Wnt3a links left-right determination with segmentation and anteroposterior axis elongation

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Summary
The alignment of the left-right (LR) body axis relative to the anteroposterior (AP) and dorsoventral (DV) axes is central to the organization of the vertebrate body plan and is controlled by the node/organizer. Somitogenesis plays a key role in embryo morphogenesis as a principal component of AP elongation. How morphogenesis is coupled to axis specification is not well understood. We demonstrate that Wnt3a is required for LR asymmetry. Wnt3a activates the Delta/Notch pathway to regulate perinodal expression of the left determinant Nodal, while simultaneously controlling the segmentation clock and the molecular oscillations of the Wnt/β-catenin and Notch pathways. We provide evidence that Wnt3a, expressed in the primitive streak and dorsal posterior node, acts as a long-range signaling molecule, directly regulating target gene expression throughout the node and presomitic mesoderm. Wnt3a may also modulate the symmetry-breaking activity of mechanosensory cilia in the node. Thus, Wnt3a links the segmentation clock and AP axis elongation with key left-determining events, suggesting that Wnt3a is an integral component of the trunk organizer.

Key words: Mouse, Wnt3a, Left-right determination

Introduction
The anteroposterior (AP) body axis is the first axis to be established during the formation of the mammalian body plan. The left-right (LR) axis is specified last, and is oriented orthogonally to the pre-existing AP and DV axes. The specification and coordination of all three vertebrate body axes is controlled by a small group of cells known as the Spemann-Mangold organizer (Niehrs, 2004). A transient structure, termed the node, is generally considered to be the murine equivalent of the Spemann-Mangold organizer; however, the node first forms at the anterior end of the primitive streak of the gastrulating embryo on embryonic day (E) 7.5, well after AP polarity has been established. The timing of node formation correlates well with LR axis specification, and with the beginning of somitogenesis and the development of the trunk. Somitogenesis generates the segmental structures of the trunk and is a major morphogenetic force driving the elongation of the AP axis. The node plays an important role in trunk development as node ablation results in the loss of LR and dorsoventral (DV) polarity, retarded somite formation and shortened trunks (Davidson et al., 1999). Thus, the node functions as a trunk organizer, coordinating axis determination with trunk elongation.

Members of the transforming growth factor β (Tgfβ) family, specifically Nodal, Lefty1 and Lefty2, are the first genes to be asymmetrically expressed along the LR axis (Hamada et al., 2002). Nodal is expressed in the periphery of the node, where it functions as the left-determinant (Brennan et al., 2002; Saijoh et al., 2003). Nodal transcription is controlled by the Notch signaling pathway. Activation of Notch receptors by the ligand Delta-like 1 (Dll1), leads to the cleavage and nuclear translocation of the Notch intracellular domain, where it acts as a transcription factor when bound to the DNA-binding protein RBP-J (Rbpsu – Mouse Genome Informatics) (Schweisguth, 2004). Loss of function mutations in components of the Notch pathway lead to loss of LR asymmetry, and RBP-J-binding sites found within the Nodal node-specific enhancer are required for Nodal expression in the node (Krebs et al., 2003; Raya et al., 2003). These data demonstrate that Nodal is a direct target gene of the Notch signaling pathway; however, the relationship between Notch activity and symmetry-breaking events in the node is not clear.

Cilia emanating from the ventral surface of the node play a crucial role in the breaking of bilateral symmetry (McGrath and Brueckner, 2003). Embryos carrying mutations in genes required for cilia formation or motility display laterality defects (Marszalek et al., 1999; Nonaka et al., 1998, Supp et al., 1999). Motile cilia generate a leftward flow of extra-embryonic fluid at the node, termed nodal flow, that is necessary for the generation of LR asymmetry (Nonaka et al., 1998; Okada et al., 1999). Artificial reversal of nodal flow is sufficient to reorient the LR axis (Nonaka et al., 2002) demonstrating that nodal flow is both necessary and sufficient for LR axis specification. These experiments led to the development of the...
morphogen flow model that proposed that nodal flow, generated by node cilia, set up a morphogen concentration gradient that directs asymmetric gene expression at the node (Nonaka et al., 1998; Okada et al., 1999).

A second population of node cilia, known as mechanosensory cilia, have been proposed to participate in LR determination, largely owing to the observation that mutations in the polycystic kidney disease 2 (Pkd2) gene cause abnormal LR development (Pennekamp et al., 2002). Pkd2 encodes polycystin 2 (PC2), a Ca2+-permeable cation channel expressed in node cilia that is necessary for the generation of asymmetric Ca2+ flux (McGrath et al., 2003). These results led to the development of the two-cilia model for LR initiation in which a centrally located population of Lrd-containing motile cilia generate nodal flow, while a second population of PC2-expressing nonmotile mechanosensory cilia sense nodal flow on the left side of the node and convert it into an asymmetric Ca2+-dependent signal transduction event (McGrath and Brueckner, 2003; Tabin and Vogan, 2003).

Activation of the Wnt/β-catenin pathway by members of the Wnt family of secreted signaling molecules elevates levels of β-catenin, a protein involved in cell-cell interactions that is normally degraded by the proteasome in response to Wnt and Li signals (Nobes and Hall, 1995). This results in the increased expression of downstream targets such as cyclin D1, c-myc, c-mos, and c-fos. The β-catenin pathway is also involved in the regulation of cell proliferation and differentiation, and its activation is thought to contribute to the development of a range of human cancers (Fero et al., 2000; Nakagawa and Nakamura, 2000; Rodriguez-Esteban et al., 2000). Wnt3a, a member of the Wnt family, is expressed in the node and is required for LR axis formation, demonstrating a requirement for Wnts in this process.

Results

Whole-mount immunofluorescence and confocal microscopy

Mouse embryos were fixed with 2% PFA for 20 minutes at room temperature, washed with PBS and stored in 0.1% sodium azide/PBS at 4°C until use. Embryos were permeabilized with 0.1% Triton X-100 and 100 mM glycine in PBS for 10 minutes at room temperature, blocked with 10% calf serum, 0.1% BSA (Sigma) and 3% normal goat serum (NGS) in TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20), and then incubated with primary or secondary antibodies diluted in TBST containing 0.1% BSA and 1.5% NGS. All blocking and antibody incubation steps were performed overnight at 4°C followed by multiple TBST washes. For imaging (Zeiss LSM510), embryos were placed in Glass Bottom Culture Dishes (MatTek Corporation) in PBS containing 50% Vectashield mounting medium (Vector Labs), or node regions were dissected and mounted on a slide glass with a spacer in SlowFade Light Antifade Kit (Molecular Probes). Four Wnt3a+/− and four Wnt3a+− 0- to 2-somite stage embryos were analyzed for expression of the cilia markers PC1, PC2 and acetylated tubulin. PC-positive cilia were quantitated manually.

Mice

To make the Batlaczz mouse, two oligos (TB3-1, 5'-AAT TCA GAA TCA TCA AAG GAC CT-3'; and TB3-2, 5'-AAT TAG GTC CTT TGA TGC TTC TG-3') containing a TcI/Lef binding site sequence flanked by EcoRI sites, were annealed and ligated to construct an 8× multimer, and then subcloned into pBluescript to generate a plasmid designated 8× TBS-pBS. A 130 bp Xenopus Xiamois minimal promoter was amplified by PCR from p01234 (kindly provided by D. Kimelman) using primers xSiamois-1 (5'-CGT GAA TCC CTC TGA TGG TTC TG-3') and xSiamois-2 (5'-AGC GGA TCA CTC TGT CCG AAA AAA AAG-3'). Then, then subcloned into the EcoRI/BamHI sites of 8× TBS-pBS. NLS-lacz from pCS-nb-gal was subcloned into the BamHI/XbaI sites of 8× TBS-xSiamois-pBS to generate the Batlacz transgene. Transgenic mice were generated in the Transgenic Core Facility by pronuclear injection following standard procedures. From the four lines that were generated, the one that most faithfully replicated domains of Wnt signaling was designated as the BATlacz line. These mice are similar in design to the BATgal mouse of Maretto et al. (2003). All animal experiments were performed in accordance with the guidelines established by the NCI-Frederick Animal Care and Use Committee.

Materials and methods

Whole-mount in situ hybridization

The original cDNA clones described in the literature were used as templates for the generation of cRNA probes. Details are available upon request. Whole-mount in situ hybridization was performed as previously described (Wilkinson and Nieto, 1993). Embryos were photographed on a Leica stereoscope or a Zeiss Axiophot compound microscope. Unless indicated otherwise, at least four mutant embryos were examined for expression of each probe, and all yielded similar results.

Antibodies

The following reagents were obtained commercially: mouse monoclonal anti-β-catenin (BD Transduction Laboratories), anti-acetylated tubulin, clone 6-11B-1 (Sigma), goat polyclonal anti-PC1 (M-20) (Santa Cruz), Rhodamine-Phalloidin, DAPI, anti-mouse IgG(H+L) goat Alexa-Fluor 488, anti-goat IgG(H+L) donkey Alexa-Fluor 488 (Molecular Probes), anti-rabbit IgG(H+L) goat Cy3, and anti-mouse IgG(H+L) goat Cy3 and Cy5 (Amersham). The YCC anti-mouse antibodies directed against amino acids 687-962 of human PC2 have been characterized previously (Cai et al., 1999).
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S2B in the supplementary material). The direction of axial rotation or embryonic turning was randomized with 47% (\(n=17\)) of Wnt3a–/– embryos correctly turning clockwise such that the tail and allantois lay on the right side of the embryo, and 53% turning in the opposite direction (not shown). The heart-looping defects were not secondary to earlier defects in cardiogenesis, as several heart markers were expressed normally observed in mutants (G), in contrast to the wild-type lungs (F, stomach not shown). lv, left ventricle; rl, right lateral lobe; c, cranial lobe; m, medial lobe; ca, caudal lobe; a, accessory lobe; l, left lobe; st, stomach.

**Fig. 1.** Loss of Wnt3a leads to laterality defects. (A-C) SEM micrographs of E9.5 wild-type (A) and Wnt3a–/– (B,C) hearts; mutants displayed normal (B) and inverted (situs inversus) looping (C). (D,E) E11.5 livers. Situs inversus was observed in the asymmetric arrangement of the Wnt3a–/– liver (E), compared with the control (D). (F,G) E11.5 lungs and stomach. A midline stomach and right pulmonary isomerism was often observed in mutants (G), in contrast to the wild-type lungs (F, stomach not shown). lv, left ventricle; rl, right lateral lobe; c, cranial lobe; m, medial lobe; ca, caudal lobe; a, accessory lobe; l, left lobe; st, stomach.

sensitive to perturbations in left-right determination as 78.9% (\(n=19\)) of mutant embryos displayed abnormalities including situs inversus (10.5%) (Fig. 1E) and situs ambiguous (68.4%). Abnormal midline (Fig. 1G), or right-sided, positioning of the stomach was noted 37.5% of the time (\(n=16\)). Thus, laterality defects were observed in all of the organs that we were able to assess, indicating that Wnt3a plays an early and crucial role in LR determination.

**Wnt3a is necessary for asymmetric gene expression**

Wnt3a–/– embryos were examined for the expression of the asymmetrically expressed genes Nodal, Lefty1, Lefty2 and Pitx2 at stages prior to the morphological manifestation of LR or AP phenotypes. Nodal transcripts were detected in the ventral node of Wnt3a–/– mutants at presomite, headfold (E7.75-8) stages; however, the spatial domain was smaller (Fig. 2B,F) compared with wild-type controls (Fig. 2A,E) (Lowe et al., 1996; Collignon et al., 1996). This domain became increasingly restricted to the posterior edge of the mutant node as development proceeded, and was approximately one-third the size of the wild-type domain (compare Fig. 2H with 2G) by the two- to four-somite stages (Fig. 2D,H,J,N). Nodal mRNA was not detected in the Wnt3a–/– left lateral plate mesoderm (LPM) at these stages (Fig. 2D) when Nodal was normally expressed there in wild-type embryos (Fig. 2C), but was bilaterally expressed in the posterior LPM and streak starting at the four- to five-somite stage (Fig. 2J,N), and remained bilaterally expressed in the LPM (Fig. 2L,P) at stages when Nodal was normally turned off in wild-type embryos (six- to eight-somite stages; Fig. 2K,O). The anterior limit of the Nodal LPM expression domain was posteriorized in mutants (arrowhead, Fig. 2L), never extending anteriorly into the heart as in earlier staged wild-type embryos (arrowhead, Fig. 2I).

Lefty1 and Lefty2 are required for proper LR patterning, functioning as negative regulators of Nodal (Hamada et al., 2002). Lefty2 is a direct target gene of Nodal, and is normally expressed in the left LPM between the three- and six-somite stages (Meno et al., 1998) (Fig. 2C,Q,R). Lefty2 expression in Wnt3a–/– embryos mirrored Nodal expression, with expression in the LPM initially delayed, then restricted to the posterior streak (Fig. 2D and not shown), and later bilateral and posteriorized (seven somites, Fig. 2Q,R). Similarly, Pitx2, a bicoid-type homebox gene expressed in the left LPM and heart (Yoshioka et al., 1998), was delayed, and then bilaterally expressed in mutant posterior LPM (Fig. 2S; data not shown). Lefty1 is asymmetrically expressed in the left prospective floor plate (PFP) in wild-type embryos (Meno et al., 1998) (Fig. 2C,G), but was never detected in the mutant PFP, being expressed in only a few individual cells in the posterior node and anterior streak (Fig. 2D,H). Loss of Lefty1 expression is not due to the physical loss of a midline barrier (Hamada et al., 2002) as several markers, including Shh, Foxa2, T, Wnt11, Gdf1 and cryptic (Cfc1 – Mouse Genome Informatics) were easily detected in the mutant node, notochord or PFP (see Fig. S3A-L in the supplementary material; data not shown). Thus, molecular marker analyses demonstrate that a cascade of genes necessary for the generation of LR asymmetry are abnormally expressed in the Wnt3a–/– node and LPM, indicating that Wnt3a functions early in the genetic hierarchy of LR determination.

If Wnt3a is upstream of left determining genes, then ectopic
activation of Wnt signaling should alter their expression. To test this, we examined the expression of left determining genes in embryos lacking Axin, a negative regulator of the Wnt/β-catenin signaling pathway (Zeng et al., 1997). Homozygous AxinTg1 embryos continued to express Wnt3a and Nodal normally in the primitive streak; however, Nodal expression in the node was slightly expanded (Fig. 2U,W) and large ectopic domains of symmetrical Nodal (Fig. 2U) and Lefty1/2 (Fig. 2W) expression were observed. Thus, both gain- and loss-of-function alleles of genes in the Wnt/β-catenin signaling pathway lead to aberrant expression of left determining genes.

**Cilia are structurally normal but display reduced polycystin 1 (PC1) expression**

To determine whether a relationship between Wnt3a and cilia structure or function exists, we first determined whether cilia were present on the Wnt3a–/– node. Scanning electron microscopy (SEM) analysis of mutant nodes at E7.75 revealed the presence of monocilia in the ventral node (Fig. 3B), similar to that observed in wild-type nodes (Fig. 3A). Immunofluorescent labeling of cilia with anti-acetylated tubulin confirmed this, and further showed that the general morphology of the node remained normal (compare Fig. 3C with 3D; data not shown). Quantitation of node cilia in Wnt3a+/– (mean=158+/–13.7, n=4) and Wnt3a–/– (mean=144+/–30.7, n=4) stage-matched embryos revealed no significant differences in total cilia number. The presence of structurally normal cilia indicates that Wnt3a does not lie upstream of genes required for ciliary structure, such as Kif3a or Kif3b as these mutants lack cilia (Marszalek et al., 1999; Nonaka et al., 1998; Takeda et al., 1999).

To assess ciliary motility, we examined Wnt3a–/– embryos for the expression of left-right dynein (Lrd; Dnahc11 – Mouse Genome Informatics), which encodes a ciliary motor protein. Lrd was easily detected in the mutant node (Fig. 3E, right embryo). Conversely, Wnt3a was normally expressed in inversus viscerum (iv) embryos (which carry a mutation in Lrd (Supp et al., 1997)), including embryos that displayed reversed or bilateral Nodal expression (Fig. 3F). Crosses between Wnt3a and iv did not reveal genetic interactions in transheterozygotes or

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Fig. 2. Wnt signaling controls LR asymmetric gene expression. (A–P) Whole-mount in situ hybridization analysis of Nodal expression (see text for details). Headfold-stage wild-type (A,E), and Wnt3a–/– (B,F) embryos express Nodal (purple) in the node. Two-color whole-mount in situ hybridization showing Nodal (orange), and Lefty1 and Lefty2 expression (purple) in wild-type (C,G) and mutant (D,H) three-somite stage embryos. (G,H) High-power ventral views of the nodes of the wild-type and mutant embryos depicted in C,D. Nodal expression in wild-type four-somite (I,M) and similarly staged Wnt3a–/– (J,N) embryos. Arrows in N indicate bilateral Nodal expression in the mutant posterior LPM. Nodal was not expressed in the wild-type LPM after the six- to seven-somite stage (K,O), but was bilaterally expressed in the mutant LPM (L,P). (Q-S) Similar abnormal expression patterns were observed for Lefty2 in the seven-somite stage mutants (right embryos in Q and R, compare with the four-somite wild-type embryos on the left), and for Pitx2 expression in six-somite mutants (S). (T,U) Two-color whole-mount in situ hybridization showing Nodal (purple) and Wnt3a (orange) expression in four-somite wild-type (T) and AxinTg1/Tg1 (U) littermates. (V,W) Lefty1/2 (purple) and Nodal (orange) expression in two-somite wild-type (V) and AxinTg1/Tg1 (W) littermates. Asterisks indicate ectopic bilateral expression of Nodal (U) and Lefty1/2 (W) in anterior domains. (A–D,I–L,Q–T) lateral views; (E–H,M,N,S) ventral posterior views; (O,R,P) anterior views; arrows indicate the LPM; arrowheads indicate the anterior limit of LPM expression. ps, primitive streak; n, node.
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compound mutants, indicating that Wnt3a and iv function in independent genetic pathways (data not shown). In addition, the axonemal dynein heavy chain gene Dnahc5\(5\) (Ibanez-Tallon et al., 2002) was also expressed in the \textit{Wnt3a}\textsuperscript{−/−} node (not shown). We suggest that ciliary motility was unaffected by the absence of Wnt3a.

The presence of mechanosensory cilia in Wnt3a mutants was evaluated by examining nodes for the expression of polycystin 1 (PC1) and PC2. The Pkd1 gene product, PC1, interacts with PC2 and is thought to control the gating of PC2 Ca\(^{2+}\) channels (Delmas et al., 2004). While cilia co-expressing PC1, PC2 and acetylated tubulin were easily found in the wild-type node (arrows, Fig. 3J,O), similarly labeled cilia were rarely found in the mutant (Fig. 3N,P,Q). Interestingly, PC1 expression was significantly downregulated (\(P<0.0001\), Welch’s \(t\)-test) in the \textit{Wnt3a}\textsuperscript{−/−} node cilia (Fig. 3K,N), with only 6.8\(±\)5.8\% (\(n=4\) embryos) of the mutant cilia expressing detectable levels of PC1, compared with the 46.9\(±\)0.9\% (\(n=4\)) of cilia that were PC1-positive in \textit{Wnt3a}\textsuperscript{+/−} embryos (Fig. 3G,J). PC2 was strongly expressed in more than 92\% of central and peripheral cilia in both wild-type and \textit{Wnt3a}\textsuperscript{−/−} nodes (Fig. 3H,L). The rare cilium that co-expressed PC1 and PC2 in \textit{Wnt3a}\textsuperscript{−/−} nodes expressed PC1 weakly and in a much smaller spatial domain (arrow, Fig. 3P) than in wild-type cilia suggesting that mechanotransduction may be perturbed in the absence of Wnt3a.

\textbf{Wnt3a signals directly to the node and presomitotic mesoderm via \(\beta\)-catenin}

Although Wnt3a is expressed in the dorsal posterior node, gene expression in the \textit{Wnt3a}\textsuperscript{−/−} mutants is perturbed in both the dorsal and ventral node. To determine which tissues respond directly to Wnt signals, we examined two independent transgenic lines that report sites of presumed Wnt/\(\beta\)-catenin activity in vivo. Both the TOPgal and BAT\textsuperscript{lacZ} (see Materials and methods) transgenes were expressed in the node, primitive streak and posterior mesoderm during LR determination stages (Fig. 4A-C,E; see Fig. S4A-C in the supplementary material) (Merrill et al., 2004). The node was the strongest site of \(\beta\)-galactosidase (\(\beta\)-gal) expression in TOPgal embryos at early somite stages (Fig. 4A), with particularly robust expression detected in the ventral node (Fig. 4B).

Although the TOPgal and BAT\textsuperscript{lacZ} reporters were both expressed in the node and streak, BAT\textsuperscript{lacZ} expression was stronger and extended further anteriorly, through the presomitotic mesoderm (psm) where Wnt3a functions to regulate segmentation (Aulehla et al., 2003), to reach an anterior limit at the base of the future hindbrain (compare Fig. 4C with Fig. S4B in the supplementary material). This domain closely paralleled the expression of Wnt8 (Bouillet et al., 1996). This suggested that the BAT\textsuperscript{lacZ} transgene was a more sensitive and accurate reporter of Wnt/\(\beta\)-catenin activity than TOPgal as Wnt8 and Wnt3a are co-expressed at these stages and probably signal via \(\beta\)-catenin.

We therefore chose to examine BAT\textsuperscript{lacZ}...
expression in the Wnt3a mutants. BATlacZ expression was downregulated in the E7.75 primitive streak in the absence of Wnt3a (Fig. 4D, inset), compared with controls (Fig. 4C, inset), and was strikingly absent from the node (curved white line) and anterior psm (black arrow). This loss of anterior Wnt/β-catenin reporter expression in Wnt3a–/– embryos suggests that Wnt3a, emanating from a posterior primitive streak source, functions at a distance to directly activate target genes in the node and anterior psm.

Alternatively, the lack of reporter expression in the mutant node and anterior psm could simply be due to downregulation of the reporter and the consequent reduced levels of the stable β-gal protein. To distinguish between these possibilities, we compared the spatial domain of β-gal activity in BATlacZ embryos with that of the lacZ mRNA itself. Whole-mount in situ hybridization revealed that lacZ mRNA expression (Fig. 4H) was coincident with β-catenin activity (Fig. 4G) and extends into the anterior psm, indicating that the β-catenin expression domain is not defined by β-catenin stability but rather by direct transcriptional activation of the transgene by Wnt/β-catenin signaling. We suggest that Wnt3a is a long-range signaling molecule capable of activating Wnt/β-catenin target genes in node and anterior psm cells at least 15-20 cell diameters away.

The observation that the Wnt/β-catenin reporters were expressed in the node in a Wnt3a-dependent manner strongly indicates that Wnt3a signals in the node via β-catenin. To examine this hypothesis directly, we analyzed the expression of the β-catenin target gene Nkd1 in four-somite stage wild-type (EF) and Wnt3a–/– (GH) embryos. Arrows indicate sites of asymmetric expression in the wild-type node. Boxed regions in E and G are represented as high-power views of the nodes in F and H, respectively. All assessments of Nkd1 distribution were performed on whole embryos by whole-mount in situ hybridization (not shown), and subsequently sectioned for confirmation and clarity.

Fig. 5. Asymmetric distribution of canonical Wnt/β-catenin signaling pathway components in the node. All images are posterior views of cross sections, the left side of the embryo is facing left. (A) Merge of confocal microscopy images of E7.75 wild-type embryo labeled with anti-β-catenin antibody (B), rhodamine phalloidin (C) and DAPI (D). (E-H) Expression of the Wnt/β-catenin target gene Nkd1 in four-somite stage wild-type (EF) and Wnt3a–/– (GH) embryos. Arrows indicate sites of asymmetric expression in the wild-type node. Boxed regions in E and G are represented as high-power views of the nodes in F and H, respectively. All assessments of Nkd1 distribution were performed on whole embryos by whole-mount in situ hybridization (not shown), and subsequently sectioned for confirmation and clarity.
homolog of the Drosophila segment polarity gene naked cuticle (Wharton et al., 2001; Yan et al., 2001), was expressed symmetrically in the primitive streak, psm and node at E7.75, but was asymmetrically distributed in the node by the two-somite stage. Elevated levels were observed on the left side of the ventral node, while expression in the psm remained symmetric (Fig. 5E,F). Analysis of Nkd1 expression in three- to six-somite stage Wnt3a−/− embryos (n=4) revealed that transcript levels and asymmetric expression were reduced in the node (Fig. 5G,H). Expression in the mutant psm was also downregulated. Thus, Wnt3a signals directly to the psm and ventral node to activate expression of the Wnt/β-catenin target gene Nkd1.

Wnt3a regulates the Dll1/Notch pathway during LR determination and somitogenesis

As the Dll1/Notch signaling pathway directly controls Nodal expression in the node (Raya et al., 2003; Krebs et al., 2003), we investigated the possibility that the abnormal Nodal expression domain in the Wnt3a mutant node may be due to aberrant Notch signaling. Using Dll1 and Lfng as reporters of Notch activity (Raya et al., 2003), we examined Notch activity in Wnt3a−/− embryos. At E8, Dll1 was expressed in the streak and in psm (Fig. 6A) in a pattern similar to the Wnt reporter (Fig. 4C). Dll1 was expressed in psm cells immediately adjacent to Nodal-expressing peripheral ventral node cells (Fig. 6C). Notably, expression of Dll1 in Wnt3a−/− psm was posteriorized such that Dll1-expressing cells only contacted the posterior-most node (Fig. 6B,D). This domain correlated well with the abnormally small domain of Nodal expression, suggesting that Wnt3a regulates Nodal expression indirectly, via Dll1 and the Notch signaling pathway.

To examine whether Dll1 might function in the same genetic pathway as Wnt3a, we crossed vestigial tail (vt) mice, carrying a hypomorphic allele of Wnt3a (Greco et al., 1996), with mice carrying a targeted allele of Dll1 (Hrabe de Angelis et al., 1997). Analysis of the progeny of Wnt3avt/+; Dll1+/– animals crossed to Wnt3avt/vt indicated that animals of the expected genotypes were born at Mendelian frequencies but reduced viability of Wnt3avt/+; Dll1+/– and Wnt3avt/vt; Dll1+/– compound mutants was observed by weaning stages (see Table S1 in the supplementary material). Examination of offspring at postnatal stages revealed that cardiac abnormalities were the likely cause of the reduced viability. On examination of the hearts, 14.8% of Wnt3avt+/-; Dll1+/– neonatal hearts (n=27; Table 1) displayed situs ambiguous or atrial or ventricular septation defects, and 41.7% of Wnt3avt+/–; Dll1+/– hearts displayed persistent truncus arteriosus (PTA) (Fig. 6F), transposition of the great arteries (TGA) (Fig. 6G), ventricular
septation defects (VSD) (Fig. 6H) or other abnormalities consistent with cardiac laterality defects (Maclean and Dunwoodie, 2004). No other visceral laterality defects were observed. These results suggest that Wnt3a and Dll1 participate in a common genetic pathway to regulate cardiac laterality.

Analysis of the Wnt3a<sup>vt</sup> allele has shown that Wnt3a and the Dll1/Notch pathways play important roles in somitogenesis at tailbud stages (E9.5 onwards), functioning as integral components of the segmentation clock (Aulehla and Herrmann, 2004; Dubrulle and Pourquie, 2004). The temporal and spatial proximity of LR determination (at E7.75-8 in the node), the onset of somitogenesis (at E8 in the adjacent psm), and the common Wnt3a/Dll1 molecular components suggests an intimate relationship between LR determination and somitogenesis. This relationship is strengthened by our demonstration that Wnt3a is required for activation of a Wnt/β-catenin reporter in both the node and psm (Fig. 4F). To explore this further, we examined the expression of segmentation clock genes in Wnt3a<sup>−/−</sup> mutants at these earlier stages. Axin2 is a direct Wnt/β-catenin target gene and negative regulator of the Wnt pathway (Jho et al., 2002; Lustig et al., 2002). It is expressed at E9.5 in a graded oscillating manner in the tailbud and in a single stripe in the anterior psm (Aulehla et al., 2003). At E7.75, Axin2 is expressed symmetrically in the wild-type node, streak and posterior mesoderm (not shown), and an additional stripe of expression in the anterior psm becomes detectable at early somite stages (Fig. 6J). Axin2 expression oscillates in the psm and is dependent upon Notch signaling (Barrantes et al., 1999) and Wnt3a (Aulehla et al., 2003) at tailbud stages. At early somitogenesis and LR determination stages when Nodal is expressed in the left LPM, Lfng is expressed in a dynamic manner that can manifest in a diffuse patch in the posterior primitive streak and as two stripes adjacent, and anterior, to the node in the psm (Fig. 6K). Interestingly, Lfng is also expressed in the node periphery, overlapping with Nodal expression in the node (Fig. 6K and not shown). Wnt3a<sup>−/−</sup> mutants (0-7 somites, n=7) displayed only a single abnormally shaped stripe of Lfng expression posterior to the node and no expression was detected in the node periphery (Fig. 6L). More than one set of stripes was never observed, suggesting that dynamic oscillating Lfng expression did not occur in the absence of Wnt3a. Together, the Axin2 and Lfng expression patterns indicate that the segmentation clock does not function properly at early somitogenesis stages in the absence of Wnt3a. Wnt3a appears to play dual roles at these stages, signaling to the node and psm to regulate LR determination and somitogenesis.

### Discussion

We have identified Wnt3a as an important new component of the molecular LR determination pathway. Wnt3a plays an early role in this process by regulating the expression of the Wnt target gene Dll1 in the psm, which in turn activates the expression of the left determinant Nodal at the psm/node boundary, and regulates somitogenesis in the psm itself (Hrabe de Angelis et al., 1997). Despite the fact that Wnt3a transcription is limited to the primitive streak and posterior node, we demonstrate that Wnt3a protein can signal over long distances to directly stimulate gene expression in the node and anterior psm. We confirm and extend the findings of Aulehla et al. (Aulehla et al., 2003) by showing that Wnt3a is required for the oscillating expression of the Wnt target gene Axin2, as well as the Notch target Lfng, in the psm at the onset of somitogenesis. We have also presented evidence that Wnt3a may regulate the function of mechanosensory cilia in the node. Thus, Wnt3a regulates multiple target genes to simultaneously control LR determination and segmentation.

### Wnt signaling and organ laterality

Although posterior organs did not develop in Wnt3a mutants because of a requirement for Wnt3a for posterior development (Takada et al., 1994), laterality phenotypes in anterior viscera such as the heart, lungs and liver were assessed. The majority of E11.5-12.5 Wnt3a<sup>−/−</sup> embryos were heterotaxic, i.e. at least one organ displayed laterality defects. The laterality phenotypes were not secondary to the posterior truncation phenotype as aberrant gene expression in the node was observed well before AP phenotypes emerged. In fact, Dll1 and Nodal are two of the earliest known genes to be affected by the Wnt3a mutation and display aberrant expression prior to somitogenesis, arguing that Wnt3a, signaling via its target gene Dll1, regulates LR determination first, and somitogenesis and AP elongation second.

The process of cardiac looping determines the relative positions of the heart chambers and their connections with the aorta and pulmonary artery. Alterations in the direction of cardiac looping lead to alignment defects that result in a range of cardiovascular abnormalities such as TGA, PTA, double outlet right ventricle (DORV) and atrioventricular septal defects (AVSD) (Maclean and Dunwoodie, 2004). Several of these anomalies were observed in the Wnt3a<sup>−/−</sup>;Dll1 compound mutants, consistent with Wnt3a and Dll1 functioning in a common genetic pathway to regulate cardiac laterality.

The aberrant expression of Nodal in Wnt3a<sup>−/−</sup> embryos presents an opportunity to examine the importance of the timing and asymmetric nature of Nodal and Lefty signaling for organ laterality. As Nodal is required in the node to activate Lefty1 expression in the dorsal node and Nodal expression in the LPM (Brennan et al., 2002), we suggest that the reduced
levels of Nodal in the Wnt3a−/− node are insufficient to activate Lefty1 or Nodal at the two-somite stage when they are normally activated, but are sufficient to account for the delayed Nodal expression observed in the LPM. Lefty1 was never detected in the mutant PFP, indicating that the midline barrier to Nodal diffusion was absent, and providing an explanation for the bilateral expression of Nodal in the 3-somite stage LPM. Interestingly, left pulmonary isomerism was not observed in Wnt3a mutants, as would be predicted from the Lefty1−/− phenotype (Meno et al., 1998). Instead, the Wnt3a mutants more closely resembled cryptic mutants, which lack Nodal expression in the LPM and display randomized situs and right isomerism (Yan et al., 1999). As Nodal is not expressed bilaterally in the Wnt3a−/− LPM until the five-somite stage, these phenotypes suggest that asymmetric Nodal expression in the LPM must be established between the two- to four-somite stages (a 6-hour window) to establish proper LR asymmetry in anterior organs. Bilateral Nodal expression in the LPM after the five-somite stage appears to be insufficient to induce left isomerisms in any of the organs examined; however, it should be noted that Nodal expression in the LPM never extended anteriorly into the heart, as it did in wild-type embryos.

**Wnts and polycystins in LR determination**

The membrane receptor PC1 colocalizes with PC2 in renal mechanosensory cilia where it senses mechanical bending of the primary cilium induced by fluid flow, transducing it into a chemical Ca2+ flux by activating PC2 (Nauli et al., 2003). Kidney cells lacking PC1 form cilia but do not display Ca2+ influx when stimulated by fluid flow (Nauli et al., 2003) or activating antibodies (Delmas et al., 2004). Our observation that PC1 is co-expressed with PC2 in node mechanosensory cilia suggests that a similar regulatory relationship between PC1 and PC2 exists in node cilia. Embryos lacking Wnt3a display structurally normal node cilia that robustly express PC2 but display reduced levels of PC1. These results predict that Ca2+ asymmetry will be perturbed in the Wnt3a−/− node, despite the presence of PC2, and this will be addressed in future experiments. As PC1 and PC2 activity appear to be mutually dependent, and mutations in either Pkd1 or Pkd2 result in identical polycystic kidney disease phenotypes (Delmas, 2004), it seems likely that Pkd1 mutants will also display laterality defects and a loss of Ca2+ asymmetry. The cardiovascular defects observed in embryos homozygous for a targeted allele of Pkd1 (Boulter et al., 2001) are consistent with a role for Pkd1 in the regulation of cardiac laterality.

Despite reports in the literature that PKD1 is a direct target gene of Wnt/β-catenin signaling (Rodova et al., 2002), our results suggest otherwise. Examination of 5 kb of the mouse Pkd1 promoter revealed five consensus Tcf1-binding sites; however, activation of the Wnt/β-catenin pathway did not activate Pkd1 promoter luciferase reporter constructs in transient transfections in vitro (data not shown). Furthermore, mutational analysis showed that the Tcf sites were not necessary for basal expression. It is unclear how Wnt3a indirectly regulates ciliary PC1 expression; however, it is tempting to speculate that the mechanism involves inversin, another ciliary protein required for proper LR determination (Watanabe et al., 2003) that has recently been shown to bind dishevelled and regulate Wnt signaling (Simons et al., 2005).

**Wnt3a signaling and target gene expression in the node**

Although much of the LR phenotype observed in Wnt3a mutants can be directly attributed to the aberrant expression of Dll1 in the psm, and consequently of Nodal in the node, our data indicate that Wnt3a also directly regulates gene expression in the ventral node. Two independent Wnt/β-catenin reporters, as well as the Wnt/β-catenin target genes Nkd1 and Axin2, were expressed there. Interestingly, Nkd1 expression was asymmetric in the ventral node. The significance of this asymmetric expression, and the mechanisms underlying it, are presently unclear. Given that Wnt3a is symmetrically expressed in the streak and node, one possible mechanism, interpreted in the context of the morphogen flow model, is that the Wnt3a ligand itself becomes asymmetrically distributed at the ventral node surface by cilia-generated nodal flow. This hypothesis would require that Wnt3a, which is expressed by the dorsal node, is able to traverse the ventral node epithelium to reach the apical surface of the node where the cilia are located. This is unlikely to occur as the transverse movement of secreted molecules across an epithelial tissue is blocked by the tight junctions of the polarized epithelium. More importantly, this postulate is not supported by our data demonstrating that neither of the Wnt/β-catenin reporters, nor Axin2, were asymmetrically expressed in the node. Perhaps a more likely scenario is one in which Nkd1 is symmetrically activated in the node by Wnt3a, but Nkd1 mRNA becomes graded because of asymmetric localization or decay. Although Nkd1 has also been shown to exhibit oscillatory gene expression in the psm (Ishikawa et al., 2004), its function remains unclear as animals lacking Nkd1 do not display embryonic phenotypes (Li et al., 2005).

**Wnt3a is a major component of the trunk organizer**

Embryological studies performed primarily in amphibians, fish and chick have demonstrated that the Spemann-Mangold organizer is a dynamic structure that can be subdivided into head, trunk and tail organizers based on their distinct cell subpopulations and differing inductive capacities (Niehrs, 2004). In the mouse, evidence for the distinction of all three organizers remains relatively scant (Robb and Tam, 2004); however, a strong argument can be made for trunk organizer activity residing in the node: (1) transplantation experiments demonstrate that the node is sufficient to induce patterned ectopic trunks, but not heads (Beddington, 1994; Tam et al., 1997); (2) surgical ablation studies show that the node is necessary for DV and LR asymmetry, and proper segmentation and AP elongation of the prospective trunk, but is not required for AP polarity (Davidson et al., 1999). The timing of node formation, which occurs after AP polarity and head structures have been specified, but before LR determination and trunk development, is also consistent with the node functioning as a trunk organizer.

Our demonstration that Wnt3a is expressed in the node and is required for LR determination and segmentation, coupled with previous studies demonstrating that Wnt3a is required in a dose-dependent manner for the formation of the entire posterior trunk and tail (Greco et al., 1996; Takada et al., 1994), suggests that Wnt3a is a major component of the trunk organizer. We present a model for how Wnt3a could function in this capacity (Fig. 7). A source of Wnt3a is established at
E7.5 in the primitive streak and node progenitors at the posterior end of the gastrulating embryo. Wnt3a specifies mesoderm fates in the streak by directly regulating T transcription (Galecrer et al., 2001; Yamaguchi et al., 1999). Wnt3a also regulates Dll1 expression in the psm directly, and indirectly via T (Galecrer et al., 2004; Hofmann et al., 2004). Dll1 expression in the psm stimulates Notch activity at the psm/node boundary, to activate Nodal transcription in the node periphery (Krebs et al., 2003; Raya et al., 2003). Activation of Nodal in the lateral aspects of the node establishes an axis of asymmetric nodal expression and the direction of embryonic turning. Elevated Notch activity also activates Lfng in the node periphery, which could serve to restrict Nodal to the node periphery by inhibiting Notch in a negative-feedback loop (Dale et al., 2003).

The indirect regulation of PC1 expression in the node by Wnt3a may regulate the ability of mechanosensory cilia to interpret symmetry-breaking leftward nodal flow and generate asymmetric Ca\(^{2+}\) flux. As alterations in local Ca\(^{2+}\) concentrations can affect the affinity of Dll1 for its Notch receptor (Raya et al., 2004), then it is conceivable that Wnt3a concentrations can affect the affinity of Dll1 for its Notch receptor (Raya et al., 2004), then it is conceivable that Wnt3a concentrations can affect the affinity of Dll1 for its Notch receptor. Axin2 also regulates AP patterning in the trunk and tail by influencing Cdx1 and Hox gene expression (Lohnes, 2003). We propose that Wnt3a functions in the trunk organizer to coordinate patterning and morphogenesis along multiple body axes.

Our work implicates β-catenin as the primary transducer of canonical Wnt signals that coordinately regulate LR specification and segmentation. The use of conditional β-catenin alleles and Cre drivers that are expressed in the node or psm will help address the specific roles that β-catenin plays in the node during LR determination, and in the psm in the regulation of oscillating gene expression during somitogenesis.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/24/5425/DC1

**References**


**Fig. 7.** Wnt3a functions as a trunk organizer. The diagram depicts the ventral view of an E8 embryo. Wnt3a (red stippling) is expressed in the streak and node where it directly activates (solid blue arrows) T (brachyury), Dll1 and Axin2 via the Wnt/β-catenin pathway. Please see the text for details. Red N, Nodal; solid blue arrow, direct gene regulation; broken blue arrow, indirect regulation; green gradient, left-sided Ca\(^{2+}\) flux; curved black line, negative feedback loop. Ax, axonal mesendoderm.


Development


(1999). T (Brachyury) is a direct target of Wnt3a during paraxial mesoderm specification. *Genes Dev.* 13, 3185-3190.

