Left-Right Organizer Flow Dynamics: How Much Cilia Activity Reliably Yields Laterality?

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http://dx.doi.org/10.1016/j.devcel.2014.04.030

SUMMARY

Internal organs are asymmetrically positioned inside the body. Embryonic motile cilia play an essential role in this process by generating a directional fluid flow inside the vertebrate left-right organizer. Detailed characterization of how fluid flow dynamics modulates laterality is lacking. We used zebrafish genetics to experimentally generate a range of fluid flow dynamics. By following the development of each embryo, we show that fluid flow in the left-right organizer is asymmetric and provides a good predictor of organ laterality. This was tested in mosaic organisms composed of motile and immotile cilia generated by dnah7 knockdowns. In parallel, we used simulations of fluid dynamics to analyze our experimental data. These revealed that fluid flow generated by 30 or more cilia predicts 90% situs solitus, similar to experimental observations. We conclude that cilia number, dorsal anterior motile cilia clustering, and left flow are critical to situs solitus via robust asymmetric charon expression.

INTRODUCTION

During embryonic development, the vertebrate left-right (L-R) organizer operates transiently. This organ is lined by motile and immotile cilia in the mouse, while other vertebrate species such as zebrafish are less well characterized. In the mouse, the motile cilia generate a directional fluid flow, known as nodal flow, whereas immotile cilia are thought to be competent to sense it (McGrath et al., 2003). Such flow is necessary and sufficient to trigger a conserved molecular cascade of gene expression in the left lateral plate mesoderm that culminates with the correct positioning of the visceral organs (Nonaka et al., 2002). Therefore, when nodal flow malfunctions or is artificially reversed, L-R asymmetric localization of internal organs is affected (Fliegauf et al., 2007; Nonaka et al., 2002).

In order to explain how such an apparently delicate fluid flow can create asymmetry in the whole embryo, a Self-Enhancement and Laterality Inhibition system, known as a SELI system, was proposed to amplify nodal expression at the left lateral plate mesoderm (Nakamura et al., 2006). This work revealed that it is not necessary to have a strong and stable nodal signal to break initial symmetry. Supporting this idea, it was recently shown that fluid flow produced by only two motile cilia is capable of creating left identity in the mouse L-R organizer (Shinohara et al., 2012). Therefore, it is the combination of fluid flow at the L-R organizer and a SELI system that enables a conserved mechanism of robust asymmetry in the mouse embryo (Nakamura et al., 2006). Still, the initial steps of L-R establishment are not clear. The nature of the signal that is produced by the fluid flow is still debated. Two alternative and nonexclusive theories have been proposed: flow as hydrodynamic force that triggers mechanosensory pathways (McGrath et al., 2003) and, alternatively, flow as a vehicle for morphogens that dock receptors on the left side (Tanaka et al., 2005). Recently, in medaka Kupfer’s vesicle (KV), which is the fish homolog of the mouse node, where all cilia are clearly motile, it was proposed that cilia have a double role in generating the characteristic fluid flow and interpreting it through Pkd1l1-Pkd2 sensor-channel complexes expressed in all cilia (Kamura et al., 2011). Zebrafish have a more spherical KV (Okabe et al., 2008) and are harder to perform live imaging on; thus, it is not clear if all cilia are motile. Nevertheless, ever since the discovery that motile cilia can be chemosensory (Shah et al., 2009), both mechanosensory and chemosensory hypotheses are plausible in the fish L-R organizer, and they need not be mutually exclusive.

However, although a unidirectional fluid flow is evident in the mouse node, flow in the spheroid organizer of zebrafish is complex. As in the mouse node (Cartwright et al., 2004), cilia lining the zebrafish KV perform a rotational motion with tilted axis of rotation. In zebrafish, such tilt is toward the already-established posterior and dorsal axes, allowing the viscous interaction of fluid with the cell surface to produce directional flow; clustering of cilia on the anterior dorsal roof also plays an important role...
(Smith et al., 2012). However, the resulting fluid movement is a continuous counterclockwise flow (Supatto et al., 2008); and the role of this flow in the establishment of L-R asymmetry is less easy to interpret than in the mouse and therefore warrants further investigation.

If we separate the initial L-R events into a fluid dynamics/biophysical module A, followed by a gene expression module B, then it becomes clear that we do not at present understand how the first module dictates the second. To study how fluid flow dynamics can break normal L-R asymmetry, we started by analyzing the first module and used wild-type (WT) and genetic mutants to generate a range of different fluid flow patterns. We used both mutant and morphant zebrafish embryos and took advantage of the fact that different genotypes have different numbers of motile cilia, which creates a range of flow patterns. Our aim was to understand how robust the flow pattern is to disturbances of cilia number and length and what level of flow attenuation is sufficient to generate normal L-R asymmetry. In order to answer this complex question, we performed a detailed analysis of flow and kept the embryos alive to be able to evaluate their organ sites. L-R early markers shortly expressed after KV flow were also evaluated. Notably, our work differs from previous studies (Supatto et al., 2008; Wang et al., 2012) in the fact that our method was completely noninvasive.

RESULTS

Experimental Studies on KV Flow

We analyzed the KV flow by filming and tracking all native particles in the KV without injecting any beads (as in Movie S1 available online). We produced flow trajectory maps for each embryo and measured the distance of the tracks from the center of the KV. We classified the KV inner space as central and peripheral, as illustrated in the cartoon in Figure 1A. We looked for native particles, scanning the whole KV in each embryo; whenever we found one particle, we filmed it for 33 s (2,000 frames at 60 frames per second [fps]). We repeated this procedure for all the particles found in each KV. In the end, we had a number of movies per embryo that provided a representation of the most relevant KV flow in different focal planes (Figures 1B–1E are two-dimensional [2D] projections of flow). The orientation of the KV was always filmed from the dorsal side of the embryo with anterior toward the top and left toward the left. We found that most native particles were seen preferentially in the KV midplane. We selected deltaD^-/- (dld^-/-) homozygous mutants (Figures 1C and 1D) because they exhibit a range of cilia number and cilia lengths but lack major KV morphology defects (Lopes et al., 2010). We next explored the knockdown of a heavy chain axonemal dynein Dnah7 in order to reduce cilia motility even further (Figure 1E). This dynein is located in the inner dynein arms of ciliary axonemes and was found to be missing in the respiratory cilia of one patient with primary ciliary dyskinesia who had a deficiency of inner dynein arms and displayed abnormal cilia motility (Zhang et al., 2002). Dnah7 was never characterized before in any animal model; we report here a description of its knockdown phenotype and show that it is expressed in the KV (Figures 1F and 1G). The dnah7 knockdown in zebrafish rendered cilia immotile without affecting their number relative to control embryos (Figures 1H and 1I, respectively; Movie S1). The dnah7 morpholino (MO)-injected embryos allowed us to scan for how many motile cilia remained in each embryo confirming it by kymograph analysis (Figures 1J and 1K).

To explore the biological significance of fluid flow, we kept the embryos alive after imaging the movement of KV native particles in four genetic backgrounds: WT, dld^-/- mutants, embryos injected with dnah7 MO, and embryos injected with the respective five-mismatch control-MO. After 30 hr postfertilization (hpf), we evaluated heart jogging; and at 50 hpf, we assessed gut laterality by observing live larvae from the transgenic lines tg(sox17;GFP)^ab70,dld^-/- and tg(sox17;GFP)^ab70 that express green fluorescent protein (GFP) in the developing gut, allowing for visualization of the liver, pancreas, and intestinal bulb position (Figures 1L–1N).

The results showed that we were successful in generating a range of flow speeds (Figure 1O). The WT larvae, dld^-/- mutants with liver on the left, and dnah7 control-MO larvae presented faster fluid flow speeds (Figure 1O; p < 0.05). All these zebrafish larvae displayed a left position of the heart and liver (situs solitus). Next, dld^-/- mutant larvae displaying left hearts but central and right livers (heterotaxy) presented a clearly slower flow speed as embryos (Figure 1O; p < 0.05). We realized that dld^-/- embryos with the weakest flow speed developed livers in central positions. Embryo knockdown for dnah7 presented even lower speed flows, most of the time characterized by what looked like Brownian motion (Figures 1E and 1O). dnah7 morphants presented either no motile cilia or few motile cilia, resulting in larvae with randomized situs outcomes.

In search for factors that could explain how relatively similar fluid flow speeds give rise to both left and right liver laterality (Figure 1O), we reexamined the flow trajectories and their speed with more detail. First, we analyzed the tracking of all native particles and particularly focused on those trajectories that were more peripheral in each KV (represented by dark blue in Figure 1A). Peripheral can be understood as any point located close to the KV cells. The rationale was to focus on the trajectories that were near the KV cilia, as these were the ones potentially able to stimulate mechanosensory cilia. We concluded that peripheral trajectories were biased toward particular directions (as denoted by the rose plots in Figures 2A, 2D, 2G, and 2J). We next asked if these trajectories corresponded to regions of slower or faster flow speeds (it could be that very slow flow was keeping particles in these regions or that very fast flow was forcing particles to these locations). For this purpose, we measured the instantaneous flow speed in each half of the KV (Figures 2B, 2E, 2H, and 2K) and made heat maps of flow speed to achieve more local detail within each KV half (Figures 2C, 2F, 2I, and 2L). The results showed that the average flow speed was significantly faster on the anterior half of the KV in all situations (p < 0.05), except for the central liver cases (Figures 2H and 2I). In the situs solitus cases (Figures 2B and 2E), the next highest speeds observed were on the left halves of the KV, which is also visualized by the corresponding heat maps (Figures 2C and 2F). Regarding the mutant dld^-/- embryos with central livers, fluid flow was weak and homogenous all over the KV (Wilcoxon test, p > 0.05). In contrast, for heterotaxic embryos with right livers, the flow was still stronger anteriorly, but between left and right sides, there was no significant difference (Wilcoxon
Figure 1. Genetically Generating a Range of Fluid Flow Speeds

(A) Cartoon representing a Kupffer’s vesicle in 3D; light blue denotes central region, dark blue illustrates the peripheral region, and the arrow shows how we scanned the KV for native particles (represented in red) according to the dorsal-ventral axis.

(B–E) Representative KV flow map (B) for a WT embryo; (C and D) for two different dld<sup>−/−</sup> mutant embryos, and (E) for a MO-dnah7<sup>-</sup>-injected embryo displaying what seems to be Brownian motion (arrows). Left is to the left and anterior to the top throughout.

(F) Zebrfish embryo with 14 hpf showing expression of dnah7 mRNA in the KV.

(G) Expression of dnah7 mRNA in the KV amplified region.

(H and I) Immunofluorescence with anti-acetylated α-tubulin to visualize the presence of KV cilia in a dnah7-MO-injected embryo that showed (H) complete absence of cilia motility during high-speed videomicroscopy and (I) a WT control embryo.

(J) Kymograph showing an example of one motile cilium from a dnah7 knockdown embryo showing a consistent beat frequency over 2 s filmed with 500 fps.

(K) Kymograph of a cilium from a dnah7 knockdown embryo that shows no active motility over 2 s at 500 fps.

(L–N) Gut laterality at 50 hpf filmed from the ventral side after dissecting the yolk. z projection showing the liver, pancreas, and intestinal bulb of a (L) WT embryo tgfβsox17:GFP<sup>+</sup>mtn, (M) dld<sup>−/−</sup> mutant in the same genetic background and developmental stage showing a gut without lateralization, and (N) another dld<sup>−/−</sup> mutant showing reversed organ position. Li, liver; Pa, pancreas; IB, intestinal bulb.

O Box plot of flow speed in WT, dld<sup>−/−</sup> embryos, and MO-dnah7+injected embryos (p < 0.05, Wilcoxon test). Left, central, and right conditions refer to liver situs. The total number of tracks (n<sub>0</sub>) and embryos (n<sub>e</sub>) followed with native particles for each condition was as follows: for WT: n<sub>0</sub> = 847, n<sub>e</sub> = 7; for dld<sup>−/−</sup> mutants with left liver: n<sub>0</sub> = 7,877, n<sub>e</sub> = 21; for dld<sup>−/−</sup> mutants with centralized guts: n<sub>0</sub> = 1,458, n<sub>e</sub> = 2; for dld<sup>−/−</sup> mutants with right liver: n<sub>0</sub> = 858, n<sub>e</sub> = 3; for dnah7 knockdown embryos with left liver: n<sub>0</sub> = 1,105, n<sub>e</sub> = 3; for dnah7 knockdown embryos with central liver: n<sub>0</sub> = 505, n<sub>e</sub> = 1; for dnah7 knockdown embryos with right liver: n<sub>0</sub> = 1,860, n<sub>e</sub> = 4; and for dnah7 mismatch control-MO: n<sub>0</sub> = 1,700, n<sub>e</sub> = 5.

Notched box plots display a “notch” or narrowing of the box around the median. Box plot whiskers represent the minimum and maximum of all of the data. See also Movie S1.

Test, p > 0.05). However, the heat maps show a stronger local increase on the right side speed values (Figure 2L).

In summary, comparing the rose plots with the heat maps from Figure 2, we concluded that most particles were spotted in regions with higher flow speed. At this stage, we started suspecting that these higher local flow forces felt at the KV periphery, where cilia are located, were likely stimulating sensory cilia (still unknown if chemically, mechanically, or both) and starting the next signaling events that lead to the asymmetric gene expression and concomitant L-R patterning.

To test this idea further, we used the dnah7 knockdown embryos, in which we could precisely select the cases with only very few motile cilia located clearly on the left or on the right side of the KV. In these embryos, the absence of one to three motile cilia results in a very localized fluid flow close to the cilia and therefore could allow us to relate this position to subsequent organ situs (Figures 2M–2O). The results showed that embryos without any motile cilia had no significant flow and preceded situs randomization, presenting 50% situs solitus, 30% heterotaxia, and 20% situs inversus (n = 10 embryos; see heat map in Figure 2M). We then focused on six embryos that had only one or two cilia clearly placed on the left or right side of the KV. We found that five of six embryos had the liver and heart concordant and on the same side as the motile cilium for which we had screened. In these embryos, cilia were positioned on the right side, the presence of native particles allowed us to measure flow and identify a localized hotspot on the right side of the KV, which preceded situs inversus (Figures 2N and 2O). These experiments support the hypothesis that a clear local fluid stimulus generated by motile cilia can influence organ situs.

The existence of mutants where heterotaxia occurs without notochord defects, such as in dld<sup>−/−</sup> mutants (Lopes et al., 2010), lead us to envisage the existence of a specific mechanism that decouples heart from liver situs. Our results show that, in
were identified as bright GFP + objects with “cone-like” shapes, RNA (mRNA) by confocal microscopy (Movie S2). Motile cilia could originate enough left southpaw expression to then reach the heart precursor field and generate a normal heart on the left side (e.g., by the amplification of induced downstream signaling events). This flow is particularly visible in the corresponding heat maps (Figures 2I and 2L) where peaks of relatively fast flow (15–20 µm s⁻¹) are also present on the left side.

Thus, our results support a model in which asymmetric flow forces dictate organ laterality in each embryo. We observed that these asymmetric and repetitive swirls of flow occur for several hours. Our prediction is that the viscous stresses closer to the ciliated walls of the KV will repeatedly stimulate sensory cilia and likely trigger a mechanosensory or chemosensory response in these cells.

Simulation Flow Studies
In our experiments, we followed as many native particles as possible; however, it was not always possible to have a perfect coverage of midplane flow fields. Therefore, in order to better characterize these flow fields and to relate more clearly the effect of observed parameters to flow behavior, we conducted in silico “experiments,” by mathematically modeling KV fluid flow. For this purpose, we updated our last published model (Smith et al., 2012), which already incorporated variables such as cilia density in different regions of KV, and a mixture of cilia dorsally and posteriorly tilted was integrated into a three-dimensional (3D), time-dependent computational simulation resolved at the level of individual cilia length and beat pattern (Smith et al., 2012). The improved model additionally included the distribution of cilia length, measured in 3D after performing immunofluorescence with anti-acetylated α-tubulin for 27 WT embryos (639 cilia in total) and for 17 dld/c0 mutants (280 cilia in total; Figure S1A). Cilia length distribution was repeated across multiple statistical samples to take into account the heterogeneity observed experimentally. Next, by filming live embryos (Figures 3A and 3B) that were transgenic for sox17:gfp, thus having the KV cells expressing GFP (Figure 3D), we could measure the real volume of several KVs both for WT and dld/c0- mutants and use it in the simulations (n = 8 for each condition; Figure S1B). In addition, to produce better simulations, we realized that the number of immotile cilia and their localization were extremely important parameters that could be extracted by filming the whole KV in 3D using live embryos. Immotile cilia were scored by filming the entire KV of live embryos injected with arl13b-GFP messenger RNA (mRNA) by confocal microscopy (Movie S2). Motile cilia were identified as bright GFP* objects with “cone-like” shapes, created by their circular rotation (which is much faster than the normal acquisition rates achieved by confocal imaging), whereas immotile cilia appeared as sharp GFP* line segments (Figure 3D; Movie S2). In WT embryos, we found 22% of immotile cilia and tested whether their localization was biased in any axis and it was not (n = 7 embryos; 300 cilia; Fisher test, p > 0.05), whereas in dld/c0- mutants, we found that only 15% of immotile cilia were present (Figures 3K and 3L). Both immotile and motile cilia were also visible by independent experiments using bright field high-speed imaging (Movie S2), which demonstrates that cilia immotility was not being caused by overexpression artifacts. This result established that, unlike medaka (Kamura et al., 2011), zebrafish has both immotile and motile cilia in the KV.

Our next goal was to input into the model the distribution of cilia beat frequencies (CBFs) for each of the genotypes, a feature that had not previously been included in models of organizing structures.

CBF Study
We analyzed CBF by filming the KV of live embryos with high-speed videomicroscopy (Figures 3A–3C). To identify the underlying frequencies of each individual cilium, we performed kymographs and applied a fast Fourier transform (FFT). Our results showed that, in the WT zebrafish L-R organizer, there are two major populations of motile cilia characterized by different CBFs; one population that beats at a single frequency as the cilium shown in Figures 3E–3G, and a second population characterized by an additional lower frequency component, meaning that each cilium beats simultaneously at say 7 Hz and 30 Hz (Figures 3H–3J). These cilia were individually confirmed by kymograph analysis, and due to their irregular motility pattern, we named them “wobbling cilia.” In WT embryos, cilia that displayed one single CBF constitute 60% of the KV cilia population and show a unimodal distribution peaking at 33 Hz (Figure S1C). The second population of motile cilia characterized by the wobbling motility ranged from 7 to 40 Hz and accounted for 14% of the total population (Figure S1D). We performed a similar CBF analysis with dld/c0- mutants, which showed that the two motile cilia populations were maintained in similar percentages (Wilcoxon test, p > 0.05; Figure 3L; Figures S1E and S1F). These results revealed that, even in the dld/c0- mutants that tend to have shorter cilia lengths (see Figure S1A), the percentage of wobbling cilia was maintained in the KV. In order to further test if wobbling motion had no correlation with cilia length, we measured the length of several cilia that were beating at a lateral position and found no significant difference in length either in WT or dld/c0- mutants (Student’s t test, p > 0.05).

Moreover, with the current resolution, we observed that single- and double-frequency cilia appeared to be distributed randomly, meaning there was no statistical bias to find them on the left versus the right side or anteriorly versus posteriorly, in both WT and dld/c0- mutants (Fisher test, p > 0.05).

Next, in order to introduce the new CBF parameters into the simulations, we had to first create a model for cilia that beat with two or more frequencies: the wobbling cilia model (Figures 4A and 4B; Supplemental Experimental Procedures). These wobbling cilia, together with single frequency and immotile cilia, were then used to seed the KV meshing in a differentiated dorsal-ventral density (Figures 4C–4E; Supplemental Experimental Procedures for the modeling). The results of this type of modeling allow for clear visualization of the flow forces within the KV in any plane we desire (Figure 4F).

Comparing Simulations and Experiments
The 40 simulations (shown in Figure S2) resulting from eight different KV cilia numbers (based on the range of cilia numbers observed in the experimental studies in Figure S1G) multiplied by five different cilia seeding arrangements allowed us to quantify the number of cilia sufficient to reproduce in simulations the experimental behaviors observed in Figure 2.
Figure 2. Experimental Fluid Flow Measurements

(A, D, G, and J) Probability of finding and tracking native particles within the KV represented as rose diagrams. Data obtained from pooled embryos with 14 hpf of each experimental condition: in (A), for WT, number of tracks followed ($n_t$) = 675, number of embryos ($n_e$) = 7; in (D), for $dld^{-/}$ mutants with left liver, $n_t$ = 4,742. 

(legend continued on next page)
Comparing the simulated flow (Figures S2 A and S2B) with the real flow (Figure 2) in the anterior and posterior halves, we observed that 24 of 25 experiments had anterior flow much faster than posterior (binomial test, p < 0.001), showing that the anterior cluster of motile cilia was retained in the mutants, except for the very low flow embryos presenting a central liver (Figure 2 H). It is clear that anterior flow becomes stronger in the \( \text{dld}^{-/-} \) mutants as more cilia are added to the simulation (Figure S2 B). This fact shows that central liver in \( \text{dld}^{-/-} \) mutants must be an event triggered by either very few cilia equally placed in the KV four quadrants or simply by very short cilia producing almost no flow, such as the predicted flow simulation 22_4 (Figures 5 J–5L) that corresponds to the experimental flow heat map on Figure 2 I. Based on this reasoning, we matched the averaged flow speed data calculated for every KV half in WT (Figure 2 B) and in \( \text{dld}^{-/-} \) mutants (Figures 2 E, 2H, and 2K) to simulations that exemplified the observed flow behaviors (Figures 5 B, 5H, 5K, and 5N, respectively) to Figure 3.

Figure 3. Characterization of the WT and \( \text{dld}^{-/-} \) KV Cilia Populations

(A) Localization of the KV (squared region) in the body of the zebrafish embryo at 14 hpf.
(B) Snapshot image of a KV of a live embryo filmed from the dorsal side.
(C) Snapshot image of a KV beating cilium in a live embryo after image improved contrast for posterior kymograph analysis.
(D) KV of a live embryo at 14 hpf, showing the KV cells labeled with the transgene \( \text{sox17:GFP} \); cilia are also labeled with \( \text{arl13b-GFP} \) so that immotile cilia are bright and sharp (arrow), whereas motile cilia appear as cones with a blurred GFP label caused by the ciliary movement (arrowhead).
(E) Kymograph of a cilium presenting one fundamental frequency.
(F) Time series over 2 s for the same beating cilium.
(G) Power spectrum analysis using FFT for the same cilium, showing that it has only one fundamental frequency of 31.5 Hz.
(H) Kymograph of a cilium with two major frequencies, which we called a wobbling cilium.
(I) Time series for the same beating cilium (2 s).
(J) Power spectrum analysis using FFT for the wobbling cilium in (H), showing that it has two fundamental frequencies (highlighted in red): one at 7 Hz and another at 30 Hz (the lower frequency peak is an FFT artifact equal to the background noise).
(K and L) Quantification of the different types of KV cilia according to motility and to CBF in (K) WT embryos; \( n_e = 16 \), \( n_c = 80 \), and in (L) \( \text{dld}^{-/-} \) mutants; \( n_e = 26 \), \( n_c = 77 \). The class “other” includes all cilia that had a random beating pattern with no specific frequency peak after FFT analysis.

KV Kupffer’s vesicle; CBF cilia beat frequency; \( n_e \) number of embryos, \( n_c \) number of cilia.

See also Figure S1 and Movie S2.
determine what features of cilia number and placement may be underlying the observed flow.

Accordingly, simulation 60_1 (Figures 5A–5C) was matched to WT with situs solitus (Figures 2A–2C), simulation 29_3 (Figures 5G–5I) was matched to dld−/− mutants with situs solitus (Figures 2D–2F), simulation 22_4 (Figures 5J–5L) was matched to dld−/− mutants with central liver situs (Figures 2G–2L), and simulation 22_2 (Figures 5M–5O) was matched to dld−/− mutants with right liver situs (Figures 2J–2L). By examining the placement of motile cilia in these simulations (Figures 5A, 5G, 5J, and 5M), we see anterior clustering for left liver situs, homogenous cilia placement in central situs, and slight right-sided clustering for right situs. These results show that the probability of producing the observed behaviors is correlated positively with cilia clustering. However, even in WT situations, if the number of cilia is below 30 (see Figure S2A), we could predict, for example, by simulation 25_1 (Figures 5D–5F) that an abnormal flow could arise if there is disruption of the dorsal anterior cluster of motile cilia. Such flow would likely generate embryos with abnormal gene expression patterns for early L-R markers, such as charon and spaw, and subsequently lead to larvae with situs inversus or heterotaxia. However, the only way to tackle the effect of lower flow conditions during earlier developmental stages would be to perform a similar experimental study, only this time aiming to evaluate the early L-R markers in individual embryos.

To further elucidate on how local flow can influence organ situs and because these are distant events, we investigated the relationship between flow and the first gene to become asymmetric and, indeed, much stronger on the right side where flow was weaker (Wilcoxon test, p < 0.05; Figures 6 A–6C; Figures S4A–S4O). Interestingly, when the flow was still weak but significantly higher on the right or left sides of the KV, it was possible to visualize a slight weakening of charon expression locally (Figure 6 J–6L; Figures S4 P–S4R, respectively). In summary, charon expression inversely correlates with higher local KV flow in the same embryos.

We next analyzed the expression of southpaw (spaw), the first gene to be asymmetrically expressed in the lateral plate mesoderm (LPM) (Long et al., 2003). In this case, the prediction was that LPM spaw expression should be on the same side as the stronger KV flow, because Charon is a negative regulator of Nodal (and Nodal is autoregulatory) within the LPM (Hashimoto et al., 2004). Our results confirmed that spaw was expressed on the left LPM when flow was significantly stronger on the left side and anteriorly (Figures 6M–6O; Figures S3 G–S3O). On the other hand, when flow was not significantly different between the left and right sides of the KV, then spaw was absent (Figures 6P–6R). This is predictable, as we learned from our results on charon expression that a homogenous flow generates symmetric charon. In turn, strong symmetric charon expression predicts absent spaw from the LPM due to the inhibitory role of Charon on Spaw.

**DISCUSSION**

We report a link between specific local flow dynamics and internal organ laterality, which implies that some sort of sensory mechanism must be in place in the KV. We know that pdk2, a gene encoding a Ca2+ channel involved in mechanosensory responses in the kidney (Nauli et al., 2003), is expressed in the
zebrafish KV cells and that cup/pkd2−/− mutants have L-R defects (Bisgrove et al., 2005; Schottenfeld et al., 2007). These observations, together with the report that Pkd1L1–Pkd2 complexes are present in all motile cilia of medaka fish (Kamura et al., 2011), suggest that all KV cilia, regardless of their motile capabilities, may be competent to respond to hydrodynamic forces. On the other hand, our results show that there is a significant number of immotile cilia in the zebrafish KV, which may disclose a preferential sensory role for these immotile cilia, as described for the crown cell cilia of the mouse node (Yoshiba et al., 2012).

Additionally, we showed the existence of two motile cilia populations in the KV, the population that was made by “wobbling cilia” exhibits two beat frequency peaks. The origin of this smaller population of motile cilia is the object of our future research. Nevertheless, its impact in the flow dynamics simulations was accounted for and becomes relevant as cilia number increases.

Our simulations were based purely on observational data and the physics of microscale flow, with no adjustable parameters or fitting used. The real system is highly complex, and simulation cannot, at present, capture all of the details of the interior geometry, cilia structure, and dynein regulation; however, our results show both qualitative and quantitative correspondence between simulated (Figure 5) and measured (Figure 2) particle velocity distributions, suggesting that the most important features of viscous fluid dynamics, KV geometry, and cilia activity have been captured by the model. The formulation of the model can be viewed as a “blueprint” for the KV architecture. Based on these findings, we propose a simplified statistical model relating cilia number to situs. For dld−/− mutants, 8 of 10 simulations with cilia numbering 29 or more produced flow profiles exemplifying situs solitus (Figure S2B). Taking an approximate threshold of 30 cilia, above which situs solitus is produced 100% of the time and below which situs solitus is produced 100% of the time, we can use experimental data on the statistical distribution of cilium numbers in mutant and WT (Figure S1G) to produce a rough estimate of the proportions of situs solitus embryos. For dld−/−, approximately 80% of embryos have fewer than 30 cilia; the percentage of situs solitus via our model is then (80% × 50%) + (20% × 100%) = 60%. For WT, approximately 20% of embryos have fewer than 30 cilia; our model predicts (20% × 50%) + (80% × 100%) = 90%. These percentages closely match the embryo fates observed before experimentally (Lopes et al., 2010). While this model is idealized and approximate, the close match to experimental observations suggest that, in WT, cilia are produced in sufficient numbers to ensure the flow dominates random effects; we remark that our suggested threshold of 30 cilia corresponds to the modal value observed in WT embryos (Figure S1G).

Dorsal anterior clustering of monociliated KV cells has been described by several authors (Kreiling et al., 2007; Okabe et al., 2008; Supatto et al., 2008). More recently, it was showed that this clustering occurs during development due to a KV cell shape remodeling involving the Rock2b–Myosin II pathway (Wang et al., 2012). Here, we confirm through flow simulations that, even in WT embryos, when this dorsal anterior cluster of ciliated cells is disturbed (Figure S2A), the flow forces produced anteriorly become lower and different patterns of “abnormal” flow may arise (Figure 5F). Experimentally, we have subsequently confirmed and quantified that abnormal symmetric charon expression arises when anterior and left-sided flow forces are not significantly higher than posterior and right-sided ones (Figures 6D–6I; Figures S4A–S4O).

We have thus identified one important factor that may discern left- from right-positioned organs in embryos producing flow: flow field asymmetry occurs prior to, and is closely related to, gene expression and subsequent organ position.

In addition, our results on KV flow dynamics provided a plausible explanation for a complex phenotype such as heterotaxia observed in dld−/− mutants. An important message that arose from the data on heterotaxic embryos was that a normal left heart correlated with less left flow than a normal left liver. We thus interpreted that, to achieve a correct gut looping, a maintained fast fluid flow may be required during the KV lifetime. As WT zebrafish embryos display both heart and liver on the left side of the midline, we propose that there might be a common signal for leftness, starting at the KV left side, by repeated fluid flow stimulation over approximately the 4 hr of functional KV lifetime (from 12 to 16 hpf). Our hypothesis is supported by others’ observations (Ober et al., 2003) and by the time course experiment (Supplemental Experimental Procedures) that shows both asymmetric heart field position and gut tube looping start on the left side of the body early in development. Markers, such as lefty2, start to label the heart field on the left side of the embryo prior to 18 hpf (Figures S5A and S5C) and foxA3 labels the first gut looping to the left at around 24 hpf (Figures S5B and S5D). Thus, heart and gut declare left sidedness within a 6 hr time window that might reflect the duration of the KV lifetime with a developmental delay due to intermediary signaling events. It will be interesting to test this hypothesis in the future by disrupting KV flow in a time-dependent manner during the KV lifetime.

To summarize our ideas, we designed a diagram (Figure 7) that aims to represent what happens over time (vertically) in the different flow scenarios (horizontally), where red cells represent the most stimulated cells by the flow and blue cells represent charon mRNA expression, which is inversely related to high local flow. In this schematic drawing, we also included the predicted spaw expression in the LPM, which occurs later in development, and finally, we show the position of the heart and liver in each flow pattern scenario. We propose that situs solitus occurs in zebrafish when anterior dorsal KV cells are stimulated together with a left-sided stimulation (Figure 7A). In contrast, situs inversus requires a shift to a right-sided stimulation (Figure 7B). Heterotaxias arise when there are unequal stimulations on the left and right sides (Figure 7C); by having different strengths, these will likely reflect asymmetric charon expression patterns (Figure 7H). These different levels of charon expression will later be reflected in the different levels of spaw expression on the left and right sides of the LPM (Figure 7M), due to the inhibitory role of Charon on Spaw protein. Finally, the different anterior levels of spaw expression in the LPM are known to affect heart positioning (Schottenfeld et al., 2007), whereas the posterior levels of spaw in the LPM were related to gut misplacement (Lopes et al., 2010). Finally, we predict that more heterogeneous situations may occur when no biased flow signals are present (Figures 7D and 7E). This situation may happen if KV cells are equally stimulated on the left and right sides (Figure 7D) or not stimulated at all (Figure 7E), giving rise to a lack of laterality. In these two scenarios,
we have indications from this work that charon expression will be symmetric (Figures 7I and 7J; see also Figures 6F and 6I). Thus, depending on how strong the inhibitory role of Charon will be on Spaw, we predict that spaw expression may be absent (Figures 6R and 7N) or weak or bilateral (Figure 7O).

For future work, we aim to explore which gene targets are downstream of flow by comparing situations of lack of flow with situations of blocking the mechanosensory pathway.

**EXPERIMENTAL PROCEDURES**

**Fish Stocks**
Zebrafish were maintained at 25°C or 28°C and staged as described elsewhere (Kimmel et al., 1995). The following zebrafish lines were used for this work: AB, Tg(sox17:GFP)z370 (Chung and Stainier, 2008) and Tg(sox17:GFP)z370; dld−/− (in this study). Procedures with zebrafish were approved by the Portuguese DGV (Direção Geral de Veterinária).

**Fluid Flow Velocity Measurements**
Embryos were collected at 2 to 3 p.m. at the one- to two-cell stage and incubated at 25°C until the next morning; they were then mounted in a 2% agarose mold in glass-bottom Petri dishes and immobilized with 1.5% low-melting agarose. Mounted embryos between 13–14 hpf were set under the 100x/1.30 NA oil immersion objective lens on a Nikon Eclipse Ti-U inverted microscope at room temperature (25°C). All images were taken with the dorsal roof of the KV facing the objective lens. Bright field images were recorded with a FASTCAM MC2 camera (Photon Europe, Limited) controlled with PFV (Photon FASTCAM Viewer) software. A region of interest was drawn around the full KV filmed at 60 fps. We used KV native particles as before (Lopes et al., 2010) to measure fluid flow speed. Our success rate in finding naturally occurring particles was one in three KVs. We have successfully imaged 26 dld−/− embryos, 7 WT embryos, 16 dnah7-MO injected embryos, and 5 mismatch dnah7 control-MO-injected embryos. For the early laterality markers charon and southpaw, we have measured the flow in 17 additional embryos. We developed a new macro for ImageJ (available on request) to systematically and reproducibly measure particle speed. For this purpose, we measured several focal planes by scanning the full KV from dorsal to ventral (Figure 1A). The trajectories were measured as 2D projections. We scanned the full KV and measured long tracks of flow in the focal midplane, which supported the hypothesis that the mean out-of-plane velocity must be small.

Flow measurements were performed by temporal projections of movies, in which we used the standard deviation of the pixel intensity over time, which corresponds to the movement of objects, e.g., debris or detached cilia, inside the KV. Each color in Figures 1B to 1D represents the total displacement of a given particle during 1 s (filmed at 60 fps). MTrack J from ImageJ (National Institutes of Health [NIH]) was used to manually track particle trajectories to then calculate velocities. The positions of all tracked particles were normalized considering their relative distance from the center to the wall of the KV to allow comparisons between different embryos aligned through their KV center (Figure 2).

**Heart and Gut Laterality**
At 30 hpf, the heart is readily visible beating under the left eye, so we evaluated heart jogging using a stereoscopic zoom microscope (SMZ745, Nikon Corporation) to observe the embryos from the ventral side. At 50 hpf, we assessed gut laterality by observing live larvae from the dorsal side on a SteREO Discovery V8 stereomicroscope (Carl Zeiss Microimaging GmbH) using a GFP filter. Transgenic lines Tg(sox17:GFP)z370; dld−/− and Tg(sox17:GFP)z370 that have GFP in the developing gut were used throughout the experimental procedure, allowing for visualization of the liver, pancreas, and intestinal bulb position. In this way, we could score organ situs in the same embryos that we measured the KV flow speed. These included 7 WT, 26 dld−/− mutant embryos, 16 dnah7 knockdown embryos, and 5 dnah7 mismatch control-MO-injected embryos.

**CBF Spectral Analysis**
Embryos were mounted and filmed as explained earlier for fluid flow measurements. A region of interest was drawn around the focused cilium and recorded at 500 fps for 2 s (binning = 1 x 1) (Figure 3C). CBFs were obtained by image filtering in ImageJ (NIH) followed by Fourier analysis in R software (R Development Core Team, 2011). (1) Immotile objects were removed by creating an average time projection of individual CBF recording (1,000 frames) that was substracted from each frame of the movie. As the result of such processing, the motile objects (such as the cilium) appear as bright objects in a dark background (as in Figure 3C). (2) All frames were cropped to a smaller region (~10 x 10 μm) enclosing the cilium of interest. (3) CBF image stacks (x, y, t) were then represented as orthogonal views (x, t) and (y, t) (the kymographs of the cilium). (4) A straight line was drawn over the time domain of the entire (x, t) kymograph, and its profile was exported to R software to perform Fourier analysis with the FFT algorithm (as in Figures 3F and 3G) (R Development Core Team, 2011). The criteria to classify wobbling cilia were for each cilium to have a higher power main frequency and a lower power second frequency and not to have the same lower frequency as background noise measured nearby.

We analyzed 60 cilia with a single beat frequency and 20 wobbling cilia in 16 WT embryos (Figures S1C and S1D). We also analyzed 60 and 17 cilia with single CBF and wobbling motion, respectively, in 26 dld−/− embryos (Figures S1E and S1F).

**Statistical Analyses**
Rayleigh tests of circular distribution of the relative particle position with respect to the center of the KV were performed for assessing the significance of finding particles moving preferentially in the anterior, posterior, left, or right regions. Potential differences in KV fluid flow speed between WT, dld−/− mutant embryos, dnah-MO-injected embryos, and control mismatch-MO-injected embryos were assessed using a Wilcoxon nonparametric test. To assess a relationship between CBF and cilia length, we measured a number of cilia for WT embryos (single CBF = 60, wobbling cilia = 11 in embryos) and dld−/− mutants (single CBF = 103, wobbling cilia = 10 in 15 embryos) and performed Student’s t tests. All statistical tests were performed using R software. The significance level was set at 95%.

**Immunofluorescence and In Situ Hybridization on Whole-Mount Embryos**
Whole-mount immunostaining and in situ hybridization were performed as before (Lopes et al., 2010). Antibodies used were anti-acetylated α-tubulin (1:400; Sigma) and Alexa Fluor 488 (Invitrogen; 1:500). A fragment of dnah7 zebrafish gene (ENSDART00000206924; Zv9) was amplified by PCR. dnah7 anti-sense RNA probe was transcribed using SP6 RNA polymerase and Sac II. dnah7 and charon in situ hybridizations at 14 hpf, southpaw in situ at the 15– to 16-somite stage, and time course in situ hybridization with lefty2 and foxa3 were performed as described elsewhere (Thiase and Thissel, 2010). charon and southpaw in situ hybridization experiments were stopped at the same time to allow comparisons for the same genes.

Figure 5. In Silico Experiments
From individual cilium kinematics to cluster distribution and flow fields. These five simulations, among a total of 40, were the ones that better matched the experimental data of Figure 2.

(A, D, G, J, and M) Seeding of different types of cilia in the KV mesh. Filled dots represent cilia on the dorsal side, crosses label cilia on the ventral side of the KV, red represents motile cilia with single CBFs, green represents motile cilia with wobbling behaviors, and blue represents immotile cilia.

(B, E, H, K, and N) Simulated flow speed in the different halves of the KV is plotted exactly in the same way as in Figure 2. Notched boxplots display a “notch” or narrowing of the box around the median. Boxplot whiskers represent the minimum and maximum of all of the data. Means are represented as small circles.

(C, F, I, L, and O) Flow forces produced by simulated cilia are represented by vectors. Scale is 2 μm/s−1 for WT and 1 μm/s−1 for dld−/−.

See also Figure S2.
mRNA and MO Microinjections

Human arl13b-GFP mRNA (gift from Dr. Helena Soares) was injected into Tg(sox17:GFP)s870 at the one-cell stage at a concentration of 400 pg. Full-length cDNA was transcribed in vitro using the mMESSAGE mMACHINE kit (Ambion). Embryos were left to develop at 25°C until 14 hpf and then were mounted for confocal fluorescent microscopy live imaging at 25°C. To evaluate motile versus immotile cilia number, we scanned the whole KV with z sections of 0.55 μm, with an acquisition rate of less than 1 fps. Movies were subsequently analyzed in ImageJ, and the number of cilia, of each cilia type, was counted based on the distinct projection shapes made by the motile cilia compared to the immotile cilia. We used a translation blocking MO for dnah7 (AGGGTCATCTTCACGGTGCATAATA) and a five-mismatch MO (AGGCTGATCTTGACGCTGGATAATA) as a negative control (Gene Tools LLC). dnah7-MO was injected at the one-cell stage at different concentrations to titrate the number of motile cilia in the KV. We used 3 ng per embryo for experiments shown in Figures 2 M and 2O and 2 ng per embryo for those in Figures 6 and S4.

In Silico Experiments: Mathematical Modeling

To interpret the effect of cillum number and distribution on the flow generated within KV, we used a modified version of the computational model of Smith et al. (2012), described in detail in the Supplemental Experimental Procedures, section 1. These modifications allow for arbitrary placement of cilia, multiple beat frequencies, and variable lengths. Cilia were modeled as whirling rods, with a diameter of 0.3 μm, programmed to perform a conical rotational motion with dorsal roof and ventral floor cilia tilted posteriorly and “equatorial” cilia tilted dorsally.

The average dimensions of KV were ascertained from confocal microscopy imaging of WT and dld−/− mutants; for each simulation, KV was modeled as a scalene ellipsoid with axes 59 μm (LR), 52 μm (anterior-posterior [AP]), and 49 μm (dorsal-ventral [DV]) for WT and 57 μm (LR), 56 μm (AP), and 43 μm (DV) for dld−/−. Following Kreiling et al. (2007), KV was divided into four regions: the dorsal-anterior (D-A), dorsal-central (D-C), dorsal-posterior (D-P), and ventral (V) sectors. Cilia were assigned random positions in each sector,
so that approximately 38%, 25%, 17%, and 20% of cilia were located in the D- A, D-C, D-P, and V sectors, respectively, matching both our own data and that of Kreiling et al. (2007) (Fisher test, p > 0.05).

Each simulation was carried out by sampling cilia lengths and frequencies from statistical distributions parameterized by experimental data (Figure S1), using the probability distributions described in the Supplemental Experimental Procedures. Wobbling cilia with two frequency peaks were incorporated as described in the Supplemental Experimental Procedures. For WT, approximately 14.4% of the cilia were randomly designated wobbling, and 22% immotile (approximated by the nearest whole number), with 11.3% wobbling and 15% immotile for \( \text{dld}^{-/} \).

The computational meshes for each embryo were generated in the same manner as in Smith et al. (2012) with the addition of these randomly sampled parameters. The flow generated within each model embryo was then calculated using the boundary element regularized stokeslet method (Supplemental Experimental Procedures).

For \( \text{dld}^{-/} \), five independent simulations with different random parameters were performed for embryos with 15, 22, 29, and 36 cilia. For WT, five simulations were performed for embryos with 25, 37, 45, and 60 cilia. These values were chosen to span the range of cilia numbers found experimentally (Figure S1D). Analysis of simulations focused on the coronal plane time-averaged velocity field, so the comparisons between experiments and simulations were between 2D projections for both experiment and simulation. We appreciate that motion of particles in \( z \) will make a contribution to the velocity field. However, most experimental observations and all mathematical calculations were performed in the midplane, where \( z \) components are relatively small. Elementary mathematical analysis shows that, if the \( z \) component is small, it changes the velocity magnitude by a “very small” amount.

Computations were coded in Intel Fortran 90 with the NAG Fortran Library Restarted GMRES (Numerical Algorithms Group); mesh generation and plotting were carried out using Matlab (Mathworks).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.04.030.

AUTHOR CONTRIBUTIONS

P.S., R.R.F., A.G., and S.S.L. performed experiments and analyzed the data; P.P., B.T., and J.A. performed experiments; A.A.S. and T.M.-J. performed in silico experiments and analyzed the data; S.S.L., D.J.S., T.M.-J., and A.G. designed experiments; S.S.L. and D.J.S. wrote the manuscript; and S.S.L., D.J.S., T.M.-J., and A.G. revised the manuscript.

ACKNOWLEDGMENTS

S.S.L. was supported by Fundação para a Ciência e a Tecnologia (FCT) SFRH/BPD/34822/2007 and FCT Investigator contract. A.G. benefited from an FCT fellowship SFRH/BPD/82420/2011 and EMBO Installation Grant to Monica Bettencourt Dias. R.R.F., P.P., and laboratory work were supported by FCT grants PTDC/SAU-OBD/103981/2008 and FCT-ANR/BEX-BID/0153/2012. A.A.S. and T.M.-J. acknowledge Engineering and Physical Sciences Research Council Doctoral Training studentships, D.J.S. acknowledges a Birmingham Science City Fellowship, and D.J.S. and T.M.-J. acknowledge support from EPSRC First Grant (EP/K007637/1). Computations were carried out with the University of Birmingham BlueBEAR High Performance Computing service, which was purchased through HEFCE UK SRIF-3 funds. We thank H. Soares and L. Saüde for reagents; M.G. Ferreira, L. Sau ´ de, M. Bettencourt-Dias, J. Cartwright, and our laboratory for critically reading the manuscript; A. Sampaio for diagram design; and J. Carneiro, O. Piro, I. Tuval, and J. Vermot for helpful discussion.
REFERENCES


