Notch signalling regulates left-right asymmetry through ciliary length control

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SUMMARY
The importance of cilia in embryonic development and adult physiology is emphasized by human ciliopathies. Despite its relevance, molecular signalling pathways behind cilia formation are poorly understood. We show that Notch signalling is a key pathway for cilia length control. In deltaD zebrafish mutants, cilia length is reduced in Kupffer’s vesicle and can be rescued by the ciliogenic factor foxj1a. Conversely, cilia length increases when Notch signalling is hyperactivated. Short cilia found in deltaD mutants reduce the fluid flow velocity inside Kupffer’s vesicle, thus compromising the asymmetric expression of the flow sensor charon. Notch signalling brings together ciliary length control and fluid flow hydrodynamics with transcriptional activation of laterality genes. In addition, our deltaD mutant analysis discloses an uncoupling between gut and heart laterality.

KEY WORDS: Notch, Cilia, Left-right, Zebrafish

INTRODUCTION
Internal body left-right (LR) asymmetry is a common trait among vertebrates. In contrast to the symmetric external body plan, the heart, digestive organs and parts of the brain display highly conserved asymmetric orientations that are essential for their correct functions. When the normal asymmetric distribution of the internal organs is compromised, human laterality disorders arise such as heterotaxy and situs inversus (Fliegauf et al., 2007; Gerdes et al., 2009).

The establishment of LR asymmetries in vertebrates occurs during early stages of embryonic development through a complex process involving epigenetic and genetic mechanisms. This culminates in the activation of a conserved Nodal signalling cascade that starts at the node and is then transferred to the left lateral plate mesoderm (LPM) (Hamada, 2008). Although the timing and mechanism responsible for breaking the initial embryonic symmetry might differ between species, it is clear that a directional fluid flow generated by motile cilia present in the node plays a fundamental role in LR determination, most likely by creating a LR information bias in the node (Levin and Palmer, 2007). Several lines of evidence support this idea, namely absent cilia in the mouse (Nonaka et al., 1998), shorter cilia in zebrafish mutants and morphants (Kramer-Zucker et al., 2005; Neugebauer et al., 2009), and immotile cilia in mouse, zebrafish and medaka (Essner et al., 2005; Hojo et al., 2007; Supp et al., 1999). All these cilia defects result in altered fluid flow in the node/Kupffer’s vesicle (KV) and ultimately in LR defects. This implies that proper cilia length regulation is crucial for the establishment of LR asymmetries.

Expression of Nodal and of its antagonist Cerl2 (Dand5 – Mouse Genome Informatics) in the mouse node starts symmetrically (Collignon et al., 1996; Marques et al., 2004). However, the mechanism that produces asymmetrical expression of Nodal and Cerl2 on the left and right sides of the node, and its potential link to the leftward nodal flow, is not fully understood. In zebrafish, there is no evidence for asymmetric expression of spaw and of its negative regulator charon in KV. Nevertheless, it has been suggested that an asymmetric Charon function inhibits the transfer of spaw to the right LPM (Hashimoto et al., 2004). To date, the relationship between the fluid flow inside KV and the asymmetric function of Charon in medaka and its homologue Coco in Xenopus have been studied (Hojo et al., 2007; Schweickert et al., 2010), but this relationship has not been explored in zebrafish.

Notch signalling has been implicated in LR patterning in vertebrates, largely through the activation of Nodal expression in the murine node (Krebs et al., 2003; Przemeck et al., 2003; Raya et al., 2003; Takeuchi et al., 2007). In zebrafish, Notch signalling seems to activate charon expression in KV (Gourronc et al., 2007). Apart from providing the first genetic evidence in zebrafish for the activation of charon expression in KV, we demonstrate that canonical Notch signalling, through the DeltaD ligand, is a key regulator of ciliary length at the KV. We show that reduction of Notch signalling produces shorter cilia, whereas hyperactivation of Notch produces longer cilia; thus, providing new insights into how cilia length is modulated. We find that the correct regulation of cilia length has a strong impact on the directionality and speed of fluid flow inside KV, and we were able to show that this biomechanical event correlates with asymmetric transcription of charon. Altogether, our results reveal a new and multistep role for the Notch signalling pathway that couples two fundamental processes in left-right determination: transcription of charon and ciliary length regulation that then culminates in breaking the symmetry of charon expression.

MATERIALS AND METHODS
Zebrafish line
AB wild type and mutant (notch1a/dei137, deltaC/beh4446, deltaD/aei233) embryos were staged according to Kimmel et al. (Kimmel et al., 1995).
Morpholino antisense oligo (MO) and mRNA microinjections
deltaD-MO as described previously (Holley et al., 2000) was injected from
0.5-1.0 mM into 512-cell stage wild-type embryos. The MO that blocks
translation of Su(H)1 and Su(H)2 was used as previously described
(Echeverri and Oates, 2007). Notch-intracellular and full-length deltaD
constructs (Takke and Campos-Ortega, 1999; Takke et al., 1999) were
injected at the one-cell stage at a concentration of 100 pg and 350 pg,
respectively. foxj1a and GFP full-length cDNAs were independently cloned
into pCS2+ and in vitro transcribed using mMESSAGE mMACHINE kit
(Ambion). The foxj1a and GFP mRNAs were injected at a concentration of
100 pg. Embryos were left to develop at 28°C until the desired stage and
then fixed in 4% PFA at 4°C overnight.

In situ hybridization
Whole-mount in situ hybridization was performed as described previously
(Thiriez and Thirse, 2008). Digoxigenin RNA probes were synthesized from
dNA templates of spaw (Hashimoto et al., 2004), pitx2 (Essner et al.,
2005), lefty2 (Bisgrove et al., 2005), foxa3 (Montero et al., 2008), ntl
(Amack and Yost, 2004), shh (Essner et al., 2005), charon (Hashimoto et
al., 2004), deltaD (Takke and Campos-Ortega, 1999), sox17 (Amack and
Yost, 2004) and foxj1a (Neugebauer et al., 2009; Yu et al., 2008).

Antibody staining and immunofluorescence
We followed the methods described previously (Neugebauer et al., 2009)
for immunostaining. In some cases, we also used TOPRO-3 (1:1000; Invitrogen) diluted in blocking solution.

Confocal microscopy
Flat-mounted embryos were examined with a Zeiss LSM 510 Meta
confocal microscope. Three-colour confocal z-series images were acquired
using sequential laser excitation, converted into a single plane projection
and analyzed using ImageJ software (LSM Reader). The length of all cilia
in each KV was measured using ImageJ Segmented Line Tool.

High-speed video microscopy
We used the confocal microscope Leica SP5 under the resonance mode,
which allows imaging at 8000 lines per second, to acquire images of ciliary
beating and debris movements inside KV of live embryos mounted in 1%
agar. High-speed movies of the debris were analyzed using ImageJ and the velocity was measured by tracing the debris frame
by frame. The orientation and speed of the debris were calculated using
Excel VBA, and vectors were drawn using the Gimp software.

Determination of KV cilia distribution
The total number of cilia and their distribution within KV were determined
from confocal z-stacks as previously described (Kreiling et al. 2007).

RESULTS
DeltaD is the Notch ligand that defines laterality in zebrafish
In order to study the impact of Notch signalling pathway on crucial
steps for LR patterning, we used several mutants and reagents. We
started by evaluating the asymmetric information in the LPM using
the left-identity molecular markers spaw and pitx2. The nodal-
related gene spaw is the earliest molecule to be asymmetrically
detected in the left LPM and is known to induce the expression of
the transcription factor pitx2 (Hamada et al., 2002; Long et al.,
2003).

We show that mutants in the Notch transmembrane receptor, des/notch1a−/−, lose their laterality identity by becoming largely
bilateral (Fig. 1A,B). Upon binding to its ligand, the intracellular
domain of Notch (NICD) is cleaved and translocates into the
nucleus. In the nucleus, NICD binds to Suppressor-of-Hairless
[Su(H)] thereby changing its repressor status to an activator (Fior
and Henrique, 2009). In zebrafish, two Su(H) gene paralogs (rhpja
and rhpjb – Zebrafish Information Network) can be knocked down
using a single antisense morpholino that blocks the translation of
both [Su(H)1+2-MO] (Echeverri and Oates, 2007). Consistent with
our results, Su(H)1+2 morphants also showed lack of laterality identity (Fig. 1A,B). To directly induce the Notch pathway, we
injected the mRNA encoding NICD and, similar to what has been
described previously (Raya et al., 2003), we again found a loss in
laterality (Fig. 1A,B). These results indicate that canonical Notch
signalling is required to define laterality in the LPM.

In order to assess which Notch ligand was the most relevant in LR
establishment, the expression of the left-sided markers was
analysed in the null mutants aei/deltaD−/− and bea/deltaD−/−. In
clear contrast to bea−/−, aei−/− mutants have a strong pitx2 laterality phenotype
that can be rescued by NICD (Fig. 1B). Thus, we identified DeltaD as the key Notch ligand for LR asymmetry in
zebrafish and we focus our analysis on this null mutant. We find
that the bilateral expression of pitx2 does not always follow the
expression of spaw (compare Fig. 1A with 1B). A lack of a precise
correlation between the expression of nodal and pitx2 in the LPM
was already described in the context of delta-like 1 (DLL1) mouse
mutants (Krebs et al., 2003; Przemeck et al., 2003; Raya et al.,
2003). Altogether, these results show that Notch signalling
regulates pitx2 expression in a Nodal/Spaw independent manner.

The axial midline is required to maintain LR asymmetry by
acting as a midline barrier (Chin et al., 2000; Hamada et al., 2002).
We checked whether the LR defects that arise upon Notch
signalling perturbation were due to lack of a midline. We found that
the expression pattern of both shh and ntl markers was normal
in all the experimental conditions (see Fig. S1A-N in the
supplementary material), with the exception of ntl downregulation
in NICD-injected embryos (see Fig. S1N in the supplementary
material), in agreement with what has been described previously
(Latimer and Appel, 2006). Therefore, LR defects arising from
reduced Notch signalling are not likely to be due to an absent
midline.

DeltaD maintains spaw expression in the
posterior left LPM
In aei−/− mutants, spaw expression is only mildly randomized (Fig.
1A), but the expression scored as being left-sided (Fig. 1A) shows
three distinct patterns that have been quantified in detail in Fig. 1C.
These patterns are: (1) reduced expression of spaw in the posterior
left LPM and possibly at the 16-somite stage in 77% of the homozygous embryos (Fig. 1C,D,D/H11032), and in a left anterior domain with bilateral posterior
expression in 19% of the cases (Fig. 1C,D,D/H11032, Supplementary
Fig. S1F,F,F). In contrast, in the aei−/− mutants, gut
laterality expression in the posterior left LPM was already described in the context of delta-like 1 (DLL1) mouse mutants (Krebs et al., 2003; Przemeck et al., 2003; Raya et al.,
2003). Altogether, these results show that Notch signalling
regulates pitx2 expression in a Nodal/Spaw independent manner.

DeltaD controls gut laterality
Next, we evaluated the impact that the abnormal expression of
spaw and pitx2 seen in aei−/− mutants has on heart and gut
positioning. We scored aei−/− mutants for heart asymmetry in vivo
and made use of the prospective heart marker lefty2 and of the gut
marker foxa3 in fixed embryos. We show that in aei−/− mutants, gut
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DEVELOPMENT

Fig. 1. Canonical Notch signalling through the Delta ligand controls left-right patterning. (A,B) Percentages of normal (Left, grey), reversed (Right, white), bilateral (black) and absent (stripes) expression of spaw (A) and pitx2 (B) in the lateral plate mesoderm when Notch signalling was challenged. Embryos were analysed between the 14- and 18-somite stages for spaw expression and between the 18- and 20-somite stages for pitx2. (C) Percentages of different domains of left-sided expression of spaw in the lateral plate mesoderm. (D,D’,F,F’,G) In aei−/− embryos, spaw may be expressed in an anterior domain only (D,D’), in an anterior with bilateral posterior domains (F,F’), and in anterior and posterior domains with competition on the right side (G). (E) A double spaw and lefty1 in situ hybridization showing the posterior gap (vertical line) of these two markers in an aei−/− embryo. (H-K) Expression of foxa3 in the gut in 50-53 hpf embryos (dorsal views). In wild-type embryos, the gut loops to the left (H). In aei−/− embryos, the gut may loop to the left (I), or to the right (J), or show no looping (K). (L) Percentages of normal (Left), reversed (Right) or no looping of the gut at 50-53 hpf. (M) Percentages of normal (Left), reversed (Right) or absent lefty2 in the lateral plate mesoderm at the 26-somite stage. (N) Percentages of normal (Left), reversed (Right) or no jogging (Centered) of the heart at 30-32 hpf.

looping is randomized (62.3% loops to the left, 18.8% failed loops and 18.8% loops to the right; n=85) (Fig. 1H-L). By contrast, heart jogging is not significantly affected when evaluated by lefty2 expression at 26-somite stage (99% on the left; n=112) or when judged by direct observation at 30 hpf (90.7% on the left; n=65) (Fig. 1M,N). These results indicate the uncoupling of laterality of an anterior organ, such as the heart, and of more posterior organs, such as the liver and pancreas. The heart is the first organ to be asymmetrically positioned in zebrafish, and it seems that its asymmetry may arise by a mechanism different from that governing other viscera, as suggested by others (Chin et al., 2000; Lin and Xu, 2009).

Our data indicate that the prospective heart domain is unaffected in the aei−/− mutants and thus provide an explanation for why heart laterality is not disturbed. Namely, the anterior left-sided LPM expression of spaw is unaffected and pitx2 does not reach the anterior-most domain when expressed on the right LPM. We propose that the premature absence of spaw expression in the posterior LPM, coupled with a bilateral pitx2 expression in this region could underlie the gut laterality phenotype observed in 40% of aei−/− mutants.

**DeltaD regulates cilia length in KV**

Increasing evidence shows that a properly formed node/KV with a directional strong fluid flow driven by the beating of motile cilia is essential for LR determination (Amack et al., 2007; Hamada, 2008). We therefore investigated whether the laterality problems observed in aei−/− mutants were due to KV morphogenesis and/or ciliogenesis defects.

To test KV integrity, we performed immunostaining with ZO-1, a tight junction marker and aPKC an apical epithelial marker (Neugebauer et al., 2009) and observed live embryos with high resolution DIC microscopy. The three approaches revealed that KV structure is normal both in aei−/− mutants and thus provide an explanation for why heart jogging is not significantly affected when evaluated by lefty2 expression at 26-somite stage (99% on the left; n=112) or when judged by direct observation at 30 hpf (90.7% on the left; n=65) (Fig. 1M,N). These results strongly suggest that Notch signalling, through DeltaD, controls ciliary length in KV.
DeltaD is expressed in the KV (Fig. 2O,P) and also in its precursors at the bud stage (10 hpf), in the somites, presomitic mesoderm and tail bud throughout segmentation stages (Hsiao et al., 2007). Therefore, we decided to test whether the short cilia phenotype observed in aei–/– mutants is caused by the lack of DeltaD function specifically in the KV precursors or in KV cells. We targeted the dorsal forerunner cells (DFC), which are the KV precursors (Essner et al., 2005), by co-injecting the lineage tracer fluorescein and a DeltaD-MO at the midblastula stage. In KV cells where fluorescein (and therefore DeltaD-MO) was successfully detected, we observed a reduction in cilia length comparable with the one quantified in aei–/– mutants [Fig. 2R; 2.25 ±0.6 μm (s.d.m.); n=29 cells; P=0.3] and different from the one quantified in KV cells that were not successfully targeted [Fig. 2Q; 3.6±0.6 μm (s.d.m.); n=21 cells; P<1.6×10⁻⁷]. This experiment indicates that DeltaD autonomously regulates cilia length at the level of the KV precursors or the KV cells and not in the somites, presomitic mesoderm and tail bud. In agreement, we found that in embryos where KV precursors were successfully targeted with DeltaD-MO the trend in spaw (84% on the left; 7% on the right; 8% bilateral; n=57) and pitx2 (33% on the left; 41% bilateral; 25% absent; n=12) expression is similar to the one observed in aei–/– mutants. Moreover, embryos injected with DeltaD-MO at the midblastula stage, showed gut looping defects (74% loops to the left, 18% does not loop and 8% loops to the right; n=123) and had no heart laterality problems similar to what we described for the aei–/– mutants.

DeltaD maintains proper cilia length through modulation of foxj1a

In order to investigate why the aei–/– mutant cilia are shorter, we analysed the expression pattern of foxj1a, a marker of KV motile cilia (Amack and Yost, 2004; Stubbs et al., 2008; Yu et al., 2008). At the bud stage, the DFC marker sox17 was detected in both aei–/–
and wild-type embryos (Fig. 3A,B), confirming presence of DFCs. By contrast, \( foxj1a \) expression was downregulated in \( aei^{-/-} \) mutants at the same stage compared with wild-type embryos (Fig. 3C,D). We further confirmed that \( foxj1a \) expression was downregulated at bud stages in 25% of the embryos that resulted from an \( aei \) heterozygous cross (\( n=157 \)). In addition, overexpression of both \( deltaD \) and \( NICD \) mRNA resulted in ectopic expression of \( foxj1a \) in the DFCs region at the bud stage (Fig. 3E,F). We thus propose that DeltaD controls KV cilia length via the regulation of \( foxj1a \).

Consistently, \( foxj1a \) morphants also show shorter or absent cilia in the KV (Stubbs et al., 2008; Yu et al., 2008).

In order to test whether Notch signalling is working via Foxj1a, we performed an epistatic test by co-injecting \( foxj1a \) mRNA together with GFP mRNA, as a lineage tracer, into one-cell stage \( aei^{-/-} \) mutant embryos. We found that cilia length was rescued in the KV cells that expressed GFP (Fig. 3H). Although in the \( KV \) cells that expressed GFP (Fig. 3H). This protein negatively regulates Nodal signalling by blocking the transfer of Spaw from the KV to the right side of the LPM in zebrafish and medaka (Hashimoto et al., 2004; Hojo et al., 2007). In addition, it has been suggested that \( charon \) might be a potential Notch signalling target in zebrafish (Gourronc et al., 2007). We confirmed this possibility by injecting \( deltaD \) or \( NICD \) mRNA into one-cell stage wild-type embryos, which resulted in clear ectopic expression of \( charon \) in the KV region (Fig. 4F,G). By contrast, the expression of \( charon \) in \( aei^{-/-} \) mutants was severely reduced in 35.4% of the embryos from eight- to 12-somite stage (Fig. 4O,P), whereas in \( beu^{-/-} \) mutants it was unaffected. Together, these data confirmed that \( charon \) is a target of the Notch signalling pathway in zebrafish.

A strong KV fluid flow promotes asymmetric \( charon \) expression

Charon is a Cerberus/Dan family secreted factor expressed specifically in the cells that line the posterior domain of the KV in a horseshoe shape (Fig. 4H). This protein negatively regulates Nodal signalling by blocking the transfer of Spaw from the KV to the right side of the LPM. We showed that charon expression becomes asymmetric after being symmetrically induced (Fig. 4H-K), as suggested in medaka and in Xenopus (Hojo et al., 2007; Schweickert et al., 2010). In contrast to wild-type embryos where there is a clear shift from symmetric to an asymmetric charon expression at the eight-somite stage (Fig. 4K), in the \( aei^{-/-} \) mutants, this shift does not occur (Fig. 4P). In fact, in \( aei^{-/-} \) mutant embryos where the expression of charon was not severely reduced, we could score it as being symmetric in 43.3% and asymmetric in only 21.3% from 8-12 somite stages (Fig. 4L-P). This suggests that the slower fluid flow in \( aei^{-/-} \) mutants characterized above is inefficient to promote the asymmetry switch of charon expression in KV cells.

Having shown that DeltaD regulates charon transcription (Fig. 4L-P), we could hypothesize that the inability to shift to the asymmetric charon expression observed in \( aei^{-/-} \) mutants may be caused by a transcriptional defect and not by the slow flow. To investigate the impact of KV fluid flow on charon expression in a transcription competent context, we repeated the experiment in left-right dynein1 (\( lrd1 \)) morphants. These morphants have a very reduced or absent flow (Essner et al., 2005) and show no problems in charon transcription (Gourronc et al., 2007). We injected \( lrd1-MO \) into wild-type embryos and scored charon expression in eight- to 12-somite stage embryos. We observed that in \( lrd1 \) morphants the asymmetric shift in charon expression does not occur (Fig. 4R), strengthening the idea that the fluid
flow inside the KV is crucial for inducing asymmetric charon expression. Our results, together with those obtained in other species, clearly show that charon transcription is sensitive to fluid flow. Therefore, we suggest that charon and its homologues may be candidates for flow-sensing genes in the KV/GRP/node environment.

**DISCUSSION**

The involvement of Notch signalling in the establishment of the left-right axis is evolutionary conserved among vertebrates. However, there are differences in the mode of action of this molecular signalling pathway between mammals and fish. In the mouse, the analysis of Notch pathway mutants reveals that nodal induction in the murine node is impaired (Krebs et al., 2003; Przemeck et al., 2003; Raya et al., 2003; Takeuchi et al., 2007), whereas the expression of the secreted Nodal antagonist cerl2 does not seem to be affected (Krebs et al., 2003; Przemeck et al., 2003; Raya et al., 2003; Takeuchi et al., 2007). By contrast, the expression of the secreted Nodal antagonist charon is severely affected in the KV (Gourranc et al., 2007). Using a genetic approach, we were able to confirm and extend these pharmacological results to conclude that the relevant Notch ligand for charon transcription in zebrafish is DeltaD. Therefore, Notch signalling in these two organisms targets the transcription of different genes: nodal in the mouse and charon in the zebrafish.

In the mouse node, the symmetric expression of nodal, induced by Notch signalling, and that of cerl2, induced by an unknown mechanism, later becomes asymmetric. nodal levels become higher on the left side, whereas cerl2 levels are higher on the right side of the node (Marques et al., 2004). In contrast to what happens in the mouse, there was no evidence for an asymmetric spaw or charon expression in the zebrafish KV. In our study, we discovered a sharp transition from a symmetric to an asymmetric expression of charon at the eight-somite stage in the KV, with higher levels of expression on the right side. This finding strengthens the evolutionary conserved feature of charon expression because this gene, and its homologues, are asymmetrically expressed on the right side in medaka KV (Hojo et al., 2007). Xenopus laevis gastrocoel roof plate (GRP) (Schweickert et al., 2010) and mouse node (Marques et al., 2004). The mechanism that triggers the left bias of nodal expression in the murine node and the right bias of charon and coco expression on the right side in fish KV and Xenopus GRP remains poorly understood.

There is increasing evidence in these organisms that the extracellular fluid flow generated by motile cilia present in the node/KV/GRP is essential to establish the LR asymmetries in the

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**Fig. 4. Short cilia reduce fluid flow inside Kupffer’s vesicle and compromise charon asymmetric expression.** (A–B’) Still images from wild-type siblings (A) and aei–/– mutant embryos (B) taken from Movies 1 and 2, respectively, in the supplementary material. Inserts A’ and B’ show vectors representing the fluid flow velocity and direction in each experimental situation. (C) Quantification of the average fluid flow velocity in wild type (n=5, 16.46±1.69 μm/s) and in aei–/– mutants (n=5, 6.74±2.41 μm/s). (D) Percentage of cilia in different regions of Kupffer’s vesicle (anterior, middle, posterior, dorsal and ventral) in wild-type (n=19) and aei–/– mutant embryos (n=14). (E) A 10-somite stage embryo showing nuclei stained with DAPI in blue and charon mRNA cytoplasmic localization in the epithelial cells that surround the lumen of Kupffer’s vesicle. (F,G) Expression pattern of charon in Kupffer’s vesicle of embryos overexpressing deltaD (F) and NICD (G). (H–J) charon expression at Kupffer’s vesicle is symmetric at the six-somite stage (H) and is asymmetric at the eight- and ten-somite stages (I,J). (K) Percentage of symmetric versus asymmetric charon expression in Kupffer’s vesicle in wild-type embryos from five- to 12-somite stages (n=18 for each stage). (L–O) Range of expression patterns of charon in Kupffer’s vesicle of aei–/– mutant embryos; charon expression may be symmetric (L), asymmetric with stronger expression on the right (M), asymmetric with stronger expression on the left (N) or reduced (O). (P) Percentages of the different charon expression patterns in Kupffer’s vesicle in aei–/– mutant embryos from the eight- to 12-somite stages (n=34 on average for each stage). (Q) charon expression pattern in Kupffer’s vesicle of one representative lrd1 morphant. (R) Percentage of symmetric versus asymmetric charon expression in Kupffer’s vesicle of lrd1 morphants from the eight- to 12-somite stages (n=27 on average for each stage).
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LPM. Therefore, this directional fluid flow is a good candidate to trigger the initial asymmetric expression at the node/KV/GRP (Hamada, 2008). We discovered that in aei<sup>−/−</sup> mutants, the switch in charon expression does not occur, possibly as a consequence of the slower fluid flow generated by the short motile cilia. In agreement with the possibility that the dynamics of the fluid flow promote a switch in gene expression in the node/KV/GRP are the phenotypes described for the axonemal dynein morphants in fish and Xenopus and the mouse mutants iv and inv. In medaka and as shown here in zebrafish embryos, the right bias in charon expression is no longer detected when fluid flow is abolished by the knockdown of lrdl (Hojo et al., 2007) (Fig. 4Q, R). In Xenopus, coco is no longer downregulated on the left side of the GRP in the absence of flow (Schweickert et al., 2010). In the iv mouse mutant, where cilia are immotile and no nodal flow is produced, the expression of nodal in the node becomes randomized (Okada et al., 1999). In addition, in the inv mouse mutant, where the flow is slow and turbulent, the expression of nodal is mostly symmetric in the node (Lowe et al., 1996; Okada et al., 1999; Oki et al., 2009).

Although no asymmetric charon expression had been detected before in zebrafish, asymmetric Charon function was suggested to be responsible for inhibiting the transference of Spaw from the KV domain to the right LPM (Hashimoto et al., 2004). However, it should be emphasized that in wild-type embryos, charon is also expressed on the left side of the KV and Spaw can still be transferred to the left LPM. This suggests that it is not the absence of charon transcripts that allow Spaw transfer to the left LPM but the relative levels of charon expression between left and right sides. Thus, we reason that Spaw is transferred to the left LPM because the levels of charon transcripts are lower on the left side of the KV in a wild-type embryo.

Applying the same reasoning to the aei<sup>−/−</sup> mutants, we propose that the severely reduced charon expression observed in 35.4% of the embryos (Fig. 4O–P) could correspond to: (1) no differences in the relative levels of charon [and therefore spaw is bilaterally transferred to the LPM as observed in 20% of aei<sup>−/−</sup> embryos (Fig. 1A)]; or (2) undetected small differences in charon expression that lead to a weak bilateral situation described as a spaw left anterior domain with bilateral posterior domains as observed in 15% of aei<sup>−/−</sup> embryos (Fig. 1A). In 43.3% of aei<sup>−/−</sup> mutants, charon expression is largely symmetric as visualized by in situ hybridization (Fig. 4L, P). In this situation, we cannot rule out that a biased difference between the left and the right side of charon transcripts in aei<sup>−/−</sup> mutants is still present as most embryos express left-sided spaw in the LPM (Fig. 1A). A clear asymmetric charon expression pattern on the right side of the KV (Fig. 4M) was observed in 18.3% of aei<sup>−/−</sup> embryos, resulting in left-sided spaw expression in the LPM (Fig. 1A). A few embryos (3%) expressed charon more strongly on the left side of the KV (Fig. 4N), matching the equivalent percentage of right-sided spaw in the LPM (Fig. 1A).

In summary, we showed that shorter cilia present in aei<sup>−/−</sup> mutants produce a slower fluid flow that leads to abnormal LR charon levels at the KV, which compromises the maintenance of spaw expression in the posterior domain of the left LPM. We show that in the same embryos where we observed a premature downregulation of spaw, we also see a reduced lefty1 expression in the posterior midline domain. We thus propose that this gap in lefty1 expression contributes, together with abnormal posterior ptf1a expression, to the gut laterality defects without affecting heart laterality. This phenotype is clinically relevant as there are reports on individuals with primary ciliary dyskinesia that develop situs inversus abdominis with no heart laterality defects (Fliegauf et al., 2007).

To our knowledge, Notch signalling is the first pathway that can both increase or decrease ciliary length, upon upregulation or downregulation, respectively. By contrast, FGF signalling has only been involved in shortening cilia length, (Hong and Dawid, 2009; Yamauchi et al., 2009). Our experiments identify foxj1a, the master motile ciliogenic transcription factor (Yu et al., 2008) as being downstream of DeltaID because it successfully rescued cilia length in aei<sup>−/−</sup> mutants in a cell-autonomous manner. These observations perhaps indicate that the motile cilia programme controlled by foxj1a, involves not only the transcription of motility genes such as dnah9 and wdr78 (Yu et al., 2008) but may also transcribe genes important for cilia growth yet to be discovered.

A systematic analysis of the experimental conditions where short and long cilia are generated by modulating the Notch activity will provide a framework to determine relevant ciliary length control genes. This will certainly bring new insights into how cilia/flagella length is regulated and may provide ways to increase ciliary length that could ultimately be used to treat ciliopathies often characterized by short cilia (Wemmer and Marshall, 2007).

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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