Defective Nodal and Cerl2 expression in the Arl13b<sup>hnn</sup> mutant node underlie its heterotaxia

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Abstract

Specification of the left–right axis during embryonic development is critical for the morphogenesis of asymmetric organs such as the heart, lungs, and stomach. The first known left–right asymmetry to occur in the mouse embryo is a leftward fluid flow in the node that is created by rotating cilia on the node surface. This flow is followed by asymmetric expression of Nodal and its inhibitor Cerl2 in the node. Defects in cilia and/ or fluid flow in the node lead to defective Nodal and Cerl2 expression and therefore incorrect visceral organ situs. Here we show the cilia protein Arl13b is required for left right axis specification as its absence results in heterotaxia. We find the defect originates in the node where Cerl2 is not downregulated and asymmetric expression of Nodal is not maintained resulting in symmetric expression of both genes. Subsequently, Nodal expression is delayed in the lateral plate mesoderm (LPM). Symmetric Nodal and Cerl2 in the node could result from defects in either the generation and/ or the detection of Nodal flow, which would account for the subsequent defects in the LPM and organ positioning.

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Introduction

The vertebrate body plan is established during embryonic development through the specification of three axes: the anterior-posterior, dorsal-ventral, and left–right (LR). In vertebrates, the LR axis is the final axis to be specified just before visceral development, and its specification is necessary for asymmetric development of organs, such as the heart, lungs, and stomach (Peeters and Devriendt, 2006; Shiratori and Hamada, 2006). Work over the last 15 years has provided tremendous advances in our understanding of how the LR axis is established, but many steps along the way, from the initial breaking of bilateral symmetry to the development of asymmetric organs, remain elusive (Bisgrove et al., 2003; Raya and Izpisua Belmonte, 2008; Speder et al., 2007; Tabin, 2005; Vandenberg and Levin, 2009, 2010).

In mouse, the first known event required for establishment of the LR axis is the leftward flow of extraembryonic fluid in the node, which is created by the rotation of motile cilia on the node surface (Nonaka et al., 2002; Nonaka et al., 1998; Okada et al., 1999). At the molecular level, two proteins are initially expressed symmetrically on the left and right side of the node: Nodal and its inhibitor cerberus-like 2 (Cerl2) (Collignon et al., 1996; Lowe et al., 1996). Once fluid flow is initiated, Nodal shows increased expression at the left node periphery while Cerl2 is downregulated on the left side of the node (Marques et al., 2004). As Nodal feeds back to induce its own expression, Cerl2 repression on the left side of the node would allow for the observed left-sided increase in Nodal expression (Kawasumi et al., 2011; Norris et al., 2002; Schweickert et al., 2010). While both Nodal and Cerl2 are likely regulated in response to flow, the exact mechanisms of flow detection and the cellular response to flow have been vigorously debated. One hypothesis posits that the flow physically enriches the left side of the node with a morphogen such as Sonic Hedgehog (Shh) (Tanaka et al., 2005). Alternatively, another hypothesis (the two cilia model) proposes that the flow generated by motile cilia at the center of the node is detected via mechanosensory cilia at the node periphery (McGrath et al., 2003). Although the debate is not settled, two recent observations are consistent with the two cilia model: first, very little flow is needed to break asymmetry within the node (Shinohara et al., 2012); and second, the putative sensors in the mechanosensory cilia at the node periphery were identified as Pkd1l1 and Pkd2 (Field et al., 2011; McGrath et al., 2003).

From the node, asymmetry is established throughout the embryo. Nodal becomes expressed in the left lateral plate mesoderm (LPM) where it is required for correct asymmetric development of visceral organs. Evidence suggests that the Nodal ligand itself diffuses from the node to the LPM to induce its own...
expression there (Oki et al., 2007). Therefore, it is important that the levels of active Nodal ligand be tightly regulated in the node and LPM to achieve expression in only the left LPM.

In addition to Cerl2, Nodal is also inhibited by the TGF-beta proteins Lefty1 and Lefty2, which are expressed at their highest levels in the midline and LPM of the embryo, respectively, and are targets of the Nodal ligand (Meno et al., 2001; Saijoh et al., 2000; Yamamoto et al., 2003). Because Nodal can diffuse over long distances to induce its own expression, Lefty1 acts as a barrier at the midline preventing Nodal from inducing expression in the right LPM (Meno et al., 1998; Meno et al., 2001). The expression of Lefty2 in the left LPM limits the amount of time Nodal is expressed there, and this expression pattern of both Lefty1 and Lefty2 creates a situation in which the level of active Nodal ligand must reach a concentration threshold in the LPM to overcome inhibition by the Lefty proteins (Nakamura et al., 2006), thus adding another layer to the tight regulation of active Nodal protein in the embryo.

Several other signaling events help mediate the left-sided enrichment of Nodal in the node and its subsequent expression in the left LPM. Wnt signaling, specifically Wnt3a, induces Notch at the node, and Notch signaling is required for Nodal to be enriched in the left node periphery (Krebs et al., 2003; Nakaya et al., 2005; Raya et al., 2003). Shh signaling is needed to induce expression of Gdf1 at the node periphery, which is required for Nodal ligand diffusion to the left LPM (Tanaka et al., 2007; Zhang et al., 2001). The genetic network of LR axis establishment involves many players, some of which are still unknown, but together they turn a localized leftward flow of extraembryonic fluid into a robust LR asymmetry in the LPM that can guide visceral organ development.

Here we describe the left–right axis defects found in the Arl13bhnn mutant. Arl13bhnn mutants lack the cilia protein Arl13b (ADP ribosylation factor-like 13b), a small regulatory GTase, which leads to shortened cilia in the node (Caspary et al., 2007). We show here that the Arl13bhnn mutants are heterotaxic following an unusual pattern of Nodal expression: in the LPM, Nodal initially shows delayed and randomized expression, with subsequent bilateral expression, while in the node there is an inability to maintain asymmetric Nodal expression. Interestingly, we found that although Nodal initially shows expression in the node in a pattern that is similar to wild-type, Cerl2 is not correctly down regulated on the left side of the node, indicating that, in our mutants, the defect in Nodal expression may be secondary to a defect in Cerl2 expression. Mouse mutants that are unable to generate flow as well as mutants that cannot detect flow also express Nodal and Cerl2 symmetrically within the node raising the possibility that one or both of these processes is disrupted in Arl13bhnn mutants.

Materials and methods

Mouse strains

The strains (Arl13bhnn, IFT172wim, BATgal, Patched-lacZ, and NodallacZ) were genotyped as previously described and bred at least 10 generations on the C57 background (Collignon et al., 1996; Garcia-Garcia et al., 2005; Goodrich et al., 1997; Huangfu et al., 2003; Maretto et al., 2003).

Phenotypic analysis

Timed matings of mice carrying the Arl13bhnn mutation were performed, and embryos were harvested using somite number for staging. In situ hybridization was as previously described, and embryos were genotyped after analyzing expression patterns.

Antisense probes were generated from the following cDNA plasmids (with sources): Nodal (Elizabeth Robertson), Gdf1 (Nancy Wall), Shh and Foxa2 (Andy McMahon), Notchl (Janet Rossant), Lefty1 and Lefty2 (Hiromi Hamada), Pitx2 (Axel Schweickart), Cerl2 (IMAGE clone ID 790229), and Dll (Rahdika Ait).

Embryos homozygous for Arl13bhnn and carrying either the BATgal, Patched-lacZ, or Nodal-lacZ reporter alleles were harvested at E8.5 and fixed in 4% PFA with 0.2% glutaraldehyde for 15 min (Goodrich et al., 1997; Maretto et al., 2003). Embryos were washed (0.1 M phosphate buffer, 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% NP-40) and then treated with 1 mg/mL X-gal in 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide overnight at room temperature.

For organ analysis, embryos were harvested at E12.5. Organs were removed from the body cavity to allow imaging of the lungs. Images were taken using a Leica DM6000B upright fluorescence microscope and processed using QCapture software.

Results

Arl13bhnn mutants have heterotaxia

Midgestation Arl13bhnn embryos display a variety of phenotypes including randomized heart looping, an indication of LR axis defects (Caspary et al., 2007; Garcia-Garcia et al., 2005). To characterize the extent of the LR axis defects in Arl13bhnn mutants, we examined the orientation of the heart, lungs, and stomach in E12.5 embryos. Normally, the left lung has a single lobe, whereas the right lung has four, so we determined the left versus right lung by the presence of lobes. We found that in Arl13bhnn mutants, the lungs most often showed left isomerism (44.4% of embryos) and normal orientation at a high rate (38.9%), although the right lung had smaller and fewer lobes (Fig. 1(A)–(F), Table 1, and data not shown). The heart and stomach orientation were almost completely randomized and were not coordinated. For example, the heart could be on the left while the stomach was on the right (Fig. 1(A)–(F), Table 1) indicating that, in addition to the global defect in specifying the LR axis, individual organs interpret the LR axis distinctly, a defect called heterotaxia. This anomaly is seen in several other mouse mutants with LR defects, as well as in human laterality disorders (Chen et al., 1998; Lin et al., 1999; Lowe et al., 2001; Meno et al., 1998; Peeters and Devriendt, 2006).

Nodal and Pitx2 are misexpressed in the LPM of Arl13bhnn mutants

Asymmetric organ development is guided by the asymmetric expression of Nodal and its downstream target Pitx2 in the LPM, both of which we assessed through in situ hybridization (Campione et al., 1999; Lin et al., 1999; Liu et al., 2001; Ryan et al., 1998; Shiratori et al., 2001). In all cases, we scored molecular expression patterns prior to genotyping alleviating any ascertainment bias. Wild-type embryos display Nodal expression in the left LPM starting at the 2–3 somite stage, and this expression is extinguished by the 6–7 somite stage (Fig. 2(A)–(D)). In Arl13bhnn mutants, we saw an initial delay in Nodal expression in the LPM until the 4–5 somite stage. Nodal began to be expressed in the LPM at the 4–5 somite stage, but still half of the embryos at this stage did not show Nodal expression (Fig. 2(H)). Those embryos with expression at the 4–5 somite stage most often showed expression in the left LPM or bilaterally (Fig. 2(H)). At the 6–7 somite stage, when most wild-type embryos have no expression in the LPM (Fig. 2(A)–(D)), almost all Arl13bhnn embryos had Nodal expression that was bilateral (Fig. 2(E)–(H)). Therefore, in Arl13bhnn mutants, not only is there a spatial defect in Nodal expression, but there is also a temporal defect. This expression pattern was in marked contrast to mutants...
lacking cilia. Consistent with previous reports, we observed bilateral Nodal expression in the LPM in IFT172\textsuperscript{wim} mutants starting at early somite stages (Huangfu et al., 2003). Because there is not a delay in Nodal expression in mutants lacking cilia, our data indicate that Nodal expression in the LPM is more severely affected in Arl13b\textsuperscript{hnn} embryos with abnormal cilia than in IFT172\textsuperscript{wim} embryos lacking cilia altogether.

In the LPM and splanchnopleure of E8.5 and E9.5 Arl13b\textsuperscript{hnn} embryos, we found that Pitx2 was most often expressed bilaterally or exclusively on the left side of the embryo (Fig. 3(A), (B)–(D)). This pattern is consistent with the laterality in the lungs of Arl13b\textsuperscript{hnn} mutants, which most often showed left isomerism or normal orientation (Table 1). Arl13b\textsuperscript{hnn} embryos lacking Pitx2 expression were most often found at early somite stages, suggesting Pitx2 expression is also delayed in the absence of Arl13b.

![Image](45x298 to 291x598)

**Fig. 1.** Organ laterality in Arl13b\textsuperscript{hnn} mutants shows heterotaxia, while Arl13b\textsuperscript{hnn}; NodahlacZ\textsuperscript{+/+} shows right isomerism. (A)–(C) Wild-type E12.5 embryos. (D)–(F) E12.5 Arl13b\textsuperscript{hnn} mutant embryos. (G)–(I) E12.5 Arl13b\textsuperscript{hnn}; NodahlacZ\textsuperscript{−/−} embryos. (B) A ventral view of the internal organs of the wild-type embryo shows normal placement of the heart with its apex towards the left. (C) A dorsal view of the organs shows the left–right asymmetry of the lungs, where there is a single left lobe and 4 right lobes. The stomach can be seen on the left side of the embryo. (E) A ventral view of the Arl13b\textsuperscript{hnn} mutant shows the heart apex toward the left side of the embryo. (F) A dorsal view of the same embryo in (D) and (E) shows the stomach on the right, as well as a single lobe on the left and right lung. (H) A ventral view of the Arl13b\textsuperscript{hnn}; NodahlacZ\textsuperscript{−/−} mutant shows the heart apex toward the left. (I) A dorsal view shows there are small lobes on both the left and right lung, with the stomach in the center of the embryo. Inset is a ventral view of the lungs, with arrowheads pointing to the lobes. rv, right ventricle; lv, left ventricle; st, stomach; l, left; r, right.

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The midline barrier in Arl13b\textsuperscript{hnn} mutants is lost

Because we saw a large number of Arl13b\textsuperscript{hnn} embryos with bilateral Nodal expression, we wanted to examine a possible defect in maintaining the midline barrier. In wild-type embryos, Nodal from the LPM turns on expression of its inhibitor Lefty1 in the prospective floor plate at the 2–3 somite stage, and Lefty1 expression is extinguished by the 6–7 somite stage due to loss of Nodal expression in the LPM (Meno et al., 1998; Yamamoto et al., 2003). Our previous analysis of Arl13b\textsuperscript{hnn} shows that by E9.5, mutants lack a floorplate in the caudal embryo (Caspari et al., 2007); however, we did see expression of the floor plate marker Foxa2, as well as Lefty1, in mutant embryos at E8.5 (Fig. 4). Interestingly, Lefty1 was not expressed in the midline at the 6–7 somite stage of our mutant embryos, although Nodal expression persisted in the LPM (Fig. 4(A)–(F), Fig. 2(G) and (H)). As there is no floorplate at E9.5 in our mutants, these results could indicate a loss of floorplate integrity in Arl13b\textsuperscript{hnn} mutants. While examining Lefty1 expression, we also observed Left2 expression and found it follows the same pattern as Nodal in the LPM (Fig. 4(A) and (B), (D) and (E), n = 13).

Nodal asymmetry is initiated but not maintained in the node of Arl13b\textsuperscript{hnn} mutants

Nodal and Cerl2 are initially expressed symmetrically in the crown cells of the node and are then asymmetrically expressed as flow commences at early somite stages (Nonaka et al., 1998; Okada et al., 1999). The expression of Cerl2, an inhibitor of Nodal, is reduced on the left while being maintained on the right, while Nodal expression is increased on the left (Marques et al., 2004; Schweickert et al., 2010). This pattern of Cerl2 expression results in an increase in active Nodal protein on the left side of the node, which leads to increased Nodal expression in the left LPM (Kawasumi et al., 2011; Oki et al., 2009). We examined Nodal and Cerl2 expression in the node, and surprisingly, we saw that almost half of Arl13b\textsuperscript{hnn} embryos had increased Nodal expression on the left side of the node at presomite stages, which was nearly identical to wild-type (Fig. 5(A) and (B)). However, that asymmetry was gradually lost in Arl13b\textsuperscript{hnn} mutant embryos at later somite stages (Fig. 5(A) and (B)). Consistent with Cerl2 inhibiting Nodal, we saw that Cerl2 expression was not down regulated on the left side of the Arl13b\textsuperscript{hnn} node to the same extent as wild-type from the presomite to 5 somite stage (Fig. 5(D) and (E)), indicating that the gradual loss in Nodal asymmetry could be due to an inability to down regulate Cerl2.

**Left biased Nodal asymmetry is not initiated in the node of IFT172\textsuperscript{wim} mutants**

We examined mutants completely lacking cilia, the IFT172\textsuperscript{wim} mutants, to see if they had similar defects in Nodal enrichment in...
the node (Huangfu et al., 2003). If Arl13bhnn mutants completely lacked fluid flow in the node, we might expect them to show a similar pattern of Nodal expression to mutants lacking cilia. In IFT172wim mutants, almost half of the embryos showed asymmetric Nodal expression in the node, but increased expression was seen more often on the right side of the node than in either wild-type or Arl13bhnn mutant embryos (Fig. 5(C)). Our finding that Arl13bhnn mutants showed Nodal expression in the node in a pattern that is distinct from mutants with no cilia suggests that the Nodal expression defect in the node of Arl13bhnn mutants is not due to a complete loss of fluid flow.

Other signaling pathways are grossly intact in Arl13bhnn mutants at e8.5

Several signaling pathways are active in the node and affect Nodal expression in the node and LPM. First, a Wnt3a null mutant shows a similar delay in Nodal expression followed by bilateral expression of Nodal in the LPM. Wnt3a is required for Notch signaling at the node, which in turn is needed for Nodal expression (Nakaya et al., 2005). There has also been a link between cilia and Wnt signaling making it a candidate for disruption in our mutants (Corbit et al., 2007; Germino, 2005; Simons et al., 2005; Lancaster et al., 2011). To examine canonical Wnt signaling activity in our mutant embryos, we incorporated the BATgal reporter allele, which expresses the lacZ gene in response to Wnt signaling activity (Maretto et al., 2003). We saw no difference in β-galactosidase activity between wild-type and mutant embryos (Fig. 6(A) and (B)). Likewise, we saw no change in the expression of Dll1, a Wnt3a target, or of Notch1 and its target, Lfng (Fig. 6(C)–(H)).

Another signaling pathway, Shh, induces expression of Gdf1 at the node periphery, which is required for Nodal diffusion to the LPM (Tanaka et al., 2007; Zhang et al., 2001). Shh signaling is also known to be required for Nodal expression in the LPM (Tsaiaris and McMahon, 2009), and we know there are Shh signaling defects in Arl13bhnn mutants at later stages (Caspari et al., 2007). At E8.5, we saw no change in Shh expression, and using Patched-lacZ to monitor Shh signaling activity, there was no difference between wild-type and mutant embryos (Fig. 6(I)–(L)). Gdf1 expression was
also normal (Fig. 6(M) and (N)). This is surprising since Gdf1 and Ptc1-1ac2 are Shh responsive genes and we previously showed Shh signaling is abnormal in the absence of Arl13b, however, our previous work shows that Shh signaling is not completely abolished in the Arl13b<sup>mm</sup> mutants and we predict that at e8.5 there is enough Shh signaling to allow for Ptc1 and Gdf1 expression. Thus, while we cannot rule out subtle quantitative differences within the pathways, in terms of the molecular events known to be critical in establishing the LR axis, Wnt, Shh and Notch activities appear grossly intact in the absence of Arl13b.

**Arl13b and Nodal genetically interact**

Our analysis shows that Arl13b<sup>mm</sup> mutants have an inability to maintain higher levels of Nodal expression in the left side of the node and also have delayed Nodal expression in the LPM. Because Nodal is in a positive feedback loop to enhance its own expression in the node and induces its expression in the LPM, the simplest interpretation of this genetic interaction is that the levels of active Nodal are reduced in the Arl13b<sup>mm</sup> mutant, although we cannot say whether this effect is direct or indirect. A reduction in active Nodal in the LPM could be the direct consequence of reduced active Nodal in the node.

We confirmed the loss of Nodal expression in Arl13b<sup>mm</sup>; Nodal<sup>lacZ+/−</sup> by examining Pitx2, and saw that it likewise was not expressed in the LPM (Fig. 3(C) and (D)). These molecular patterns were reflected by the organ laterality in the Arl13b<sup>mm</sup>; Nodal<sup>lacZ+/−</sup> mutants, which showed right isomerism in the lungs, with the lobes being smaller than wild-type, as well as a midline stomach (Fig. 1(G)–(I)).

To determine whether there was a defect in Nodal expression at earlier stages in Arl13b<sup>mm</sup> mutants, we also examined the Arl13b<sup>mm</sup>; Nodal<sup>lacZ+/−</sup> at E7.5 and saw that its expression using β-galactosidase detection was indistinguishable from wild-type (Fig. 7(A) and (D)). Similarly, at E12.5 in Arl13b<sup>mm</sup>; Nodal<sup>lacZ+/−</sup> mutants, the only Nodal signaling-dependent defect we saw was in the visceral LR axis, and not in mesoderm or endoderm derivatives, which are specified by Nodal in the pre-gastrulation embryo.

**LPM Nodal expression requires cilia in Arl13b<sup>mm</sup> mutants**

In mutants that lack cilia and therefore have no flow, Nodal asymmetry in the node is randomized and Nodal in the LPM is bilateral. This predicts that removing cilia in combination with Arl13b should rescue Nodal expression in the LPM. To this end, we crossed Arl13b<sup>mm</sup> mutants to IFT172<sup>mm</sup> and made double homozygous mutants that lack Arl13b as well as cilia. The double mutant embryos showed bilateral expression of Nodal starting at the 2–3 somite stage, reminiscent of IFT172<sup>mm</sup> mutants (Fig. 2(I)–(P)) indicating that without cilia, Arl13b mutants can express Nodal at the correct time.

**Discussion**

Here we characterized the left–right patterning defects in mice lacking the small regulatory GTPase, Arl13b. We found that Arl13b<sup>mm</sup> embryos are heterotaxic due to misexpression of several genes known to be critical in L–R patterning. In the node we showed that Nodal and Cerl2 are expressed symmetrically in the absence of Arl13b. As Arl13b is highly enriched in cilia and its loss leads to short cilia in the node, it is easy to imagine that the symmetric gene expression arises from abnormal nodal flow. However, we cannot rule out the possibility that Arl13b<sup>mm</sup> may disrupt the ability for flow to be detected and this inability leads to the symmetric expression of Nodal and Cerl2 in the node.

**Organ laterality and the dose of Pitx2 in Arl13b<sup>mm</sup> mutants**

The heterotaxia we observed in Arl13b<sup>mm</sup> mutants corresponded to the abnormal expression of the molecules that guide organ laterality. This phenotype could yield important clues to help us understand congenital defects in humans. In several different LR axis-defective mutants and human syndromes, the visceral organs may have different lateralities, even though a single global LR axis decision has been specified. The cause of this has been perplexing, but the suggestion is that individual organs may require different doses of Pitx2, in terms of either the amount of Pitx2 the developing organ is presented with or the length of time the developing organ sees Pitx2, or both (Liu et al., 2001).
The complex pattern of Nodal and Pitx2 expression in Arl13b<sup>hmn</sup> mutants supports this idea. Because the lungs follow almost exactly the Pitx2 expression pattern we saw in Arl13b<sup>hmn</sup> mutants, they may only require a small dose of Pitx2 for LR specification to occur. However, the heart and stomach obviously require more precise amounts of Pitx2, as they developed with a random orientation in Arl13b<sup>hmn</sup> mutants and chose a left or right side independently of one another.

Patients with mutations in Arl13b display Joubert syndrome, which is characterized by brain malformations (Cantagrel et al., 2008). In the context of our analysis it is interesting to note that laterality defects in Joubert patients are rare. Since Arl13b is a GTPase and likely has multiple effectors, it will be interesting to see whether any effectors are specific to LR specification. This would also predict that distinct alleles of Arl13b may be identified in non-Joubert patients who only exhibit laterality defects.

**Defective Nodal expression in the LPM**

In the Arl13b<sup>hmn</sup> LPM, we have shown that Nodal expression is delayed and randomized followed by late bilateral expression. This is most likely an indirect consequence of the other molecular...
defects we observed. The late bilateral expression of Nodal in the LPM may result from the late loss of midline integrity in our mutants. The Nodal inhibitor Lefty1 is normally expressed in the midline in response to Nodal from the left LPM and prevents Nodal from diffusing to the right LPM to induce expression there (Meno et al., 1998; Sakuma et al., 2002; Yamamoto et al., 2003).

Fig. 5. Arl13b<sup>mm</sup> embryos show Nodal and Cerl2 expression defects in the node. (A)–(E) in situ hybridization for Nodal and Cerl2. The graphs show the expression patterns broken down by somite stage. (A) wild-type embryo showing left-sided asymmetry of Nodal. (B) Arl13b<sup>mm</sup> mutant embryo with symmetric Nodal expression in the node. (C) IFT172<sup>mm</sup> embryo showing right-sided enrichment of Nodal. (D) wild-type embryo showing Cerl2 has higher levels of expression on the right side of the node. (E) Arl13b<sup>mm</sup> mutant showing slightly higher levels of Cerl2 expression in the left side of the node.

Fig. 6. Wnt and Shh signaling is intact in Arl13b<sup>mm</sup> mutants. (A) and (B) whole mount β-galactosidase assay shows no difference in Wnt signaling between wild-type (A) and Arl13b<sup>mm</sup> mutant (B) embryos. (C)–(H) Whole mount in situ hybridization for Notch1 (C) and (D), Dll1 (E) and (F), and Lfng (G) and (H) shows that Notch signaling is intact in Arl13b<sup>mm</sup> mutants compared to wild-type. (I) and (J) Whole mount in situ hybridization for Shh is identical between wild-type and Arl13b<sup>mm</sup> mutants. (K) and (L) Patched-lacZ shows no change in Hedgehog response between wild-type and Arl13b<sup>mm</sup> mutant embryos. (M) and (N) the Shh signaling target Gdf1 is expressed normally in Arl13b<sup>mm</sup> mutants.
We found that Lefty1 was initially expressed in the midline, but was lost at late somite stages. This loss of expression occurred even though Nodal was still expressed in the LPM, which would likely allow for Nodal to diffuse to the opposing LPM to induce its expression there, thus causing bilateral LPM expression only at late somite stages.

Another interpretation for the late bilateral expression of Nodal in our mutants is that the inability to down regulate Cerl2 and the loss of asymmetric Nodal expression in the node leads to equal levels of active Nodal protein on both sides of the node at later somite stages causing activation of Nodal expression in both the left and right LPM. This inability to down regulate Cerl2 could also be the cause for delayed expression of Nodal in the LPM, as there would be less active Nodal protein in the node to induce its expression in the LPM. The lack of Nodal in the LPM of Arl13b<sup>hnn</sup>; Nodal<sup>lacZ</sup> embryos supports the model that there is less active Nodal protein in the LPM to activate its expression there. One possibility is that Arl13b functions in Nodal signaling from the node to the LPM, however we cannot determine this definitively as the decreased Nodal expression in the node could also cause abnormal expression of Nodal in the LPM.

The fact that mutants lacking cilia can effectively signal from the node to the LPM while Arl13b<sup>hnn</sup> mutants do not is reminiscent of other L–R patterning mouse mutants possessing cilia. In the node, Pkd2 and Pkd111 mutants display normal cilia but in the LPM they lack a response to the Nodal signaling cascade (Field et al., 2011). inv mutants have cilia in the node that generate slow abnormal flow towards the left, however they display delayed Nodal expression in the right LPM (Lowe et al., 1996; Okada et al., 1999). For inv mutants the delay may be due to altered levels of Nodal signaling from the node as Cerl2 and Lefty1 expression there is abnormal. Such a mechanism may underlie the Arl13b<sup>hnn</sup> phenotype although it is unclear whether the direct comparison can be made. Analysis of the Pkd111, Pkd2 and inv mutants revealed that asymmetric gene expression is highly sensitive to genetic background and the inv work was performed on a FVB strain (Field et al., 2011; Lowe et al., 1996; Meno et al., 1996). We work in the C3H strain background where the Pkd alleles were also analyzed so can make direct phenotypic comparisons to those. Pkd2 and Pkd111 mutants have normal cilia within the node yet the Nodal signaling cascade is not activated in the LPM of either mutant whereas the cascade is activated late in the absence of Arl13b. The simplest model to reconcile these phenotypes would posit that in the Pkd mutants, there is not enough active Nodal being produced in the node to allow for Nodal induction in the LPM, whereas in Arl13b<sup>hnn</sup> mutants, there is just enough Nodal in the node to allow for activation of Nodal expression, albeit delayed, in the LPM.

The fact that removing cilia in the context of Arl13b mutants can rescue the activation of the Nodal signaling cascade suggests that cilia remove positive and negative regulators important for activation of Nodal. For example, we know that removal of cilia causes loss of Lefty1 expression in the midline (Nakamura et al., 2006), so removal of cilia in the Arl13b<sup>hnn</sup> mutants would likely remove the Lefty1 inhibitor allowing for higher levels of active Nodal in the LPM (Nakamura et al., 2006). It would be interesting to delete Lefty1 in the Arl13b<sup>hnn</sup> mutants to determine if we see the same phenotype that we found from removing cilia.

It is also possible that Arl13b has roles in the LPM independent of its function in the node. A precise understanding would require us to spatially delete Arl13b in the LPM which has proven technically challenging as Arl13b protein requires 42 h to turn over (our unpublished data). Regardless, the idea that Arl13b affects the balance of positive and negative inputs to Nodal signaling parallels its function in Shh signaling where we showed it controls activation but not repression of Shh signaling (Caspary et al., 2007).

**Defective Nodal expression in the node**

The primary LR axis specification defect in Arl13b<sup>hnn</sup> mutants is the symmetric expression of Nodal and Cerl2 in the node as this can explain all subsequent observations. Both Cerl2 and Nodal are predicted to be downstream targets of fluid flow (Hirokawa et al., 2006; Schweickert et al., 2010), and our mutants have stunted cilia that, if motile, would likely be unable to generate the same quality of leftward flow that would be found in wild-type embryos. It was recently shown that very little fluid flow is required in the node to generate asymmetric expression of Cerl2, and that in the absence of flow, Cerl2 is symmetric (Shinohara et al., 2012). We saw that almost 50% of Arl13b<sup>hnn</sup> mutant embryos had higher levels of Nodal expression in the left side of the node at presomite stages, a pattern that was nearly identical to wild-type. However, this pattern gradually shifted to symmetric expression between the 2 to 5 somite stage. This may indicate that our mutants generate a fluid flow that is too weak to allow for the signaling events required for down regulation of Cerl2, but this weak flow is sufficient for initial Nodal enrichment. Consistent with this, we found that the inhibitor of Nodal, Cerl2, was not down regulated normally in the left side of the node, and most often showed equal levels of expression in both sides of the node. An inability to down regulate Cerl2 could lead to less active Nodal protein in the node, preventing the Nodal feedback loop necessary for increasing Nodal expression there (Norris et al., 2002). To confirm...
that reduced expression of Nodal expression are due to the defect in Cerl2 expression, it will be necessary to generate Cerl2−/−; Arl13b−/− double mutants and observe Nodal expression.

The symmetric Nodal and Cerl2 we observed in the Arl13b−/− node could also be explained if Arl13b is required for detection of flow in the node. The two cilia hypothesis invokes a Pkd2-dependent mechanism for flow detection. Mutations in Pkd2 or its binding partner, Pkd1l1 have normal cilia but LR defects stemming from symmetric expression of both Nodal and Cerl2 in the 3 somite node. As this mimics the Arl13b−/− phenotype, it raises the possibility that Arl13b−/− mutants may also be unable to detect flow. Arl13b is a member of the Arf family whose members have well-established roles in protein transport including the transport of Pkd2 to the cilium by Arf4 (Ward et al., 2011).

Furthermore, both Arl13b and Pkd mutants possess cilia yet Nodal is delayed or absent in the LPM. This has always been difficult to reconcile with the phenotype of mutants lacking cilia which express Nodal in the LPM, albeit bilaterally. One model to explain this would posit that cilia are required for both positive and negative regulators that induce Nodal in the LPM and that removing cilia ablates both, resulting in bilateral Nodal expression by the 2–3 somite stage. In contrast, in Pkd or Arl13b mutants where cilia are present (albeit distinctly) perhaps the balance of the positive and negative regulation is distorted resulting in distinct spatial and temporal patterns of Nodal expression in the LPM. The bilateral Nodal we saw in the Arl13b−/− show Arl13b−/− double mutants is consistent with this interpretation.

Taken together our analysis shows that Arl13b controls left-right patterning initially by disrupting the normal asymmetric expression of Nodal and Cerl2 in the node. It remains possible that this is due to distinct roles of Arl13b in both the generation and detection of flow. In support of this, mutants lacking cilia do not display preasymmetric node asymmetry of Nodal as Arl13b mutants do. Mutants that cannot detect flow cannot activate the Nodal signaling cascade in the LPM as Arl13b mutants do (Field et al., 2011). We have recently been able to bypass the Arl13b dependent ciliogenesis defects in a cell culture model indicating that Arl13b functions via distinct effectors (Mariani and Caspary, personal communication). As we identify specific Arl13b effectors, it will be interesting to determine if they distinguish roles of Arl13b in generating and detecting flow in the node.

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