morpholino (MO) with random sequence (Std MO: CCTCTTAC- GACAGTG; MO2: TAGCAGATGTTGTGCCTGGCCAT) and a standard control morpholino (MO) with random sequence (Std MO: CCTCTTAC-GACAGTG; MO2: TAGCAGATGTTGTGCCTGGCCAT) were custom-synthesized by Gene Tools. Full-length coding sequences of atx and lpar3 with or without 5′UTR MO-binding region were cloned from zebrafish cDNAs into the pCS2+ vector (supplementary material Fig. S1A) and is 62% identical to its human and mouse homologs. Phylogenetic tree analysis grouped zebrafish Lpar3 with human and mouse Lpar3 and obtained its 5′ and 3′ sequences by rapid amplification of cDNA ends (RACE; sequence submitted to GeneBank, number JQ400025). Sequence analysis revealed that Lpar3 contains seven transmembrane domains (supplementary material Fig. S1A) and is 62% identical to its human and mouse homologs. Phylogenetic tree analysis grouped zebrafish Lpar3 with human and mouse Lpar3.

**RESULTS**

**Loss of Atx and/or Lpar3 results in organ laterality defects**

We cloned zebrafish *lpar3* (NCBI number XM689898) by RT-PCR and obtained its 5′ and 3′ sequences by rapid amplification of cDNA ends (RACE; sequence submitted to GeneBank, number JQ400025). Sequence analysis revealed that Lpar3 contains seven transmembrane domains (supplementary material Fig. S1A) and is 62% identical to its human and mouse homologs. Phylogenetic tree analysis grouped zebrafish Lpar3 with human and mouse Lpar3.
concentration) into the yolk syncytial layer at the 512-cell stage was examined and quantified (treatment is shown on top of each column for all bar graphs. a, b, c, groups denoted with different lettering refer to statistical significance in three trials, the percentages of embryos with different heart jogging (O, 24 hpf) and looping phenotypes (P, 48 hpf) are presented. (Q) Embryos were injected with 2.5 ng Std MO, lpar3 MO or atx MO with or without 100 pg lpar3 mRNA. The percentages of embryos with different heart jogging phenotypes are shown (n=3). (R) Cardiac jogging of wild-type embryos and atx morphants injected with 2.3 nl of buffer or 0.25 mM LPA (pipette concentration) into the yolk syncytial layer at the 512-cell stage was examined and quantified (n=3). Total number of embryos used in each treatment is shown on top of each column for all bar graphs. a, b, c, groups denoted with different lettering refer to statistical significance (P<0.05).

Fig. 1. The Atx-lpar3 axis mediates cardiac laterality. (A-P) Zebrafish embryos injected with 5 ng of different MOs were examined for cardiac jogging at 24 hpf (A-G) or cardiac looping at 48 hpf (H-N) by WISH against cmlc2. Representative heart phenotypes are shown for embryos injected with MOs as follows: (A,B,E) left-jogging, (C,F) no-jogging, (D,G) right-jogging, (H,J,L) D-looping, (J,M) no-loop and (K,N) L-looping hearts. In three trials, the percentages of embryos with different heart jogging (O, 24 hpf) and looping phenotypes (P, 48 hpf) are presented. (Q) Embryos were injected with 2.5 ng Std MO, lpar3 MO or atx MO with or without 100 pg lpar3 mRNA. The percentages of embryos with different heart jogging phenotypes are shown (n=3). (R) Cardiac jogging of wild-type embryos and atx morphants injected with 2.3 nl of buffer or 0.25 mM LPA (pipette concentration) into the yolk syncytial layer at the 512-cell stage was examined and quantified (n=3). Total number of embryos used in each treatment is shown on top of each column for all bar graphs. a, b, c, groups denoted with different lettering refer to statistical significance (P<0.05).

(supplementary material Fig. S1B). Whole-mount in situ hybridization (WISH) analysis of lpar3 showed ubiquitous expression patterns during early development up to 24 hours post fertilization (hpf; supplementary material Fig. S1C); lpar3 mRNA was present at all developmental stages as shown by RT-PCR analysis (supplementary material Fig. S1D), suggesting an important role of lpar3 during early embryogenesis in zebrafish. We also cloned the zebrafish atx gene (enpp2) (Nakanaga et al., 2010) and found that it shows a similar ubiquitous expression pattern as lpar3 during early embryogenesis (supplementary material Fig. S2A). To investigate the role of Atx and Lpar3 in early embryogenesis, we used antisense morpholino oligonucleotides (MOs) against lpar3 (Chiang et al., 2011) and atx. Knockdown efficiency of the translational blocking MO was verified by western blotting (supplementary material Fig. S2B). MO-injected embryos will be referred to as morphants hereafter. lpar3 morphants were grossly normal with a mild developmental delay at 24 hpf (supplementary material Fig. S3A). However, at 48 hpf, cardiac edema and blood pooling appeared at the ventral site of the yolk sphere of lpar3 morphants (supplementary material Fig. S3B), unlike embryos injected with a control MO (Std MO). atx morphants exhibited similar but more severe cardiac defects (supplementary material Fig. S3A,B). We stained erythrocytes with o-dianisidine in both atx and lpar3 morphants (supplementary material Fig. S3C). Erythrocytes in normal embryos were visible in the peri-cardiac cavity and major blood vessels at 72 hpf (supplementary material Fig. S3C, top). By contrast, atx and lpar3 morphants showed blood cell pooling as evidenced by the expansion of erythrocyte staining to the ventral side of the yolk sphere (supplementary material Fig. S3C, middle). The morphants showed up to 30% less erythrocytes and severe hemorrhage in the brain and eyes (supplementary material Fig. S3C, bottom). The alterations in erythrocyte staining were MO dosage dependent. Embryos were injected with various amounts of Std MO, lpar3 splicing blocking MO (Lee et al., 2008), lpar3 MO and atx tMO, and the blood pooling defects were quantified (supplementary material Fig. S3D). Both atx and lpar3 MOs showed graded MO dosage responses, whereas hardly any effect was observed in lpar3 MO- or Std MO-injected embryos. A synergistic effect of atx and lpar3 MOs (at a threshold dosage of 1.25 ng per embryo) was also observed (supplementary material Fig. S3E).

The phenotypes of lpar3 and atx morphants suggest a defect in cardiovascular development. We examined cardiac development by WISH against cardiac myosin light chain 2 (cmlc2). At 24 hpf, untreated control and lpar1 MO-injected embryos had a left jogging heart, as did most of the Std MO-treated embryos (Fig. 1A; data not shown). By contrast, no jogging, left jogging or right jogging hearts appeared in lpar3 (Fig. 1B-D) or atx (Fig. 1E-G) morphants (Fig. 1B-G,O). At 48 hpf, most of the Std MO or lpar1-injected embryos showed a heart tube looping to the right (D-looping) as observed in untreated embryos (Fig. 1H). By contrast, lpar3 (Fig. 1I-K) and atx (Fig. 1L-N) morphants showed cardiac looping laterality defects (Fig. 1I-N,P). To confirm that the lpar3 MO-induced laterality defects are due to loss of Lpar3, we performed lpar3 mRNA rescue experiments using lpar3 mRNA without its 5' untranslated region. Injection of 100 pg lpar3 mRNA did not induce a notable phenotype by itself; however, co-injection with lpar3 MO led to a significant increase in the percentage of normal cardiac jogging (L jogging heart) from 75% to 96%. Moreover, the percentage of normal cardiac jogging in atx
morphants was partially rescued (from 73% to 89%) by lpar3 mRNA co-injection (Fig. 1Q). Amack and Yost (Amack and Yost, 2004) showed that microinjecting materials into the yolk syncytial layer (YSL) of 512-cell stage embryos, the injected reagents can be localized to DFCs. Using the same method, we microinjected LPA into the DFCs of atx morphants to examine whether the defects were due to the specific loss of LPA in DFCs. LPA by itself had no effect on cardiac jogging in control embryos. By contrast, it significantly increased the percentage of normal L jogging hearts from 60% to 81% (Fig. 1R). Based on these results, we conclude that the Atx/Lpar3 signaling axis is necessary for regulating cardiac laterality.

Defects in heart laterality are often accompanied by impaired gut laterality (Hamada et al., 2002, Raya and Izpisúa Belmonte, 2006). We therefore examined gut looping by WISH against sonic hedgehog (shh) and no tail (ntl). Control, atx and lpar3 morphants showed continuous and straight shh, ntl and lefty1 expression at the midline, even in those morphants with bilateral or reversed spaw expression (Fig. 2A-E; supplementary material Fig. S6). It thus appears that atx and lpar3 are required for establishing asymmetric gene expression.

**Atx and Lpar3 mediate Kupffer’s vesicle formation and ciliogenesis**

Organ laterality in zebrafish is established by Kupffer’s vesicle (KV), an embryonic organ equivalent to the mammalian node. Deformation of KV and/or malfunction of KV cilia can affect organ laterality (Essner et al., 2005; Oishi et al., 2006). Sox17 is expressed in DFC/KV (Kikuchi et al., 2001; Aamar and Dawid, 2010). To examine the effect of Atx and Lpar3 on DFC/KV development, we used two DFC/KV reporter lines, Tg(sox17:dsRED) and Tg(sox17:GFP), which express DsRED and GFP, respectively. At the 6-10 somite stage, control Tg(sox17:dsRED) embryos showed a normal button-like KV at the terminus of the notochord under bright-field microscopy (Fig. 3A). By contrast, lpar3 and atx morphants both displayed deformed or reduced KVs (Fig. 3B-E). Under dark-field microscopy, KV cells showed a DsRED-labeled fluorescent ring structure (Fig. 3F). Some of the KV cells were dispersed, and the KV ring was disorganized or smaller (Fig. 3G-J). We also used Tg(sox17:GFP) embryos to investigate the KV defects in further detail. We found

**Fig. 2. Asymmetric gene expression is disturbed in lpar3 and atx morphants.** (A-O) WISH of asymmetry genes spaw (A-E), pitx2 (F-J) and lefty1 and lefty2 (K-O) in zebrafish embryos injected with designated MOs are shown. Open arrowheads, lateral plate mesoderm; arrow, diencephalon; solid arrowheads, heart precursor. (P-R) Asymmetry genes spaw (P), pitx2 (Q) and lefty (R) were quantified in MO-injected embryos. For each treatment, embryos from two independent batches were analyzed. Gene expression in diencephalon (labeled B) and heart precursor (labeled H) were analyzed separately in R.
KVs to be present in all embryos examined. The majority of the defective KVs was deformed, but very few of them were reduced in size (Fig. 3N). KV formation was also examined by WISH against charon (cha), another KV marker gene (Hashimoto et al., 2004). Unlike the typical cha horseshoe expression pattern surrounding the KV in control embryos, with a stronger expression at the right side of the embryo (Fig. 3K), cha expression in lpar3 and atx morphants was strongly reduced or missing (Fig. 3L,M,O). Collectively, these results indicate that loss of Atx/Lpar3 signaling leads to aberrant KV formation.

Dispersed DFCs/KV cells resemble the KV phenotype resulting from aberrant DFC clustering and coalescence (Schneider et al., 2008; Matsui et al., 2011). We set out to determine DFC specification and allocation by examining DFC-specific genes. The integrity of the DFC cluster was confirmed by WISH against sox17 (Fig. 4A-C) in wild-type embryos and by probing GFP in Tg(sox17:GFP) embryos (supplementary material Fig. S5). During epiboly progression, DFCs remained as a compact cluster in control embryos (Fig. 4A,D,G), whereas spread-out or fragmented DFC clusters were detected in atx and lpar3 morphants (Fig. 4B,C,E,F,H,J). Despite the distortion of DFC clusters, DFC specification was unaffected in the atx and lpar3 morphants, as shown by DFC-specific expression of sox17 (Fig. 4A-C), ntl and lrdr1 (supplementary material Fig. S6A-I), and DFC migration in transgenic Tg(sox17:GFP) embryos (supplementary material Movies 1-6). Next, we examined DFC formation by incubating embryos in media containing 10$$\mu$$m SYTO-11. SYTO-11 staining showed that atx and lpa3 morphants contained the same number of well-specified DFCs displaying active endocytosis (supplementary material Fig. S6M-O). Defective DFC clustering was again detected in the atx and lpar3 morphants (supplementary material Fig. S6M-O). These data indicate that DFC specification does not depend on Atx/LPA signaling.

To monitor the dynamic changes in DFC clustering and migration, we imaged Tg(sox17:GFP) embryos treated with or without MOs against lpar3 or atx (supplementary material Movies 1-6). DFCs in both morphants were dispersed, and formed linear or fragmented cell clusters during migration. They failed to coalesce and to form an intact KV lumen at the tail bud stage (Fig. 4D-I; supplementary material Movies 1-6).
Calcium signaling in the shield/DFC region

Calcium signaling in the DFC region during mid-epiboly stages regulates KV formation and organ laterality by antagonizing β-catenin function (Schneider et al., 2008). As LPA is a potent calcium-mobilizing agonist (Moolenaar et al., 2004), we examined whether the laterality defects observed in atx and lpar3 morphants might be due to loss of LPA-induced calcium mobilization. We coinjected fura-2 dextran with the respective MOs and monitored calcium signals in the shield region at the 60% epiboly stage. We observed a local rise in calcium around the shield region with a peak level in DFCs in control embryos, in agreement with previous findings (Schneider et al., 2008) (Fig. 6A). This localized calcium signal was abolished in lpar3 and atx morphants (Fig. 6A,B). Compared with control embryos, the basal calcium levels in the morphants were not altered (ventral counterpart as an example). As shown in Fig. 6B, the dorsal/ventral fura-2 fluorescence ratios in control embryos were significantly higher than those in the atx and lpar3 morphants. Ubiquitous expression of lpar3 (supplementary material Fig. S1C) and atx (supplementary material Fig. S2A) seems difficult to reconcile with the localized regulation of the shield/DFC calcium signal. We therefore examined the localization of Atx and found that it was sporadically distributed in the blastomeres, but with higher levels in the shield region (supplementary material Fig. S7A,C). Atx accumulation was time dependent, as it disappeared before the 80% epiboly stage (supplementary material Fig. S7B). In agreement with the western blotting results (supplementary material Fig. S2B), total Atx was lower in the atx morphants, and there was no Atx accumulation in the shield region (supplementary material Fig. S7B,C). In addition, DFC/YSL-targeted delivery of LPA (Fig. 1R) in atx morphants restored the calcium signal at the DFC region (Fig. 6C). We double stained nuclei and β-catenin using DAPI (red) and β-catenin antibody (green, with FITC-conjugated goat anti-rabbit secondary antibody), respectively. Pseudocolor image analysis showed that the nuclear localization of β-catenin (supplementary material Fig. S8, white) was significantly higher in the atx and lpar3 morphants, consistent with the notion that loss of LPA-induced DFC calcium signaling leads to Wnt activation. We conclude that Atx/Lpar3 signaling is required for calcium mobilization leading to DFC cohesive migration and coalescence during KV formation in a Wnt-dependent manner.

The Atx-Lpar3 axis regulates calcium signaling in the shield/DFC region

To investigate the significance of LPA signaling in DFCs, we injected FITC-conjugated lpar3 MOs into the YSL of 512-cell-stage embryos and detected fluorescent MO in DFCs and the YSL (supplementary material Fig. S9). This allowed us to perform targeted knockdown of Atx and Lpar3 in DFCs. Embryos with targeted gene knockdown in DFCs are referred to as DFC<sup>gene</sup> MO embryos hereafter. Surprisingly, >80% of DFC<sub>lpar3</sub> MO<sub>lpar3</sub> embryos showed normal cardiac jogging unlike those embryos injected MO at the 1-cell stage (Fig. 7A). Instead, the DFC<sub>lpar3</sub> MO<sub>lpar3</sub> embryos exhibited a delay in heart primordium fusion and cardiac bifida in an MO dose-dependent manner (supplementary material Fig. S2E).

By contrast, DFC<sub>atx</sub> MO embryos showed impaired laterality, with only a few embryos having heart primordium fusion and bifida defects (Fig. 7A). Co-injection of lpar3 mRNA significantly rescued the defects in DFC<sub>lpar3</sub> MO embryos (Fig. 7A), but not in DFC<sub>atx</sub> MO embryos. However, it appeared that primordium fusion was reduced by lpar3 mRNA in DFC<sub>atx</sub> MO embryos. Although the rescue effect was not statistically significant, it is notable and more obvious in morphants injected with 2.5 ng atx MO in which reverse jogging (R jog) was significantly reduced (Fig. 7). The disturbed migration and fusion of heart primordium eventually recovered, but these DFC<sub>atx</sub> MO embryos still had cardiac looping defects at 48 hpf (Fig. 7B). Similarly, DFC<sub>atx</sub> MO embryos showed fewer laterality defects than did DFC<sub>lpar3</sub> MO embryos in cardiac looping (Fig. 7B).

To determine the active window for LPA during DFC formation, we treated embryos from time points during epiboly...
with the Atx inhibitors HA51 and HA130 (Albers et al., 2010). First, we confirmed that zebrafish Atx has lysoPLD activity, using recombinant myc-tagged zAtx expressed in HEK293 cells, and is inhibited by HA51 and HA130, with HA130 being the more potent drug (supplementary material Fig. S2D). Embryos were incubated with HA130 from the 50% epiboly stage until the bud stage (marked as ‘E’ in supplementary material Fig. S2G). The drug-treated embryos showed prominent dose-dependent defects in cardiac primordium migration at 24 hpf. At 48 hpf, HA130-treated embryos showed defective cardiac looping and blood pooling, as shown by cmlc2 and o-dianisidine staining. Embryos with a ‘no-loop’ heart, but not embryos with a reversed L-loop heart, showed more severe expansion of blood cell staining at the ventral side of the yolk sphere (supplementary material Fig. S2F). We also incubated embryos with HA130 from 60% epiboly (marked as ‘L’ in supplementary material Fig. S2G). Those embryos showed only very mild defects in cardiac primordium migration and jogging. These results indicate that LPA is required between 50 and 60% epiboly, whereas a high calcium flux is required for DFC formation (Schneider et al.,

Fig. 6. Calcium rise in the DFCs is abolished in lpar3 and atx morphants. (A) Zebrafish embryos were injected with the indicated MOs and fura-2 dextran, incubated to the shield stage and photographed under bright and dark field. The dark-field images were converted to 16-color intensity images (intensity bar is shown in the bottom right corner of A) using Image J software. The shield showed higher calcium levels in control embryos (with peak levels in DFCs; black arrowhead) than those in lpar3 and atx morphants. (B) The peak 340/380 nm ratio in the shield/DFCs region (cycles at the dorsal side) and the ventral blastoderm region (cycles at the ventral side) were measured using Image J software (n=10 for each treatment from three independent experiments). The dorsal, ventral and dorsal/ventral 340/380 nm ratios are shown. (C) Similar analysis was applied in atx morphants with additional DFC-targeting LPA delivery. LPA restores the calcium signal in the shield/DFC region. a, b, c, groups denoted with different lettering refer to statistical significance (P<0.05).

Fig. 7. Targeted knockdown of lpar3 or atx in DFCs causes cardiac bifida in addition to laterality defects. (A) Zebrafish embryos at the 512- to 1000-cell stage were injected with 2.5 or 5 ng of the indicated MOs with or without 100 pg of lpar3 mRNA, as shown at the bottom of each column. Treated embryos were cultured until 26-28 hpf and then subjected to cardiac jogging analysis. The percentages of each phenotype in all treatments are shown in the lower graph. a-f, groups denoted with different lettering refer to statistical significance (P<0.05). (n=3). (B) Embryos with the indicated treatments were incubated to 48 hpf and then subjected to cardiac looping analysis.
Deficiency of both atx and lpar3 altered spaw expression at a similar penetrance level, whereas atx deficiency showed much higher impact on pitx2 expression than did lpar3 deficiency. Also, lefty2 expression in the left heart primordium and lefty1 in diencephalon are largely missing in atx and lpar3 morphants. This agrees with the fact that expression of nodal, pitx2 and lefty2 are not always correlated. lefty2 expression can be ectopically induced in the absence of nodal expression, or it can be abolished in the presence of nodal (Chocron et al., 2007; Shu et al., 2007). The expression of lefty1 and lefty2 is sometimes abolished in the presence of spaw in zebrafish morphants (Schneider et al., 2008; Lin and Xu, 2009; Francescato et al., 2010; Zhang et al., 2012). The differential expression of lefty1 in notochord and lefty1 and lefty2 in brain and heart has been reported previously (Chocron et al., 2007). Our results further support the uncoupling of different organ laterality.

Embryos defective in L-R asymmetry often show altered visceral laterality (Hamada et al., 2002). The laterality uncoupling between gut and heart that we observed in lpar3 morphants also occurs in zebrafish embryos overexpressing Wnt3 and Wnt8 (Lin and Xu, 2009), deltaD mutants (Lopes et al., 2010) and the laf/alk8 (acvr1l) mutant (Chocron et al., 2007). This suggests that additional LPA receptors and/or other signaling pathways are involved during later organ morphogenesis, or that LPA signaling through Lpar3 is required at a later developmental stage, resembles Bmp signaling in the regulation of L-R asymmetry. Our results, along with previous findings, suggest that complex signaling cross-talk underlies asymmetric gene expression and subsequent control of organ laterality.

Calcium signaling in the DFCs region has been implicated in KV formation (Schneider et al., 2008) and occurs downstream of LPA receptor stimulation through activation of phospholipase C (Moolenaar et al., 2004; Schneider et al., 2008; Choi et al., 2010). We observed localized calcium signals at the shield/DFCs region, which was abolished in embryos lacking atx or lpar3. Although atx mRNA was ubiquitously expressed in early embryos, Atx protein was enriched in the shield region. Thus, part of LPA produced by the shield blastomeres might activate neighboring DFCs in a paracrine fashion. Consistent with this, DFCs in lpar3 MO embryos showed less severe defects than did DFCs in lpar3 morphants. As a consequence, atx knockdown has higher penetrance than lpar3 knockdown in other loss-of-function experiments. LPA signaling induces a local calcium release in shield/DFCs region, presumably through Lpa3-mediated calcium mobilization.

Deficiency in L-R patterning lead to abnormal organ positioning, skeletal malformation, neural tube closure failure and complex congenital heart defects (Bisgrove et al., 2003; Ramsdell, 2005). This is the first study to demonstrate a role of the Atx-Lpar3 axis in regulating L-R asymmetry during embryogenesis. The involvement of G protein-coupled receptors (GPCRs) in L-R asymmetry has been reported previously. In particular, Wnt and Fgf pathways might mediate L-R patterning via GPCRs (Oishi et al., 2006; Hong and Dawid, 2009; Lin and Xu, 2009). Wnt signaling controls KV formation and ciliogenesis via β-catenin-dependent and -independent pathways (Oishi et al., 2006; Lin and Xu, 2009).

It has been suggested that LPA might crosstalk with Wnt signaling (Malbon, 2005). Consistent with this, we found a significant increase of nuclear β-catenin in lpar3 and atx morphants, possibly due to reduced calcium signals in the DFC region (Schneider et al., 2008).
Fig. 8. Atx/Lpar3 signaling in the regulation of L-R asymmetry during zebrafish development. In our model, Atx-produced LPA signals through Lpar3 to induce calcium mobilization in the shield, leading to KV formation from DFCs and to ciliogenesis. Asymmetric gene expression induced by KV subsequently regulates organ laterality and morphogenesis.

Taken together, our results reveal a unique role for Atx and Lpar3 in mediating L-R asymmetry via regulation of calcium signaling in the shield/DFC region, which regulates KV formation and ciliogenesis. As illustrated in Fig. 8, proper KV formation and ciliogenesis then establishes asymmetry in gene expression and organ laterality during zebrafish development.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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Fig. S1. Sequence analysis and expression profile of zebrafish lpar3 gene. (A) Domain architecture of the G protein-coupled receptor Lpar3 in zebrafish. Lpar3 possesses evolutionarily conserved seven transmembrane domains (TMs) and translation-blocking MOs (tMO1 and tMO2) binding sites are indicated. Amino numbers flanking each transmembrane domain are shown. (B) Phylogenetic analysis of LPA receptors 1-3 from human (hLPAR1-3), mouse (mLpar1-3) and zebrafish (drLpar1-3). (C,D) WISH expression patterns (C) and RT-PCR analysis (D) of lpar3 in zebrafish embryos at the designated stages. ef1-α was used as an internal control.
Fig. S2. Characterization of zebrafish Atx. (A) Expression patterns of zebrafish Atx during early development was observed by WISH. (B) Embryos injected with 5 ng Std MO, or 2.5 or 5 ng atx MO were cultured to shield stage and subjected to western blotting analysis. Expression of Atx was dose-dependently inhibited by atx MO. Molecular weight is shown in KD on the left-hand side. (C) Uninjected embryos and embryos injected with 2.5 ng of an atx splicing blocking MO (sMO) were incubated to 26-28 hpf and subjected to cardiac jogging analysis by WISH against cmlc2. Total number of embryos used in each treatment is shown on top of each column hereafter. (D) Human ATX (hATX) and zebrafish Atx (drATX) were expressed in HEK293T cells. LysoPLD activity of secreted Atx in the medium was measured by hydrolysis of FS3 (2.5 μM) as described in Materials and methods. (E) Representative patterns of cardiac jogging phenotypes in Atx-LPAR3 loss-of-function conditions. (F) Embryos were injected with or without HA51 at the one-cell stage, incubated and fixed at 48 hpf. Fixed embryos were subjected to o-dianosidine staining and WISH against cmlc2. Representative images are shown. (G) Cardiac jogging of embryos incubated with various concentrations of HA130 from 50% epiboly to tail bud stage (marked by ‘E’ for early) or embryos incubated with various concentrations of HA130 from 60% epiboly to tail bud stage (marked by ‘L’ for late) was examined and quantified (n=3). (H) Cardiac jogging of embryos incubated with various concentrations of Ki16425 from 50% epiboly to tail bud stage was examined and quantified (n=3).
Fig. S3. Knockdown of atx and/or lpar3 causes cardiac edema and blood pooling. (A,B) Embryos injected with atx or lpar3 MOs were examined and photographed at 24 (A) and 48 (B) hpf. Cardiac edema (filled arrowheads) and blood pooling (open arrowheads) appeared at the ventral side of the yolk in both atx and lpar3 morphants were observed (B). (C) Embryos injected with atx or lpar3 MOs were fixed and stained with o-dianisidine to reveal erythrocytes (brown staining). The treated embryos were positioned and photographed from the lateral view or ventral view. In the normal condition (top row), erythrocytes concentrated in pericardiac cavity (open arrowhead) and major vessels; however, in moderate defective embryos (middle row), erythrocytes spread out to the ventral side of the yolk (filled arrowhead); in the most severe condition (lower row), were reduced erythrocytes and hemorrhage in brain and/or eyes were sometimes observed (ventral view). (D) Dose-dependent disturbance of erythrocyte distribution was found in embryos injected with 1.25, 2.5 and 5 ng per embryo of lpar1, lpar3 or atx MOs. Std MO at 5 ng was used as a control (n=3). (E) Embryos were injected with single or different combinations of 1.25 ng of the indicated MOs.
**Fig. S4. Atx-Lpar3 axis mediates gut looping.** Embryos injected with 5 ng Std MO, lpar1 MO, lpar3 MO or atx MO were incubated until 48 hpf, fixed and subjected to WISH against foxA3. (A) Three representative gut looping phenotypes, including L-loop gut liver bud (Left), defected midline gut (Midline) and reversed R-loop gut (Reverse), were shown by WISH against foxA3 and insulin (ins). Insets showed corresponding cardiac looping patterns. (B) The percentages of each phenotype in all treatments are shown (n=3). Total number of embryos used in each treatment is shown on top of each column.
Fig. S5. KVs were deformed and dispersed in lpar3 and atx morphants. (A-F) GFP and DAPI merged images (A-C) and GFP image 3D stack model (D-F) of KV in control embryo (A), and lpar3 (B) and atx (C) morphants at the tail bud stage were examined and analyzed for cell dispersion and cell number under confocal microscopy. KV shown by GFP was from immunostaining of sox17:GFP embryos.
Fig. S6. DFC specification and cell number were not altered in lpar3 and atx morphants. (A-I) Wild-type (Wt) embryos, lpar3 and atx morphants were subjected to WISH against the DFC-specific marker ntl (A-F) at 60% epiboly and the six-somite stage (6 SS) and lrd1 + lefty1 (G-I) at the ten-somite stage (10 SS; as indicated). Formation of DFCs and sustained expression of DFC-specific genes were observed in Wt embryos as well as in lpar3 and atx morphants at the stages indicated (open arrowheads). Notochord expression of both ntl and lefty1 were also observed (filled arrowheads). (J-O) Embryos were stained with vital dye SYTO-11 (10 μM) and examined at 6.5 hpf for DFCs endocytosis activity, cell clustering and cell number quantification. DFCs in lpar3 (K,N) and atx (L,O) morphants formed as early as those in Wt embryos (J,M), with similar numbers, but are more dispersed as linear pattern and sometimes even separate into two groups.
**Fig. S7. Atx localizes in the shield during mid-epiboly stage.** Atx was examined by immunofluorescence staining at the shield stage. (A) Atx was localized sporadically in blastomeres and concentrated in shield region (arrowheads) in control embryos. (B) The overall expression of Atx protein was notably reduced in atx morphants compared with that of control embryos. (C) When observed from the animal top view, Atx was found to be localized in shield blastomeres (arrowheads) in control embryos, but not those of atx morphants.
Fig. S8. Knockdown of Atx-Lpar3 signaling activates β-catenin. (A-C) Wild-type (Wt) embryos (A), and lpar3 (B) and atx (C) morphants were fixed at the 60-70% epiboly stage and immunostained with anti-β-catenin (green) and DAPI (red). Forty-five stacks ranging from 8.3 to 12.45 μm below the surface was calculated for colocalization (as indicated by white signal) using ImageJ software, merged and shown. Nuclear β-catenin signal was stronger in lpar3 and atx morphants than in Wt embryos. (D) Forty-five stacks in five embryos with respective treatments were quantified. Groups denoted with different lettering refer to statistical significance (P<0.05).
Fig. S9. DFC-targeted delivery of MOs. (A-H) Embryos at the 512-cell stage were injected with \textit{lpar3} MO with a 3’ fluorescein modification into the YSL, cultured to mid-epiboly and examined at different magnifications under bright field (A-C) and dark field with a GFP cube (D-F). In addition to YSL, fluorescent MOs were enriched in DFCs (arrowheads in A-F), and also found in yolk (Y) and some tail cells at 24 hpf (arrowhead in H).