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

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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 lysosphatidic 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 K116425. 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.

Lysosphatidic 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 lysophosphatidylcholine by a secreted lysophospholipase D (lysoPLD), 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; Chun 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 sucking (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

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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−/− knockout mice die at E9.5 with severe vascular defects (Tanaka et al., 2006; van Meeteren et al., 2006b). Similarly, Lpar4−/− 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 (MO1: CTGGTG-GCTCTCTTCCACTGAC; MO2: TTTGACAAACCACACTAG-GACAGTG; MO2: TAGCAGATGGTTGGCAGCCAT) and a standard control morpholino (MO3) were injected into one-cell embryos. MOs were injected in 1× HH buffer at a final concentration of 2 nM.

Whole-mount in situ hybridization

DNA fragments of atx and lpar3 were cloned from zebrafish cDNAs by RT-PCR and subcloned into pGEMT-easy vectors for probe synthesis. southpaw, lefty1, lefty2, pitx2, charon (dand5), cmil2 (my17), atl, ldrd (dnak9) and sox17 were kindly provided by Sheng-Ping Huang (Academia Sinica, Taiwan); foxA3 was obtained from Chin-Hwa Hu (National Taiwan Ocean University, Taiwan). Whole-mount in situ hybridization was performed as described (Thiess and Thiess, 2008). Stained embryos were mounted in 1% methylcellulose, observed under a Leica MZ75 stereomicroscope (Leica Microsystems, Wetzlar, Germany) and photographed using a Canon 7D DSLR camera (Canon, Lake Success, NY, USA).

Antisense morpholinos designed against lpar3, lpar4, lefty1, lefty2, sox17, pitx2, charon, cmil2, atl, ldrd, foxA3, sox17, my17, southpaw, and cmlc2 were injected into one-cell embryos. MOs were injected in 1× HH buffer at a final concentration of 2 nM.

Immunofluorescence staining of KV cilia and atypical protein kinase C (aPKC) was performed as previously described (Essner et al., 2005) using an acetylated tubulin antibody (1:400, Sigma) and an aPKC antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Whole-mount immunofluorescence staining of β-catenin and GFP was carried out using a β-catenin antibody (1:250, Sigma) and a GFP antibody (1:250, GeneTex International Corp., Hsinchu, Taiwan) as described (Essner et al., 2005). Embryos were mounted in SlowFade with DAPI (Invitrogen). Colocalization was analyzed and quantified using ImageJ software. Images were collected using a Leica TCS SP5 confocal microscope system (Leica Microsystems) and a Nikon Eclipse Ti (Nikon, Tokyo, Japan).

Western blotting

Whole-mount zebrafish embryos were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 4 hours at room temperature. Embryos were washed and then lysed in RIPA buffer containing 0.05% sodium deoxycholate with 2× of protease inhibitors. Lysates were boiled for 5 minutes and centrifuged at 10,000× g for 10 minutes. Whole-mount lysates were normalized against the cmlc2 transcript. Lysates were separated using 4%–15% gradient Tris-HCl acrylamide gel and electroblotted onto PVDF membranes. Blots were probed with a rabbit anti-Atx antibody (1:250, Abnova) and a mouse anti-β-catenin antibody (1:250, Sigma) followed by secondary antibodies conjugated to horseradish peroxidase (HRP) (1:2500, Sigma). Densitometric analysis was performed using ImageJ software. Cilia number and volume were also analyzed using ImageJ software. Images were collected using a Zeiss LSM 780 laser scanning microscope (Carl Zeiss MicroImaging, Göttingen, Germany), and processed using ImageJ software. Images were collected using a Leica TCS SP5 confocal microscope system (Leica Microsystems) and an LSM 780 confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany). Atx immunostaining was observed under a Leica DM2500 DIC microscope (10× objective; Leica Microsystems) and photographed using a CoolSNAP fx CCD camera (Roper Scientific, Ely, France). The Atx antibody was used at 1:400 for western blotting as described (Lai et al., 2005).

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...
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, lpar1 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 lpar1 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 foxA3, a marker of the digestive system. Std MO-injected embryos and lpar3 morphants showed a normal left-looped gut, whereas the atx morphants contained gut also in the midline and right side (supplementary material Fig. S4). However, 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**

Organic 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

**A**

![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 leftys (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.](Image 83x507 to 529x732)
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 μM SYTO-11. SYTO-11 staining showed that *atx* and *lpar3* 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). This led to a deformed
Calcium signals in the shield region at the 60% epiboly stage. We injected fura-2 dextran with the respective MOs and monitored whether the laterality defects observed in \( \text{atx} \) and \( \text{lpar3} \) morphants, \( \text{ubiquitous expression of \( \text{lpar3} \)} \) (supplementary material Fig. S1C) and \( \text{atx} \) (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 addition, \( \text{DFC/YSL-targeted delivery of \( \text{LPA} \)} \) (Fig. 1R) in \( \text{atx} \) morphants restored the calcium signal at the DFC region (Fig. 6C). We double stained nuclei and \( \beta \)-catenin using DAPI (red) and \( \beta \)-catenin antibody (green, with FITC-conjugated goat anti-rabbit secondary antibody), respectively. Pseudocolor image analysis showed that the nuclear localization of \( \beta \)-catenin (supplementary material Fig. S8, white) was significantly higher in the \( \text{atx} \) and \( \text{lpar3} \) 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.

\section*{The Atx-Lpar3 axis regulates 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 \( \beta \)-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 \( \text{atx} \) and \( \text{lpar3} \) morphants might be due to loss of LPA-induced calcium mobilization. We co-injected 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 \( \text{lpar3} \) and \( \text{atx} \) 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 \( \text{atx} \) and \( \text{lpar3} \) morphants.Ubiquitous expression of \( \text{lpar3} \) (supplementary material Fig. S1C) and \( \text{atx} \) (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 addition, DFC/YSL-targeted delivery of LPA (Fig. 1R) in \( \text{atx} \) morphants restored the calcium signal at the DFC region (Fig. 6C). We double stained nuclei and \( \beta \)-catenin using DAPI (red) and \( \beta \)-catenin antibody (green, with FITC-conjugated goat anti-rabbit secondary antibody), respectively. Pseudocolor image analysis showed that the nuclear localization of \( \beta \)-catenin (supplementary material Fig. S8, white) was significantly higher in the \( \text{atx} \) and \( \text{lpar3} \) 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.
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 cmle2 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.
2008). Next, we tested Kii16425, an antagonist of zebrafish Lpar1-3 (Yakiura et al., 2011). Embryos incubated with Kii16425 from the 50% epiboly to the tail bud stage showed similar effects to those observed with the Atx inhibitor HA130, although somewhat less pronounced (supplementary material Fig. S2H).

**DISCUSSION**

Establishment of L-R asymmetry is crucial for embryogenesis. Directional fluid flow generated by specialized ciliated epithelial cells is a conserved mechanism underlying L-R asymmetry and occurs at the node in mice and the KV in zebrafish (Nonaka et al., 1998; Essner et al., 2005; Kramer-Zucker et al., 2005). In this study, we discovered a novel mediator of KV formation and L-R asymmetry, namely the Atx-Lpar3 axis. We found that the Atx-Lpar3 axis regulates calcium signaling in the shield/DFCs region to control cohesive migration and clustering of DFCs for proper KV formation and L-R patterning.

LPA signaling has been extensively studied in numerous mammalian cell systems (Moolenaar et al., 2004; Choi et al., 2010). Yet the in vivo functions of LPA signaling are still incompletely understood, despite many studies on Atx and LPA receptor knockout mice (Contos et al., 2000; Contos et al., 2002; Ye et al., 2005; Tanaka et al., 2006; van Meeteren et al., 2006b). Here, we use antisense MOs and Atx inhibitors to dissect the possible roles of LPA signaling during early embryogenesis and found that Atx and Lpar3 regulate L-R patterning. Consistent with previous knockout studies in mice (Tanaka et al., 2006; van Meeteren et al., 2006b; Sumida et al., 2010), we observed defects in cardiovascular development by disturbing Atx/LPA signaling. In addition to vascular defects, the disturbed blood circulation and blood pooling in atx and lpar3 morphants are partly due to heart malformation, resulting from impaired cardiac jogging and looping. This LPA-mediated laterality control is a novel observation, as none of the Atx or LPA receptor knockout studies in mice has hinted at laterality defects during embryogenesis. Atx-deficient mice die at E9.5 with profound vascular defects (van Meeteren et al., 2006a), which might have masked any laterality defect. Furthermore, embryos with L-R defects might be morphologically ‘normal’, thereby escaping detection. Therefore, it would be interesting to re-examine L-R asymmetry phenotypes in Atx knockout embryos. It is of note, however, that the origin, development and ultrastructure of the node in mice versus the KV in zebrafish are different (Supatto and Vermot, 2011). It would therefore be interesting to investigate the possible roles of Atx/LPA signaling in node formation and ciliogenesis during mouse embryogenesis.

Defects 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).

Deficiency of both atx and lpar3 altered spaw expression at a similar penetration 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). 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; Francescatto 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; 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, DFC-Lpar3MO embryos showed less severe defects than did DFC-Lpar3MC embryos, whereas 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.

LPA signaling also leads to activation of Rho family GTPases that regulate cell shape (Moolenaar et al., 2004; Choi et al., 2010). The shape of ciliated KV cells is asymmetric along the AP axis. Anterior KV cells are long and narrow with small apical surfaces, whereas posterior KV cells are short and wide and have a larger apical surface. AP asymmetry of KV cell arrangement thus results in more cilia in the anterior half than the posterior, and produces directional nodal flow to establish L-R asymmetry (Wang et al., 2011). We observed a similar feature, in that KV cells are irregularly shaped and lost their AP morphologies around the KV in atx and lpar3 morphants. As a consequence, asymmetric distribution of cilia number along the AP axis was abolished. It remains possible that LPA signaling is also involved at later steps to establish AP asymmetry of KV cells, for example through activation of Rho GTPases. Although KV formation may not be comparable to node formation in mice, and ciliogenesis defects in atx and lpar3 morphants could be secondary to malformed KV, further studies on ciliogenesis and laterality defects in Enpp2 and Lpar knockout mice are warranted.
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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References
Aamir, E. and Dawid, I. B. (2010). Sox17 and chordin are required for formation of Kupffer's vesicle and left-right asymmetry determination in zebrafish. Dev. Dyn. 239, 2980-2988.
Amack, J. D. and Yost, H. J. (2004). The T box transcription factor ttx3 in mice is required to sustain left-right asymmetry. Genes Dev. 18, 685-690.


