Laminin β1a controls distinct steps during the establishment of digestive organ laterality

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SUMMARY
Visceral organs, including the liver and pancreas, adopt asymmetric positions to ensure proper function. Yet the molecular and cellular mechanisms controlling organ laterality are not well understood. We identified a mutation affecting zebrafish laminin β1a (lamb1a) that disrupts left-right asymmetry of the liver and pancreas. In these mutants, the liver spans the midline and the ventral pancreatic bud remains split into bilateral structures. We show that lamb1a regulates asymmetric left-right gene expression in the lateral plate mesoderm (LPM). In particular, lamb1a functions in Kupffer’s vesicle (KV), a ciliated organ analogous to the mouse node, to control the length and function of the KV cilia. L-R asymmetry is an important aspect of organogenesis and involves a sequence of molecular and morphogenetic events. L-R asymmetry initiates at gastrula stages via activity of motile cilia in the organizer/node, or Kupffer’s vesicle (KV) in zebrafish (Capdevila et al., 2000; Levin, 2005), and its maintenance depends on the integrity of midline structures such as the notochord. Organ chirality ultimately emerges as the morphological outcome of this sequence of signaling cues (Danos and Yost, 1996; Ryan et al., 1998; Bisgrove et al., 2000; Chin et al., 2000; Horne-Badovinac et al., 2003; Long et al., 2003). Disruption of these events results in congenital conditions such as situs inversus and heterotaxy.

Fluid flow generated by nodal ciliary movements initiates L-R asymmetry (Nonaka et al., 1998) that is transferred to the lateral plate mesoderm (LPM), as revealed by Nodal expression in the left LPM (Levin et al., 1995; Collignon et al., 1996; Lowe et al., 1996; Lustig et al., 1996; Long et al., 2003). Expression of the Nodal-related gene southpaw (Long et al., 2003), and of the Nodal-induced genes pitx2 (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998; Campione et al., 1999; Essner et al., 2000) and lefty1 (Meno et al., 1996; Bisgrove et al., 1999; Branford et al., 2000), is restricted to the left LPM. The notochord not only serves as a midline physical barrier (Danos and Yost, 1996), but also plays an active role in the consolidation of the asymmetric expression of Nodal in the LPM via midline expression of the inhibitor factor Lefty (Nakamura et al., 2006; Tabin, 2006), thereby ensuring organ chirality.

The downstream cellular mechanisms underlying asymmetric organ morphogenesis take place substantially later in development. In amniotes, expression of Pitx2 is restricted to the left dorsal mesentery that connects the midgut to the body wall (Danos and Yost, 1996; Heckscher-Sørensen et al., 2004; Davis et al., 2008; Kurpios et al., 2008), where it locally translates the L-R signals into the differential cellular organization between the left and right LPM, resulting in leftward tilting of the gut tube. Similarly, in zebrafish, asymmetric migration of the LPM displaces the underlying gut endoderm to adopt an asymmetric configuration (Horne-Badovinac et al., 2003). Little is known about the effector mechanisms ultimately connecting early asymmetric signals with later tissue morphogenesis.

There is increasing evidence supporting the importance of the extracellular matrix (ECM) during the establishment of L-R asymmetry. For example, disruption of integrinβ1 or fibronectin during gastrulation affects the development of the node/KV in zebrafish and Xenopus, resulting in defective L-R asymmetry (Ablooglu et al., 2010; Pulina et al., 2011). Studies in mouse and Xenopus have shown that proteoglycans facilitate and provide directional cues for rapid propagation of Nodal and Lefty signaling from the node to the left LPM (Oki et al., 2007), and subsequently from the left to the right LPM (Oki et al., 2007; Marjoram and Wright, 2011). ECM proteins also play crucial roles in downstream morphogenetic events underlying organ asymmetry. In silico analysis of the formation of gut asymmetry in amniotes predicts that a synergy between epithelial cell-cell adhesion and ECM deposition between left and right sides leads to asymmetric cell compaction and movement in the dorsal mesentery (Kurpios et al., 2008). In zebrafish, the LPM cells degrade the basement membrane at the LPM-gut boundary. Such LPM-ECM interaction is necessary for
the asymmetric migration of the LPM and gut-looping morphogenesis (Yin et al., 2010).

Here, we report that laminin plays an essential role in the establishment of L-R asymmetry of the zebrafish liver and pancreas. Laminins are large heterotrimeric glycoproteins, comprising of α, β and γ chains assembled into a cross-shaped molecule with a long arm and three short globular arms (Engel et al., 1981; Miner, 2008). We present a novel mutant allele of the laminin β1a (lamb1a) gene, which encodes a subunit of the ECM protein laminin 1. In this mutant, the liver spans the midline, and the ventral pancreatic bud remains split into bilateral structures. We find that laminin is necessary for controlling cilia length and fluid flow in the KV, and for restricting southpaw expression to the left LPM. Subsequently, lamb1a deficiency disrupts the dynamic deposition of laminin 1 in the LPM epithelium, resulting in a severely disorganized epithelium with abnormal protrusions into the gut, and a failure of asymmetric gut looping. Thus, laminin 1 participates in sequential events that are important for the establishment of L-R asymmetry of visceral organs, including an early role in the KV and a later role in the LPM.

MATERIALS AND METHODS

Animals

Adult fish and embryos were maintained as described previously (Westerfield, 1995). We used the following lines: Tg(XlEef1a:GFP)s854 (Field et al., 2003) and Tg(hand2:EGFP)°°° (Kikuchi et al., 2011). The grumpy/lamb1a°°° mutant line (Parsons et al., 2002) was used to test complementation. The lamb1a°°° mutant was identified in an ENU mutagenesis screen (Öber et al., 2006).

Cloning and genotyping of lamb1a°°°

Mapping showed that the s804 mutation was on LG25 near lamb1a. The known lamb1a mutant allele grumpy°°° failed to complements the s804 allele. The lamb1a gene was amplified by PCR for sequencing: block A (5'-GGTACAACTGCGACGCCTTTT-3', 5'-ACTTGGCTCTCCTCTGC-TTCA-3'), block B (5'-TACCGGAAGGAACCTGTGACC-3', 5'-GCCGTAATAGCCAAGTCTGT-3'), block C (5'-TTTTTTGCTGTGCAACACAGG-3', 5'-GCGCTGATAATCTCCAGGTTC-3') and block D (5'-TGCTGGACAGCTAGACACA-3', 5'-TGCTGCTGTAGACGGTC-ACCTT-3'). We PCR amplified each block from cDNAs obtained from pooled s804 mutant embryos, or from their wild-type/heterozygous siblings.

The alternative splice variants of lamb1a generated by the s804 mutation were detected by PCR amplification of Block C. Identity of different splice variants was confirmed by direct sequencing.

A G-to-T mutation in the splice donor site of exon 24 of lamb1a was identified in s804 mutant embryos, resulting in the loss of a SnaBI restriction site. Embryos were genotyped by PCR-RFLP: a 286 bp genomic region spanning exons 24-25 is PCR-amplified (5'-CGAAAACA-GTCCAACACTCAA-3', 5'-TGACGGCTCAGTGTGGT-3'), and subsequent digestion with SnaBI produces two bands in wild type (93 bp and 193 bp), while the mutant fragment (286 bp) is not cleaved.

RESULTS

Characterization of s804 mutants

The s804 mutant was identified in a forward genetic screen using Tg(XlEef1a:GFP)s854, a transgenic zebrafish line expressing green fluorescent protein (GFP) throughout the developing endoderm (Field et al., 2003; Öber et al., 2006). The mutant exhibits lateral expansion of the visceral organ region and loss of asymmetric L-R positioning of the pancreas and liver. At 30 hours post fertilization (hpf), using expression of Tg(XlEef1a:GFP) and the gut marker foxa3 to label the entire gut primordium (Chen et al., 2001), we observed the gut loops to the left in wild-type embryos (95%, n=71/75) (Fig. 1A,B), but stays in the midline in s804 mutants (76%, n=37/49) (Fig. 1A,C). Although animals with impaired gut laterality often exhibit defects in cardiac asymmetry (Danos and Yost, 1996; Ryan et al., 1998; Bisgrove et al., 2000; Chin et al., 2000; Horne-Badovinac et al., 2003; Long et al., 2003), over 85% of s804 mutants examined showed normal cardiac asymmetry at 35 hpf, as revealed by the left-sided expression of the cardiomyocyte marker myl7 (Fig. 1B,B,C) (Yelon et al., 1999). At 72 hpf, the liver and pancreas are situated on the left and right sides of wild-type larvae, respectively (92%, n=57/62) (Fig. 1D). In s804 mutants, however, the ventral pancreatic bud splits into bilateral organs (71%, n=26/37) and the liver spans the midline (79%, n=29/37) (Fig. 1D) (P<0.001). Although we occasionally observed inversion of visceral
organ sidedness (n=3/62) in wild-type embryos, we never found this phenotype in s804 mutants.

To further assess endodermal organ development in s804 mutants, we examined the expression of various endodermal marker genes. In zebrafish, endodermal progenitors are specified around the margin at the late blastula stage (Stainier, 2002) and undergo convergent extension movements to form a sparse monolayer along the anteroposterior axis, then moving medially to form a solid multicellular rod at the midline by 20 hpf. This endodermal rod forms the endodermal components of the alimentary canal and its connected organs, including the liver and pancreas (Field et al., 2003). Early endodermal morphogenesis appears unaffected in s804 mutants, as assessed by sox17 (Alexander and Stainier, 1999) and foxa1 (Ober et al., 2003) expression; s804 mutants form an endodermal rod by 20 hpf (data not shown). The endocrine pancreas and liver are properly specified in the mutants by 26 hpf (not shown), with normal insulin (Argenton et al., 1999; Biemar et al., 2001) and hhex (Ho et al., 1999; Ober et al., 2006) expression. However, expression of islet 1 and insulin proteins at 72 hpf revealed isolated pancreatic endocrine cells that failed to home to the principal islet and were instead randomly dispersed within the hepatopancreatic ductal system (Fig. 1D').

Cloning of the s804 mutation
s804 mutants present a shortened body phenotype, similar to other laminin-deficient mutants (Parsons et al., 2002). Genetic mapping placed the s804 mutation on linkage group 25, which contains the genes encoding Lamb1a and Lamb4. Moreover, we found that the s804 mutation failed to complement the grumpy/lamb1am189−/− allele (Parsons et al., 2002).

We thus cloned lamb1a from a pool of s804 mutant embryos, or their wild-type and heterozygous siblings (Fig. 2A). The s804 mutant carries a G-to-T point mutation that disrupts the splice donor site (bold, ACGT) of exon 24 of the lamb1a gene (Fig. 2B), producing alternative splice variants (Fig. 2C) and a frame-shift causing loss of the coiled-coil domains that are necessary for assembly of the trimeric lamin 1 protein. PCR amplification of Block C from wild-type cDNA produces a band of 1.54 kilobases (kb) (Fig. 2A,D). The s804 mutation predominantly causes aberrant excision of exon 24, resulting in a loss of 1.3 kb. When Block C was amplified from cDNA obtained from a pool of phenotypically normal embryos (s804 +/-) and (+/+), the 1.54 kb fragment was the most abundant PCR product (Fig. 2D). Traces of alternative size bands were not detected in mutant or wild-type embryos, suggesting that these bands were due to the mixed nature of the s804 sample rather than to naturally occurring splicing variants. Finally, injection of a splice blocking morpholino (MO) designed against the junction of intron 24 (Fig. 2F,G). At 30 hpf, Lamb1 is also expressed in the basement membrane of the dorsal aspect of the LPM (Fig. 2H, arrows) and at the boundary between the LPM and gut endoderm (Fig. 2H, arrowheads) (Yin et al., 2010).

Laminin protein expression is impaired in multiple tissues in lamb1a s804 mutants
Laminins are an integral part of the scaffolding of basement membranes. Lam1 is formed by the specific assembly of the α1, β1 and γ1 chains at the coiled-coil long arm (Beck et al., 1990). In zebrafish, Lam1 is localized to the basement membranes associated with the neural tube, notochord, somites and skin at 16 ss (Fig. 2F,G). At 30 hpf, Lam1 is also expressed in the basement membrane of the dorsal aspect of the LPM (Fig. 2H, arrows) and at the boundary between the LPM and gut endoderm (Fig. 2H, arrowheads) (Yin et al., 2010).
In lam1as804 mutants, Lam1 deposition was greatly reduced (Fig. 2F, H11032, G, H11032), with only patches of residual expression around the neural tube and vestigial notochord at 30 hpf (Fig. 2H, H11032). The diffuse signal seen by immunodetection of Lam1 in lam1as804 mutants may result from the failure of laminin monomers to assemble in the absence of the coiled-coil domain of the β1 subunit, which causes them to accumulate intracellularly.

lam1as804 mutants exhibit defects in both the notochord and KV cilia

The striking organ laterality defects observed in lam1as804 mutants prompted us to investigate whether the expression of L-R genes was impaired in these embryos. We examined the expression of the Nodal-related factor gene southpaw (spaw), one of the early factors in the establishment of L-R asymmetry (Long et al., 2003). In wild-type embryos, expression of spaw is restricted to the left LPM at 21 ss. However, in lam1as804 mutants, spaw is expressed bilaterally (70.5%, n=12/17), suggesting that Lamb1a is required for restriction of spaw expression to the left LPM (Fig. 3A, B).

Establishment of L-R gene expression depends on the integrity of the KV cilia and notochord: whereas ciliary movements in the KV contribute to the initial establishment of asymmetry, midline structures function as a chemical and/or physical barrier between the right and left sides of the embryo (Danos and Yost, 1996; Meno et al., 1998; Nakamura et al., 2006). Analysis of Lam1 deposition during the stages of KV morphogenesis (Oteíza et al., 2008) shows that Lam1 is detected as scattered puncta associated with epiblast cell membranes during gastrulation (Fig. 3E) (Latimer and Jessen, 2010). Concurrently, a fibrillar assembly of Lam1 is also observed in the mesendoderm-yolk boundary near the embryonic margin (Fig. 3F). From the 1 ss, strong homogeneous expression of Lam1 is also observed in the chordamesoderm and in the basement membrane of the ectoderm. In the KV, however, discrete foci of Lam1
deposition are generally associated with the lumen of the vesicle, at the junctions between cells (Fig. 3G). Interestingly, at 2-3 ss, the cilia tend to localize at the intercellular junctions where Lam1 deposition is detected (Fig. 3H).

As Lam1 is expressed in both the notochord and KV, we asked whether development of either tissue was impaired in s804 mutants and perhaps contributed to the bilateral expression of spaw. To investigate notochord formation in lamb1as804 mutants, we examined the expression of not tail/ntl at 21 ss (Fig. 3C). Integrity of the notochord is assessed by expression of no tail/ntl at 21 ss. Reduction of ntl expression in lamb1as804 mutant (B) embryos is restricted to the level of the visceral organ-forming region (white bracket). (A-D) Dorsal views. (E-H) Single-plane confocal imaging of wild-type embryos stained for Lam1 (green), acetylated tubulin (red) and phalloidin (blue). Dorsal views, anterior towards the top. Expression of Lam1 during KV morphogenesis in the epiblast (E) and at the margin (F) of 80% epiboly stage embryos. Discrete foci of Lam1 deposition are associated with the lumen of the vesicle (G, arrowheads) and where cilia formation takes place (H, arrows). Scale bars: 10 µm. (I,J) Confocal imaging projection of KV cilia at 8 ss, as revealed by acetylated tubulin staining (red) in wild-type (I) and lamb1as804 mutant (J) embryos. Counterstaining by phalloidin (blue). Scale bars: 10 µm. Dorsal views, anterior towards the top. (K) Quantitative analysis (mean±s.e.m.) of KV cilia length in wild type and lamb1as804 mutants. Asterisks indicate statistical significance, *P<0.0001 and **P=0.031.

To examine KV cilia in lambβ1s804 mutants, we performed immunostaining using an anti-acetylated tubulin antibody in embryos at 7-8 ss (Piperno and Fuller, 1985; Essner et al., 2005) (Fig. 3I,J). Cilia length was significantly reduced in lamb1as804 mutants, with an average±s.e.m. of 3.95±0.09 µm (n=23 embryos, 1179 cilia) in contrast to 4.44±0.11 µm in wild-type siblings (n=14, 880 cilia) (P<0.0001) (Fig. 3K). The shortened cilia length in lamb1as804 mutants is associated with a shift in the distribution of cilia length (P=0.031). For each embryo, the cilia were classified according to their length. At 8 ss, 71.4% of the wild-type embryos contained KV cilia that were predominantly 4-6 µm long (10/14 embryos), followed by a second class of embryos (28.6%) with predominance of cilia length in the range of 2-4 µm. By contrast,
cilia length was predominantly shorter (2-4 µm) in 65% of the 
lamb1a<sup>s804</sup> mutants examined (n=15/23), with the other 35% of the embryos (n=8/23) showing cilia length mainly in the range of 4-6 µm (Fig. 3L). Overall cilia number was not significantly affected in lamb1a<sup>s804</sup> mutants (data not shown).

To determine whether this difference in the KV cilia length affected their function in lamb1a<sup>s804</sup> mutants, we examined the generation of fluid flow in the KV of wild-type and mutant embryos. Analysis of the dynamics of fluorescent beads injected into the KV at 8-10 ss showed that deficiency in Lamb1a expression did not halt fluid flow in the KV and that directionality was also largely preserved in the mutants (data not shown). Instead, we observed variable defects in the size of the KV and the speed of flow in the mutants (Fig. 3M-P). In all the mutants, tracking of the beads showed more erratic trajectories and increased tendency to cross the center of the vesicle (Fig. 3N), in contrast to the regular pattern of fluid flow in the KV of wild-type embryos, where the beads mostly concentrated in the periphery of the vesicle (n=5/6) (Fig. 3M). In 50% of the lamb1a<sup>s804</sup> mutant embryos analyzed (n=3/6), the KV appeared smaller, yet the average flow speed (7.99±0.13 µm/s) was not significantly affected, when compared with the average traveling speed of 7.65±0.11 µm/s (average±s.e.m.) measured in wild-type and heterozygous embryos (n=6). In one-third of the mutants analyzed (n=2/6), whereas the KV size was normal, the speed of fluid flow was reduced, with an average speed of 4.58±0.11 µm/s (Fig. 3O). Overall, our analysis of the KV fluid flow indicates that the average flow speed in wild-type embryos ranges between 6 and 10 µm/s (n=6). By contrast, in lamb1a<sup>s804</sup> mutant embryos, we observed increased prevalence of embryos with reduced flow (average speed of 4-5 µm/s) (P=0.02) (Fig. 3P).

Taken together, our analyses suggest that loss of Lamβ1 function in lamb1a<sup>s804</sup> mutants impairs the integrity of the KV cilia and notochord, both of which are essential for L-R gene expression.

**Knockdown of lamb1a in the KV causes randomization of gut L-R asymmetry**

Given that lamb1a<sup>s804</sup> mutants show defects in both the notochord and KV, we examined which defect directly accounted for impaired L-R patterning. To test the role of Lamb1a in the KV, we injected the lamb1a MO into the yolk of embryos at midblastula stages to specifically target the dorsal forerunner cells that will form the KV (Amack and Yost, 2004) (Fig. 4A).

We assessed the morphology of the digestive organs based on Tg(XlEef1a:GFP) expression at 52 hpf (Fig. 4B-E). Embryos injected with control MO displayed a normal gut phenotype, with the liver and pancreas located on the left and right sides of the embryo, respectively, and the gut displaying an S-shape curvature (85.7%, n=36/42). By contrast, only 47.6% (n=20/42) of embryos injected with lamb1a MO showed a similar leftward gut looping, and 28.6% of the lamb1a MO-injected embryos exhibited bilateral liver and pancreas accompanied by the absence of gut looping (n=12/42) when compared with 4.8% (n=2/42) of the control MO-injected embryos. In addition, we observed an increased occurrence of inverted liver and pancreas position in lamb1a MO-injected embryos (23.8%, n=10/42), compared with 9.5% in the control MO group (n=4/42) (P=0.001) (Fig. 4F). Injections of lamb1a MO after

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**Fig. 4. Knockdown of lamb1a in the KV results in randomization of liver and pancreas asymmetry.** (A-E) Confocal imaging of the gut in Tg(XlEef1a:GFP) embryos injected at 3 hpf (A) with control (B) or lamb1a (C-E) MO. Effect of KV knockdown of lamb1a on the L-R asymmetry of the liver (Li) and pancreas (Pa) was analyzed at 52 hpf. Resulting phenotypes of the liver and pancreas after KV-specific lamb1a knockdown include: wild type (C, blue); bilateral organs, midline gut (D, red); inversion of L-R asymmetry, with the liver and pancreas localized to the right and left sides of the embryo, respectively (E, yellow). Ventral views. (F) Percentages (mean±s.e.m.) of embryos displaying distinct liver and pancreas L-R asymmetry phenotypes. Forty-two embryos were analyzed for each treatment. Asterisks indicate statistical significance, *P=0.001. (G) Percentages (mean±s.e.m.) of embryos showing distinct spaw expression patterns at 20 ss. Wild-type embryos were injected with control (n=28) or lamb1a (n=36) MO at 3 hpf.
midblastula stages did not significantly affect L-R asymmetry of the liver and pancreas.

Knocking down lamb1a in the KV led to randomization of the gut laterality, rather than failure of gut looping, as in most lamb1a<sup>804</sup> mutants. Whereas the majority of lamb1a<sup>804</sup> mutants showed bilateral spa2 expression during somitogenesis (Fig. 3B), sidedness of spa2 expression after lamb1a knockdown in the KV was rather random, with 44% of the embryos showing normal left-sided spa2 expression (Fig. 4G). These data suggest that lamb1a deficiency in the KV alone only partially impairs L-R gene expression and that lamb1a also functions in other tissues, probably the notochord, to regulate L-R gene expression. Interestingly, lamb1a knockdown in the KV resulted in a significant increase of inverted liver and pancreas phenotype. However, complete inversion of liver and pancreas sidedness was never observed in lamb1a<sup>804</sup> mutants, despite bilateral expression of spa2. The differences between lamb1a<sup>804</sup> mutants and embryos with lamb1a knockdown in the KV thus suggested that dysregulation of L-R gene expression alone could not account for the gut-looping defects in the lamb1a<sup>804</sup> mutants.

**Asymmetric migration of the LPM is impaired in lamb1a<sup>804</sup> mutants**

We have previously shown that the leftward gut-looping morphogenesis results from the asymmetric migration of the LPM, and that LPM migration relies on the remodeling of laminin distribution in the basement membrane at the gut-LPM boundary (Horne-Badovinac et al., 2003; Yin et al., 2010). To analyze the dynamics of the LPM during gut looping in lamb1a<sup>804</sup> mutants, we tracked LPM migration in the gut looping region between 24 and 30 hpf, using the Tg(hand2:EGFP) line combined with phalloidin staining to visualize the LPM and adjacent tissues. At 25 hpf, the left and right LPM in both wild-type and lamb1a<sup>804</sup> mutant embryos have converged to the midline, dorsal to the gut endoderm and Tg(hand2:EGFP)-expressing cells located in some mutants (Fig. 5D,G). Based on the migration pattern and morphogenesis results from the asymmetric migration of the LPM, and that LPM migration relies on the remodeling of laminin distribution of the LPM (Fig. 5A,B). At 30 hpf, asymmetric morphogenetic movements result in the asymmetric configuration of the LPM in wild-type embryos, with the right LPM extending ventrally and across the left LPM (Fig. 5C,C’). In lamb1a<sup>804</sup> mutants, however, the left and right LPM remain dorsal to the gut, lacking asymmetry after converging to the midline. Moreover, the morphology and distribution of the Tg(hand2:EGFP)-expressing cells is abnormal in some mutants (Fig. 5D-G). Based on the migration pattern and morphology of LPM cells, we classified four types of LPM phenotypes. In class I, the LPM is composed of two rows of epithelial cells with the Tg(hand2:EGFP)-expressing cells located in the ventral half. The left and right LPM undergo asymmetric migration and the gut loops to the left (Fig. 5D). In class II, the majority of the LPM cells exhibit epithelial polarity, but there are more than two rows of cells in the left and right LPM. Moreover, the left and right LPM fail to undergo asymmetric migration, remaining dorsal to the gut (Fig. 5E). In class III, the LPM cells round up and the two-row organization of the LPM is also compromised (Fig. 5F). Last, in class IV, embryos exhibit aberrant intrusion of Tg(hand2:EGFP)-expressing cells into the gut, in addition to defects in epithelial morphology and LPM organization (Fig. 5G).

In wild types, over 90% of the embryos exhibit the class I phenotype (n=10). Among the mutants examined here (n=24), 40% were class IV, 25% were class III and 15% were class II. Only 10% had normal asymmetric LPM migration and leftward gut-looping (Fig. 5H). In wild types, the left and right LPM are composed of equal numbers of Tg(hand2:EGFP)-expressing and non-expressing cells throughout the time course of gut-looping morphogenesis. Whereas lamb1a<sup>804</sup> mutants maintain equal numbers of Tg(hand2:EGFP)-expressing and non-expressing cells prior to gut looping (Fig. 5I), later there is an increased number of Tg(hand2:EGFP)-expressing cells at the expense of Tg(hand2:EGFP)-non-expressing cells (Fig. 5I), with the total number of LPM cells comparable with wild type (data not shown). The severe disorganization of Tg(hand2:EGFP)-expressing cells raised the possibility that their LPM cell identity might be compromised. Arguing against this possibility, the LPM markers wnt2b (Fig. 5J) (Ober et al., 2006) and bmp2b (data not shown) (Chung et al., 2008) were expressed in the gut-looping region in lamb1a<sup>804</sup> mutants. Alternatively, complete loss of epithelial polarity also cannot explain the LPM phenotype in the mutants, as localized expression of the tight junction protein ZO-1 (Horne-Badovinac et al., 2001) (Fig. 5K) is still detected in the mutants, even though the mutant LPM is extremely disorganized and some cells protrude into the gut (Fig. 5K’).

**Deficiency in L-R patterning alone cannot account for the LPM migration defects in lamb1a<sup>804</sup> mutants**

We asked to what degree the disruption of L-R patterning due to impairment of the KV and notochord during somitogenesis was linked to the late LPM migration defects in lamb1a<sup>804</sup> mutants. A majority of lamb1a<sup>804</sup> mutants fail to undergo asymmetric LPM migration (Fig. 5I). Upon loss of lamb1a in the KV, however, the direction of LPM migration was randomized: 53% of the embryos showed normal asymmetric LPM migration (n=30), 33% showed reversed migration and 13% showed no asymmetric migration with both left and right LPM remaining dorsal to the gut (Fig. 6A-D,G). Therefore, Lamb1a deficiency in the KV is not solely responsible for the defective LPM morphogenesis in lamb1a<sup>804</sup> mutants.

We also examined other mutants that are deficient in notochord development. In floating head/flh and no tail/ntl mutants, specification and differentiation, respectively, of the notochord is defective (Schulte-Merker et al., 1994; Halpern et al., 1995; Talbot et al., 1995). As a consequence, these mutants show bilateral expression of L-R genes, similar to that observed in lamb1a<sup>804</sup> mutants (Bisgrove et al., 2000; Chin et al., 2000). We injected MO against flh or ntl at the one-cell stage. By 32 hpf, most control MO-injected embryos had undergone leftwards LPM migration, with only 2% showing no asymmetric migration (n=1/53). For the flh and ntl MO-injected embryos, 65% (n=32/49) and 51% (n=18/35), respectively, exhibited no asymmetric LPM migration, with both the left and right LPM remaining dorsal to the gut. Notably, LPM migration defects in ntl- and flh-deficient embryos were significantly less severe than those in lamb1a<sup>804</sup> mutants, where 80% fail to undergo asymmetric LPM migration. Interestingly, the LPM of embryos with lamb1a knockdown in the KV as well as in those with global knockdown of flh or ntl function failed to show impairment of epithelial morphology or LPM composition. In summary, disrupting L-R patterning alone yields less severe LPM migration defects than those observed in lamb1a<sup>804</sup> mutants.

**DISCUSSION**

Here, we report a zebrafish lamb1a mutant allele that displays previously uncharacterized defects in asymmetric positioning of the liver and pancreas. Lamb1a function is required in the KV and notochord for early establishment of embryonic L-R asymmetry. Interestingly, KV-specific knockdown of lamb1a expression results
in typical randomization of embryonic L-R asymmetry, with phenotypes including inversion of LPM asymmetry and liver/pancreas position. By contrast, loss of laminin function in the basement membrane associated with the LPM disrupts its epithelial organization and impedes its asymmetric migration. Consequently, gut-looping morphogenesis fails to occur properly and the liver and pancreas remain symmetric.

Lamb1a controls cilia length in the KV

Establishment of L-R asymmetry is a progressive process. At early stages, a directional fluid flow generated by the beating of ciliated cells in the node in mice, or the KV in zebrafish, induces cells on the left side of the vesicle to activate asymmetric gene expression (Nonaka et al., 1998; Okada et al., 1999; Amack and Yost, 2004). Integrity of the KV and ciliary activity is necessary to generate this L-R asymmetry. ECM proteins have been implicated in morphogenesis of the organizer. Fibronectin participates in the development of embryonic L-R asymmetry, by controlling the organization of cells into a single layer in the mouse node, without affecting cilia length (Pulina et al., 2011). In zebrafish, integrin α5 is expressed in the dorsal forerunner cells and is required for the morphogenesis of a single intact KV (Ablooglu et al., 2010).

Our data show that Lamb1a controls cilia length in the KV. We identify a distinctive distribution of Lam1 that is associated with sites of cilia formation during KV morphogenesis. Interestingly, in mammals, Lam-511 regulates primary cilia formation during dermal papilla development (Gao et al., 2008), suggesting that the role of laminin in ciliogenesis may be conserved in different organs and
across species. Yet the mechanism by which Lamb1a modulates cilia formation is not clear. Studies of primary cilia of chick chondroblasts indicate that the ECM directly signals to cells via integrins α2, α3 and β1, which anchor the cilium to collagen fibers within the ECM (Jensen et al., 2004; McGlashan et al., 2006). It is possible that Lamb1a is involved in establishing the cellular property of the ciliated cells essential for cilia formation.

Previous studies in zebrafish have identified signaling molecules that regulate KV cilia formation. For example, defects in cilia length associated with deficiency in FGF or Notch signaling pathways have been described to affect fluid flow in the KV, which consequently results in defects in L-R asymmetry (Albertson and Yelick, 2005; Neugebauer et al., 2009; Yamauchi et al., 2009; Lopes et al., 2010). Activation of Wnt/β-catenin signaling results in loss of cardiac asymmetry, and affects morphology of the KV, with larger KV and increased cilia number. By contrast, reduction of Wnt3 or Wnt8 results in shorter cilia and randomization of spaw expression, with effects on both heart and digestive organs sidedness (Nakaya et al., 2005; Lin and Xu, 2009; Caron et al., 2012). In addition, the Rho kinase Rock2b controls anterior-posterior asymmetric placement of ciliated cells in the KV, and subsequently L-R patterning of the embryo (Wang et al., 2011). In lamb1aΔ804 mutants, we observed that impaired cilia length and movements partially contribute to defects in L-R patterning, resulting in variable defects in the dynamics or directionality of the fluid flow in the KV. It is intriguing to speculate that Lamb1a and its integrin receptors might serve as mediators of the signaling pathways described above, to modulate cilia formation.

Regulation of notochord differentiation by Lamb1a is crucial for establishment of L-R gene expression

After initiation of left-sided signaling, the notochord generates a midline barrier that restricts the cascade of gene expression to the left side of the embryo (Lee and Anderson, 2008). Mutants defective in notochord formation often exhibit randomization of organ laterality (Danos and Yost, 1996). The notochord of lamb1aΔ804 mutants appears atrophic and collapsed, consistent with the role of laminins in the formation of this structure. In zebrafish, laminin subunits β1 and γ1 are essential for establishment of the perinotochordal basement membrane and organization of the notochord sheath, which provides physical resistance against the pressure exerted by the inflated notochord vacuoles (Parsons et al., 2002). Importantly, differentiation of the notochord is also impaired in the lamb1a and lamg1 mutants, suggesting that there may be signals emanating from the ECM in the basement membrane to regulate chordamesoderm differentiation (Stemple, 2005).

Analysis of ntl expression showed that the notochord defect in lamb1aΔ804 mutants does not distribute along its full extension, but rather is restricted to the liver and pancreas-forming region. Coincident with the intact ntl expression in the more anterior region of the notochord, heart sidedness was not affected in the lamb1aΔ804 mutants. The independent phenotypes of the heart and gut laterality in lamb1aΔ804 mutants suggest that heterotaxy of organ laterality caused by midline defects is dependent on distinct domains along the anteroposterior axis (Bisgrove et al., 2000; Chin et al., 2000). Moreover, our observation that fewer ntl- and flh-deficient embryos fail to undergo asymmetric LPM migration compared with lamb1aΔ804 mutants indicates that impairment in the notochord alone cannot account for the gut laterality defects seen in lamb1aΔ804 mutants.

Localized expression of Lam1 controls asymmetric migration of the LPM

Asymmetric migration of the left and right LPM is essential for leftward gut-looping morphogenesis (Horne-Badovinac et al., 2003). Lam1 is expressed in a dynamic pattern at the interface between the LPM and gut endoderm during LPM migration, and remodeling of the ECM has been associated with establishment of proper contacts between these tissues, which are necessary to drive
morphogenetic movements (Yin et al., 2010). \textit{lamb1a}^{s804} mutants lack the coiled-coil domain of the β1 chain, and expression of Lam1 protein is detected as intracellular speckles, instead of the discrete continuous expression observed in the basement membranes of wild-type embryos. This observation is consistent with the model proposed for assembly and secretion of Lam1, in which secretion of the functional trimeric laminin requires a first step of intracellular dimerization of subunits β1 and γ1, followed by assembly with an α1 chain (Peters et al., 1985; Kammerer et al., 1995; Kumagai et al., 1997; Yurchenco et al., 1997; Goto et al., 2001; Urbano et al., 2009). In the absence of proper laminin deposition, the left and right LPM fail to undergo asymmetric migration, and remain aligned dorsal to the gut.

By tracking the LPM migration at cellular resolution, we uncovered distinctive phenotypes in the LPM of \textit{lamb1a}^{s804} mutants that may account for the failure of LPM migration. Laminins have been implicated in the establishment of cell polarization in embryonic cells (Li et al., 2003). Establishment of epithelial polarity in the LPM is absolutely essential for asymmetric LPM migration and gut looping (Horne-Badovinac et al., 2003; Yin et al., 2010). Mutant LPM cells are correctly specified and the organization of the LPM is unaffected prior to LPM migration. However, as the LPM migrates to the midline, in majority of \textit{lamb1a}^{s804} mutants the LPM failed to align into two rows of cells and its epithelial architecture is compromised. Interestingly, we detected localized expression of ZO-1 in mutant LPM cells, indicating that their epithelial polarity is not completely abolished. In the absence of laminin in the basement membrane, the mutant LPM cells may be partially depolarized and undergo epithelial-to-mesenchymal transition, contributing to the failure of LPM migration.

In addition to the defects in epithelial cell morphology, the composition of the LPM is impaired in \textit{lamb1a}^{s804} mutants, as demonstrated by an increased number of Tg(hand2:EGFP)-expressing cells at the expense of the non-expressing cells. We also found that in some of \textit{lamb1a}^{s804} mutants, the LPM cells aberrantly protruded into the gut. During gut-looping morphogenesis, the LPM cells coordinate with one another and migrate as a coherent sheet. In \textit{lamb1a}^{s804} mutants, the lack of basement membrane at the LPM-gut boundary allows some LPM cells to escape from the LPM and protrude into the gut. Such cell behavior could disrupt the normal communication between the LPM cells and impede the collective migration of the LPM. An intriguing possibility is that the basement membrane may either sequester or mediate the migration cues secreted from the LPM or the gut endoderm. In \textit{lamb1a}^{s804} mutants, such regulation of migration cues was absent due to the deficiency in the basement membrane. Supporting this notion, a recent report on L-R asymmetry in \textit{Xenopus} showed that the ECM is a principal surface of Nodal and Lefty accumulation and that sulfated proteoglycans facilitate long distance movement of Nodal (Paraskevas et al., 2000; Schroeder et al., 2006); however, their role in organogenesis is not clear. Understanding their normal function in development and their link to signaling pathways is crucial for their future use in a therapeutic context.

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Author contributions
T.H.-H. and C.Y. designed and performed experiments, analyzed data and wrote the manuscript. D.E.S.K. performed experiments, analyzed data and edited the manuscript. M.E.B. and D.Y.R.S. supervised the work, including helping with experimental design, data, analysis and manuscript preparation.

References


