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and Axial Patterning Defects

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Patterning along the left/right axes helps establish the orientation of visceral organ asymmetries, a process which is of fundamental importance to the viability of an organism. A linkage between left/right and axial patterning is indicated by the finding that a number of genes involved in left/right patterning also play a role in anteroposterior and dorsoventral patterning. We have recovered a spontaneous mouse mutation causing left/right patterning defects together with defects in anteroposterior and dorsoventral patterning. This mutation is recessive lethal and was named *no turning (nt)* because the mutant embryos fail to undergo embryonic turning. *nt* embryos exhibit cranial neural tube closure defects and malformed somites and are caudally truncated. Development of the heart arrests at the looped heart tube stage, with cardiovascular defects indicated by ballooning of the pericardial sac and the pooling of blood in various regions of the embryo. Interestingly, in *nt* embryos, the direction of heart looping was randomized. *Nodal* and *lefty*, two genes that are normally expressed only in the left lateral plate mesoderm, show expression in the right and left lateral plate mesoderm. *Lefty*, which is normally also expressed in the floorplate, is not found in the prospective floorplate of *nt* embryos. This suggests the possibility of notochordal defects. This was confirmed by histological analysis and the examination of *sonic hedgehog*, *Brachyury*, and *HNF-3β* gene expression. These studies showed that the notochord is present in the early *nt* embryo, but degenerates as development progresses. Overall, these findings support the hypothesis that the notochord plays an active role in left/right patterning. Our results suggest that *nt* may participate in this process by modulating the notochordal expression of *HNF-3β*.

Key Words: **notochord; floorplate; *nodal; sonic hedgehog; Brachyury; HNF-3β; lefty;* left/right patterning; laterality; heart looping; mouse embryos; cardiovascular defects.**

INTRODUCTION

Patterning along the left/right (L/R) axes helps establish the orientation of visceral organ asymmetries, a process which when perturbed can lead to functional defects and mortality (Wood and Blalock, 1940; Zlotogora and Elian, 1981; Burn, 1991; Icardo and Sanchez de Vega, 1991; Britz-Cunningham *et al.*, 1995). At present, little is known about the mechanisms underlying the specification of the L/R axes (Yost, 1991; Fujinaga, 1997; King and Brown, 1997; Levin, 1997). In rodents, one of the early indicators of laterality is a clockwise axial rotation which converts the embryo from a lordotic to fetal position, a process occurring over the 8- to 13-somite stage and referred to as embryonic turning (Kaufman, 1992; Fujinaga, 1997). This is accompanied by the formation and right-sided looping of the heart tube, and followed much later, by the L/R asymmetric orientation of other forming visceral organs.

Although only a few genes have been identified that are likely important in L/R patterning, several of these are also known to play a role in patterning along the A/P and D/V axes. Thus several genes involved in A/P and D/V patterning are L/R asymmetrically expressed, and alteration in their expression is associated with defects in L/R patterning.

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This was first demonstrated in chick embryos, where asymmetric expression of *nodal, HNF-3β,* and *sonic hedgehog* (*shh*) as well as *activin receptor IIa* (*cAct-R11a*) was observed in or near Hensen's node, and/or in the lateral plate mesoderm (Levin *et al.,* 1995). When *shh* or activin was ectopically expressed, this disrupted the pattern of *nodal* expression, and also resulted in the randomization of heart looping (Levin *et al.,* 1995). In mouse embryos, *shh* is not asymmetrically expressed, and apparently is not required for left/right patterning (Chiang *et al.,* 1996). However, two genes, *nodal* and *lefty,* are expressed only in the left lateral plate mesoderm, with *nodal* also showing asymmetric expression in the node (Collingnon *et al.,* 1996; Lowe *et al.,* 1996; Meno *et al.,* 1996). Significantly, this distribution is altered in embryos with laterality defects, such as in the *inv* and *iv* mouse mutants with an inversion or randomization of heart looping, respectively (Hummel and Chapman, 1959; Layton, 1976; Yokoyama *et al.,* 1993; Collignon *et al.,* 1996; Lowe *et al.,* 1996; Meno *et al.,* 1996; Lohr *et al.,* 1997). Similarly, randomized heart looping induced by retinoic acid was found to perturb the asymmetric pattern of *nodal* expression (Smith *et al.,* 1997). Interestingly, a recent study reported laterality defects in mouse embryos doubly heterozygous for null mutations of *nodal* and *HNF-3β* (Collignon *et al.,* 1996), the latter being a transcription factor required for node and notochord formation (Ang and Rossant, 1994).

Together these results suggest that patterning along the A/P and D/V axes may be closely coordinated with the establishment of laterality. Studies in *Xenopus* and zebrafish embryos indicate that this may be mediated through the notochord, a structure already known to play an important role in A/P and D/V patterning. The zebrah fish mutant, *no tail,* harbors a mutation at the *Brachyury* (*T*) locus and exhibit randomized heart looping (Danos and Yost, 1996). *Brachyury* is a transcription factor normally expressed in the notochord and has been shown to be required for notochord maintenance (Herrmann and Kispert, 1994; Weinstein *et al.,* 1994). In such embryos, changes in the pattern of *nodal* expression preceded the heart looping defects (Lohr *et al.,* 1997). Other experiments using *Xenopus* embryos further showed that notochordal defects arising from various experimental manipulations also led to a randomization of heart looping (Danos and Yost, 1996).

Our present study suggests a role for the notochord in L/R patterning in the mouse embryo. We have recovered a mouse mutation affecting laterality, named *no turning* (*nt*). *nt* mutants exhibit randomization in the direction of heart looping and a number of other developmental defects. Examination of *nodal* and *lefty* expression shows expression in both the left and right lateral plate mesoderm. Histological analysis and the examination of *shh, Brachyury (T), lefty,* and *HNF-3β* expression showed that the notochord is formed, but undergoes degeneration. Based on these and other findings, we suggest that *nt* is required for the maintenance of the notochord and for the proper functioning of the notochord in early events involved in L/R patterning. We suggest that this may be mediated through *nt* modulation of *HNF-3β* expression. Overall, these findings further indicate a close linkage between L/R and axial patterning.

MATERIALS AND METHODS

Mouse Breeding, Embryo Collection, and Histology

CD-1 mice were obtained from Charles River Laboratory (Wilmington, DE). To obtain embryos, female mice were caged with males and checked daily for vaginal plugs (plug date, E0.5). To test new male *nt* carriers, offspring of a known male carrier were mated with at least three female littermates. At E8.0 through E11.5, pregnant females were sacrificed by cervical dislocation and embryos were collected. Embryos were then screened for the presence of *nt* mutant characteristics and photographed on a Wild stereomicroscope using Agfa200 color print film. For whole-mount *in situ* hybridization, embryos were fixed in 4% paraformaldehyde for 2 h at 4°C, while embryos used for histology were fixed in modified Carnoy's fixative (MacDonald and Tuan, 1989) for 3–4 h to overnight at −20°C. For histology, the embryos were paraffin-embedded, sectioned, and stained with hematoxylin and eosin.

Riboprobe Transcription and Whole-Mount in Situ Hybridization

Digoxigenin-labeled riboprobes for whole-mount *in situ* hybridization were generated in the following manner. T3 or T7 RNA polymerase was used for antisense probes according to the instructions of the supplier (Promega, Madison, WI). The polymerase used for each probe depended on the orientation of the fragment in the plasmid.

Whole-mount *in situ* hybridization was performed as previously described (Lowe *et al.* 1996). Paraformaldehyde-fixed embryos which had been stored for up to 2 weeks at −20°C in methanol were rehydrated in changes of 75, 50, and 25% methanol in PBT, treated with 10 mg/ml proteinase K and postfixed in 4% paraformaldehyde/0.1% glutaraldehyde. Embryos were then hybridized overnight at 68°C with digoxygenin-labeled riboprobe in 50% formamide, 0.75 M NaCl, 10 mM Pipes, 1 mM EDTA, 100 mg/ml tRNA, 0.05% heparin, 0.1% BSA, and 1% SDS. The next day, embryos were put through a series of washes, RNase treated, and blocked with goat serum. Then embryos were incubated overnight with preabsorbed alkaline phosphatase-conjugated sheep anti-digoxigenin Fab fragment (1:2500) and the detection of alkaline phosphatase activity was carried out histochemically using the NBT/BCIP color reaction.

RESULTS

No turning is a spontaneous mutation found in embryos collected from sibling matings of mice in the outbred CD-1 mouse background. We named this mutation *nt* since these embryos failed to undergo or complete embryonic turning (Fig. 1). It should be noted that the failure of *nt* embryos to undergo turning is not simply a matter of being developmentally arrested because these embryos typically can develop to the 20-somite stage, beyond the stage when

embryonic turning is normally completed (see Kaufman, 1992; also see below). In some embryos, there is an initiation of embryonic turning (Fig. 2A), but this never proceeds to completion (as indicated by lordotic position of E10.5 and 11.5 *nt* embryos; see Fig. 1).

The earliest time when *nt* mutants can be unambiguously identified is at E8.0 (Fig. 3). At this stage of development, they have a distinct rough appearance, such that the outlines of individual cells are clearly visible. In contrast, normal embryos usually exhibit a rather smooth contour (compare Figs. 3A and 3C). *nt* embryos are generally smaller in size than their normal littermates, even those at the same somite stages of development (Figs. 1 and 3). They appear to developmentally arrest at E9.5–10.0 (see below), but remain viable up to E11.5 (as indicated by a beating heart). Fusion of the allantois to the chorion is likely defective in *nt* embryos because the allantois readily detaches from the embryonic membranes during embryo dissection (see Fig. 1A). Breeding of putative *nt* carriers showed that this mutation was transmitted in a manner consistent with a completely penetrant-recessive mutation (Table 1). Thus approximately 25% of embryos from crosses of heterozygous carriers exhibited the *nt* phenotype (described below).

Neural Tube Defects

nt mutants exhibit a marked delay in neural tube closure. Typically neural tube closure is not yet initiated in *nt* embryos with as many as 10–12 somites (Fig. 3D). There is also a considerable delay in the elevation of the neural folds, with the rostral neural plate usually exhibiting a somewhat flattened appearance (Fig. 3D). Examination of older embryos (E9.5 and 10.5) revealed neural tube closure defects, with some showing a failure of the neural folds to fuse along the midbrain/anterior hindbrain region (Figs. 2B and 2D). In these older embryos, otic vesicles can be seen, but optic eminences could not be detected (Figs. 1C, 2D, 2C, 2E, and 2F). Also evident is a reduction in the size of the branchial arches and the frontonasal process (see Figs. 1C and 1D, compare with Figs. 1A and 1B; see Figs. 3C, 3D, 3H, and 3I, compare with 3A, 3B, 3E, and 3F).

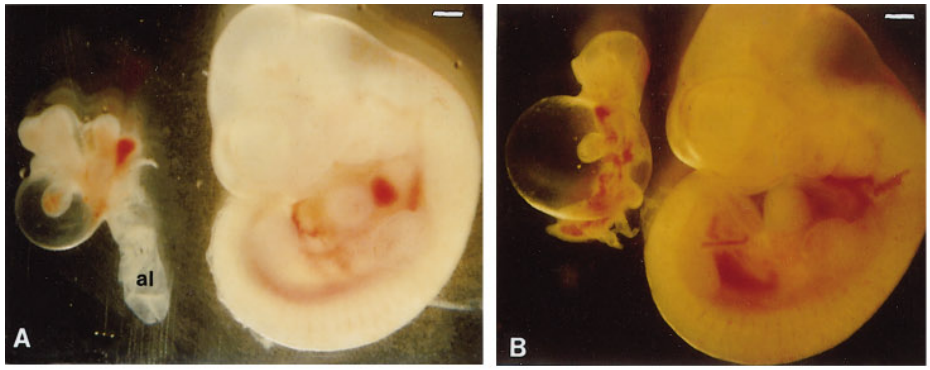
The rostral neural tube in *nt* embryos shows subdivision into the primary brain ventricles, including a recognizable forebrain, midbrain, and hindbrain (Figs. 1–3). Histological analysis indicates that this included rhombomeric segmentation of the hindbrain (Figs. 4H–4J). Examination of *Otx2* expression in embryos at E8.0 (Figs. 3E–3J) and E10.5 (data not shown) showed the expected localization of *Otx2* to the presumptive forebrain and midbrain (Simeone *et al.*, 1992, 1993). Nevertheless, it should be noted that the overall shape of the brain ventricles in *nt* embryos appeared abnormal (Figs. 1–3, and 4H–4J), with some having a ''Snoopy''-like appearance as a result of the expansion of the rostral cranial cavities (Fig. 2E). Also, in E10.5 *nt* embryos, histological analysis showed no evidence of a floorplate (Figs. 4A and 4B; also see below), even though a floorplate was observed in E8.5 embryos (not shown). Examination of histological sections further revealed notochordal defects in the E10.5 embryos. Thus, a rod-like notochordal structure was found only in some regions of the embryo (Fig. 4C). Because an intact notochord was apparent earlier in development (E8.5–9.0; see below), it is likely that the notochord degenerates and becomes discontinuous as development progresses.

Abnormal Somites in nt Embryos

nt embryos contained a maximum of 20 somites, even at E10.5 and E11.5 when 25–35 somites would have been expected (Fig. 1). This would suggest that *nt* embryos de-

FIG. 1. *nt* mutant embryos at E10.5 and E11.5. (A, B) *nt* mutant (left) and a normal littermate (right) at E10.5 (A) and E11.5 (B). The *nt* embryos show little change in size or overall appearance between E10.5 and E11.5. Note that the *nt* embryos in (A) and (B) were both lordotic in position while in the amniotic sac. Very striking is the swelling of the pericardial sac. Unlike normal embryos, the allantois of *nt* embryos easily detaches from the placenta (see al in A). (C and D) Enlarged views of *nt* embryos showing normal (C) and reversed (D) direction of heart looping (as indicated by curved arrows). Note the bird-like appearance of the embryos as a result of the failure to undergo embryonic turning. The *nt* embryo is caudally truncated, with no axial development posterior of the forelimb bud (lb), except for a primitive tailbud (t). forebrain (f), midbrain (m), and hindbrain (h), otic vesicle (o), branchial arch (ba). forelimb bud (l). Scale bars, 500 mm.

FIG. 2. Craniocaudal development in *nt* embryos. (A). An E9.5 *nt* embryo still enclosed within the amniotic sac shows partial turning. (B, C) Side (B) and frontal (C) view of a E9.5 *nt* embryo showing failure of neural tube closure in the midbrain/anterior hindbrain region (black arrowheads in B). A blister can be seen along the edge of the neural tube (white arrows in B). Note the reduced size of the frontonasal process (see f in C). This embryo exhibited more extensive caudal development, with some evidence of hindlimb bud (l) formation (indicated by open arrowheads in C). The forelimb extends in a dorsal rather than ventral direction (indicated by white arrowheads in C). (D) Frontal view of a E9.5 *nt* embryo showing failure in neural tube closure associated with an abnormal elevation of the neural folds (black arrowheads). (E) An E9.5 *nt* embryo with a ''Snoopy''-like appearance. Notice the absence of the cephalic flexure spanning the dorsal forebrain/midbrain region. Such embryos typically exhibit very severe caudal truncation, such that there is little axial development beyond the forelimb bud. (F) The midbrain (m), hindbrain (h), and otic (o) vesicles can be clearly observed in a dorsal view of this E9.5 *nt* embryo. This perspective shows the unusual orientation of the forelimb (l), which appears to project dorsally rather than ventrally (white solid arrows). This embryo also shows a bump indicative of hindlimb bud formation (see open white arrow). B, C, and E are of the same magnification, and D and F are of the same magnification. Scale bar, 500 mm.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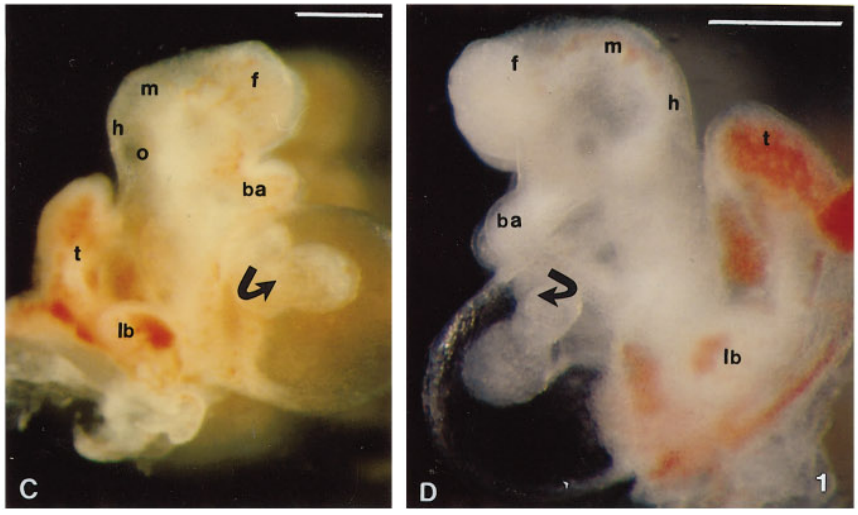
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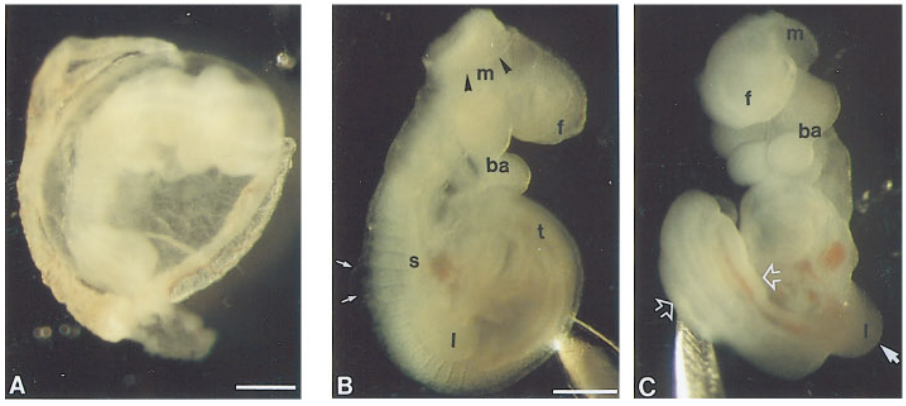
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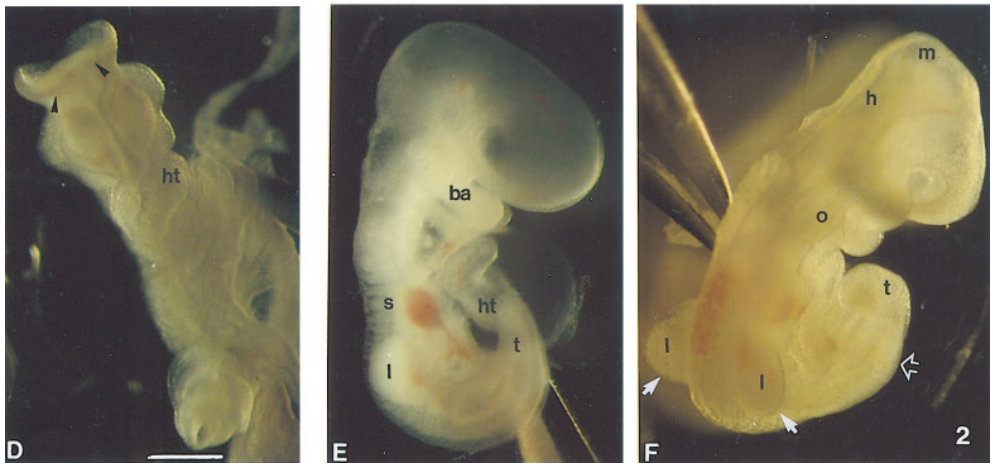
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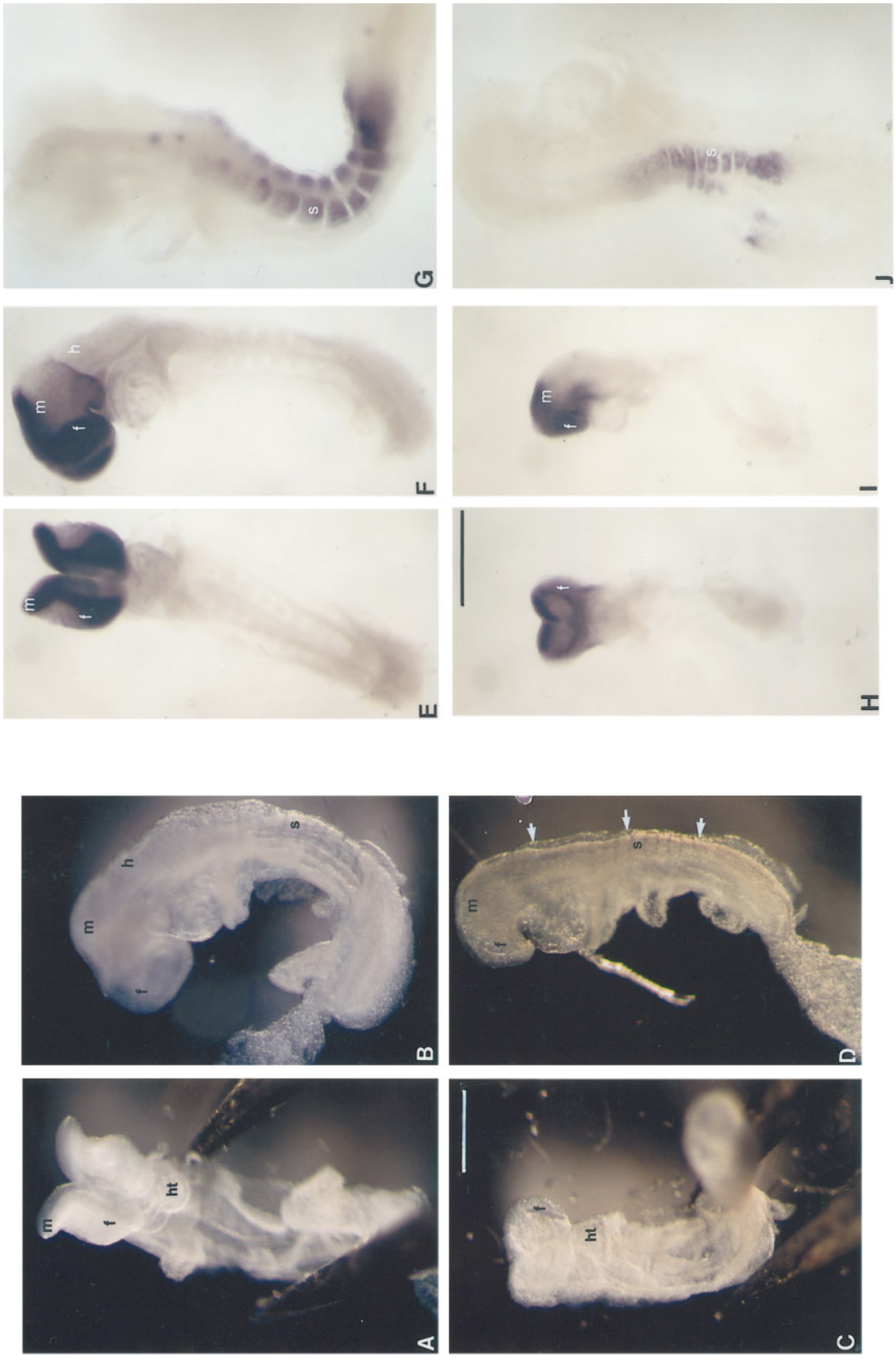

TABLE 1
Incidence of the *No turning* Mutation

Stage	No. of litters	No. of embryos	No. of *nt* embryos	% *nt* embryos
E8.0	12	128	32	25.0
E8.5	6	82	15	18.3
E9.5	6	65	14	21.5
E10.5	4	47	17	36.2
E11.5	1	10	3	30.0
Total	30	332	81	24.4

velopmentally arrest at E9.5. Somites in *nt* embryos were typically irregular in shape and appeared smaller in size compared to somites of normal embryos at the same somite stage of development (see Fig. 3B vs Fig. 3D; also see Fig. 3J). Nevertheless, the somites of *nt* embryos exhibited a normal pattern of expression of the somite marker, *paraxis* (Burgess *et al.*, 1995) (see Fig. 3J, compare with 3G). Histological analysis of E10.5 *nt* embryos showed that the somites remained as epithelial somites and showed no evidence of dermamyotome or scleratome differentiation (Fig. 4D).

Caudal Truncation of nt Embryos

Examination of embryos at E9.5 to 11.5 showed that the posterior region of *nt* embryos was truncated to varying extent (Figs. 1 and 2). In the most severely affected embryos, there was only a small undifferentiated tail bud, with no distinguishable structures found caudal of the forelimb bud (see t in Fig. 2E; Figs. 1C and 1D). At E10.5, these embryos exhibited a distinct bird-like posture (Figs. 1C and 1D). It is interesting to note that the forelimb buds of *nt* embryos typically were flexed dorsally rather then ventrally (Figs. 1C, 1D, 2C, 2E, and 2F).

In some embryos, there appears to be more extensive caudal development (Figs. 2B, 2C, and 2F). One indication of this is the finding of bumps laterally along the body wall where presumptive hindlimb buds might be expected (Figs. 2C and 2F). These differ from normal hindlimb bud swelling, as usually there were multiple bumps (Figs. 2C and 2F). Interestingly, embryos with more extensive caudal development were associated with those that initiated embryonic turning (Fig. 2A). Embryos exhibiting this modified phenotype were found only in recent matings of *nt* carriers. Given that the *nt* mutation originated in the outbred CD1 background, it is possible that this variability in caudal development reflects the segregation of genetic modifiers upon continued inbreeding of the *nt* carriers.

Defects in Heart Development

Even though *nt* embryos survive to E11.5, heart development is arrested at the looped heart tube stage (Fig. 1). They exhibit a sporadic heartbeat, and in many embryos, there is a ballooning of the pericardial sac. Pools of blood are often seen in various parts of the embryo, such as in the limb and tail buds (Figs. 1C and 1D). This is observed in living embryos with beating hearts. Histological analysis demonstrated that *nt* hearts have a normal organization, including the presence of an endocardial, myocardial, and epicardial layers (Fig. 4F). However, an unusual fibrous matrix deposit was observed in the heart (Fig. 4G). Overall, these observations suggest cardiovascular function is abnormal, and may be accompanied by defects in vasculogenesis. These abnormalities may account for death of the *nt* embryos at E11.5.

Another anomaly in heart development was the finding of left rather than right-sided looping of the heart tube in some *nt* embryos (Fig. 1C vs Fig. 1D). Screening of several litters of *nt* embryos showed that heart looping was randomized, since approximately 50% of embryos displayed a reversal in the direction of heart looping (Table 2). This laterality defect led us to investigate expression of *nodal* and *lefty*. Both genes are normally expressed transiently in the left lateral plate mesoderm, a region containing the cardiac mesodermal precursors (Collignon *et al.*, 1996; Lowe *et al.*, 1996; Meno *et al.*, 1996). Previous studies showed that *nodal* is expressed at the 2- to 8-somite stage, while *lefty* can be seen in embryos with a few somites (Collignon *et al.*, 1996; Lowe *et al.*, 1996; Meno *et al.*, 1996). In addition, *lefty* is also expressed in the floor plate over this same window of time in development (Meno *et al.*, 1996). In *nt* embryos, *nodal* expression is variable, as individuals may show expression on the right side, left side, or on both sides (Figs. 5A and 5B) (Table 3). *lefty* expression was found bilaterally

FIG. 3. Phenotype of early *nt* embryos. (A–D) At E8.5, *nt* embryos (C, D) are smaller in size and exhibit a grainy overall appearance compared to normal littermates (A, B) at the same somite stage of development. Note the reduced size of the head, especially in the frontonasal region (f). Also striking is the smaller size of the somites (s) in *nt* embryos (compare somites in B vs D). The neural folds along the entire craniocaudal axis of the *nt* embryo are wide open (see white arrows in D). In contrast, neural tube closure is almost complete in the normal littermate (except over the midbrain/anterior hindbrain; B). In this *nt* embryo, heart looping is reversed from that seen in the normal embryo (compare ht in C vs A). (E–J) *Otx2* and *paraxis* expression in *nt* embryos at E8.0. (E–I) Whole-mount *in situ* hybridization analysis revealed *otx2* expression in the rostral neural tube of *nt* embryo (H, I). The pattern of expression is similar to that of normal embryos (E, F), indicating expression associated with the presumptive forebrain (f) and midbrain (m). (G and J) Expression of *paraxis* in somites was examined by whole-mount *in situ* hybridization analysis. Note the reduced size of somites, and their irregular shape in the *nt* embryo (J) as compared to the normal embryo (G). Scale bar, 500 mm. All images are at the same magnification.

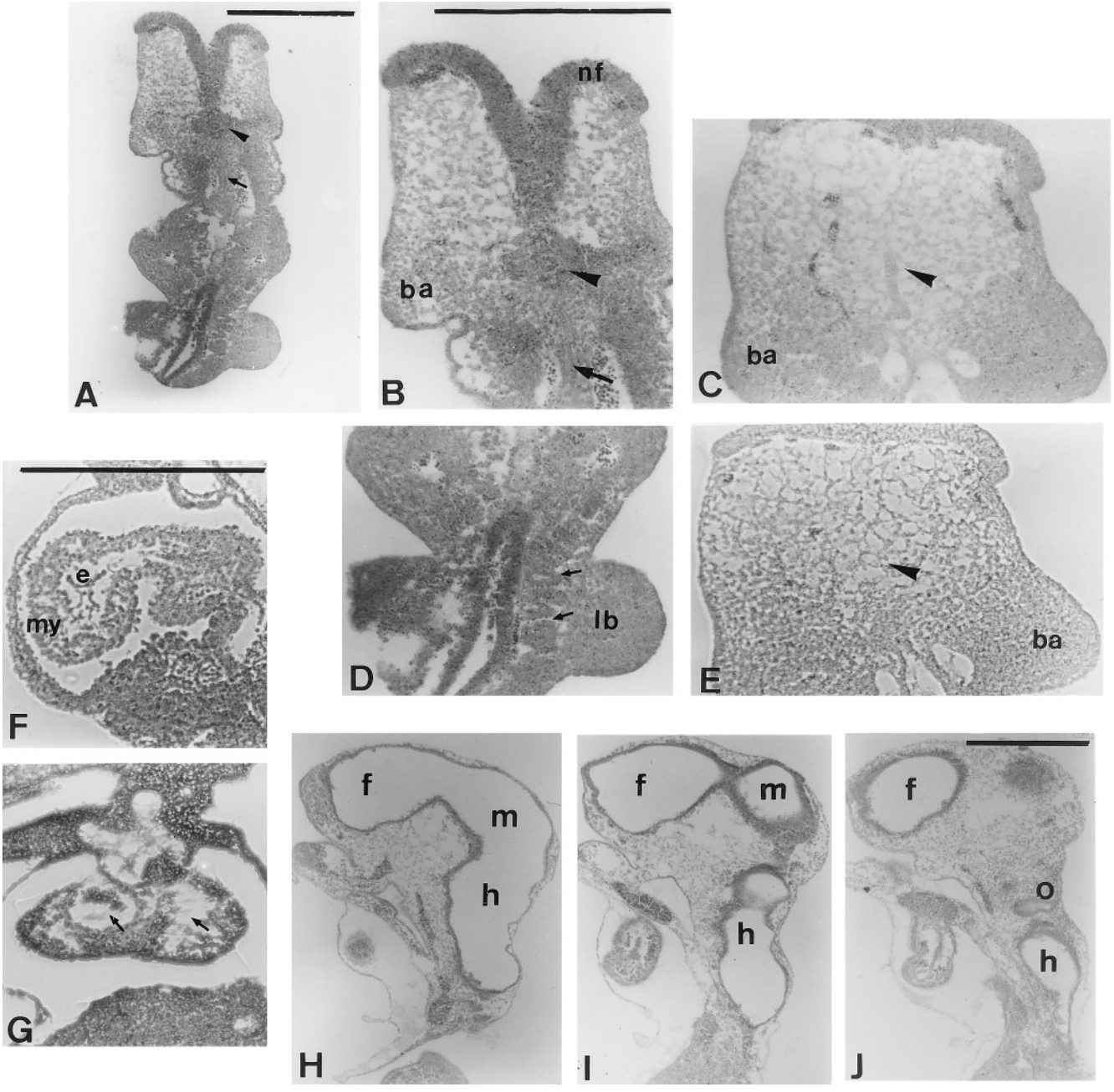

FIG. 4. Histological analysis of E10.5 and E11.5 *nt* embryos. (A, B, D) Frontal section of an E10.5 *nt* mouse embryo (A) showing an open neural plate spanning the midbrain region. At higher magnification in B the head region is shown, and in D, the tail region of the same embryo. In the ventral region of the neural fold (nf) (see arrowhead in B), there is an aggregate of disorganized tissue, which is neither recognizable as a floorplate nor as the notochord. A rod-like notochordal structure is visible just ventral to this disorganized tissue (indicated by arrow in A and B). Caudally, the forelimb (lb in D) and irregularly shaped somites (arrows in D) can be seen. (C, E) Two sections of the same *nt* embryo as in A, but at a more caudal level. A rod of tissue (arrowhead in C) is observed that appears to extend from the disorganized knot of tissue seen more anteriorly in A. Notice that in E this rod is no longer visible (region indicated by arrowhead). (F and G) Sections of hearts from E11.5 (F) and E10.5 (G) *nt* embryos. The primitive heart tube shown in F shows the presence of trabeculated myocardium (my) and endocardium (e). Very unusual was the the presence of fibrous deposits which criss-cross the cavity of the heart (indicated by arrows). (H, I, J) Nonadjacent sagittal sections of an E11.5 *nt* embryo exhibit a neural tube morphology consistent with the presence of a forebrain (f), midbrain (m), and hindbrain (h) compartment. Note the presence of the otic vesicle (o). Scale bar, 500 mm. F and G are of the same magnification, and C, D, E, H, I, and J are of the same magnification as B.

TABLE 2
Direction of Heart Looping in *nt* Embryos

Developmental stage	No. of *nt* embryos	*nt* embryos with left-sided looping
E8.5	30	15
E9.5	4	2
E10.5	12	3
E11.5	3	2
Total	49	22

TABLE 3
Expression of *nodal* in *nt* Embryos

Lateral plate mesoderm	No. of embryos
Right side only	1
Left side only	1
Both sides	5
Neither side	4

in 4 of 7 *nt* embryos examined (3- to 5-somite stage). In two of these, *lefty* expression was asymmetric, one showing higher expression in the left (Fig. 5E) and the other in the right lateral plate mesoderm (Fig. 5D). In the remaining three *nt* embryos, no *lefty* expression was observed. However, in the prospective floor plate, no *lefty* expression was detected in any *nt* embryos, even those with *lefty* expression in the lateral plate mesoderm (Figs. 5D and 5E; compare to Fig. 5C).

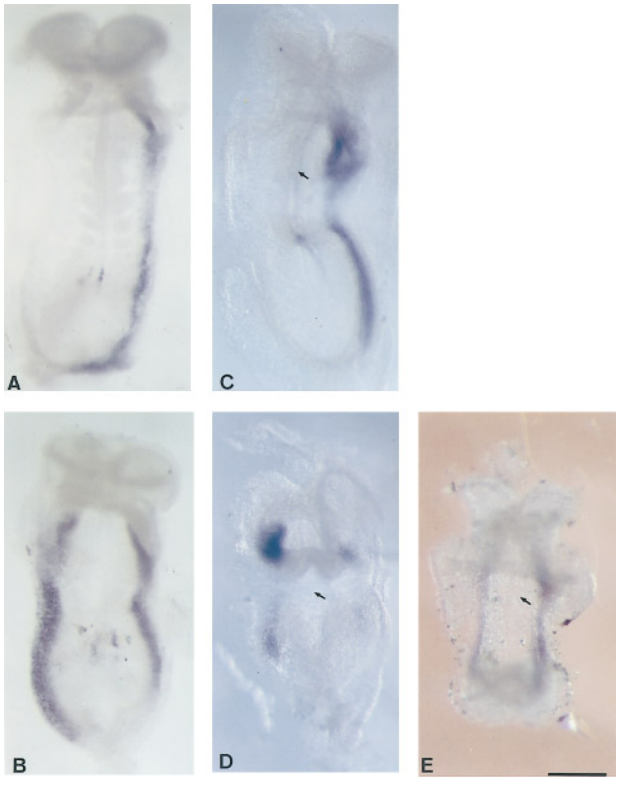

FIG. 5. Whole-mount *in situ* hybridization analysis shows perturbation of *nodal* and *lefty* expression in *nt* embryos. (A, B) Expression of *nodal* in the lateral plate mesoderm of normal (A) and *nt* (B) embryo (ventral view). Notice the bilateral expresion of *nodal* in the *nt* embryo. (C–E) *lefty* is bilaterally expressed in the lateral plate mesoderm of *nt* embryos (D, E), but notice the absence of *lefty* expression in the presumptive floorplate of these same embryos (region denoted by arrows). In contrast, in the normal embryo (C), *lefty* was expressed in the left lateral plate mesoderm and in the presumptive floorplate (see black arrow in C). All images are at the same magnification. Scale bar, 250 mm.

Notochordal Defects in nt Embryos

Given the importance of the notochord in floor plate induction and L/R patterning, we further examined expression of a number of genes known to be important in notochord development, including *Brachyury*, *shh*, and *HNF-3β*. *Brachyury* is also of interest because it is involved in the formation of the tail bud (Herrmann and Kispert, 1994). *Brachyury* expression in *nt* embryos appears normal in E8.0 embryos (<7–10 somites) (Fig. 6D). However, by E8.5 (>7–10 somites), *nt* embryos displayed a bifurcation and fragmentation or ''beading'' of *Brachyury* expression from the level of the heart to about the third somite, suggesting that the notochord in this region may be disintegrating (Fig. 6B, compare to 6A). This may account for the histological sections showing little or no notochord at E10.5 (see Figs. 4A, 4B, and 4D). The same expression pattern was observed for *shh*, a signaling molecule expressed in the notochord and floorplate (Fig. 6C) (Echelard *et al.*, 1993; Krauss *et al.*, 1993; Riddle *et al.*, 1993; Roelink *et al.*, 1994). The analysis of histological sections of E8.5 *nt* embryos hybridized with the *shh* probe showed *shh* expression in the presumptive floorplate. Interestingly, *HNF-3β*, a transcription factor expressed in the notochord and floorplate (Figs. 6E and 6G), was not detectable in the notochord or presumptive floorplate of E8.0 *nt* embryos (Figs. 6F and 6H). This is in contrast to normal *Brachyury* and *shh* expression in *nt* embryos at these same early stages of development (Figs. 6B and 6C; also data not shown).

DISCUSSION

Our studies show that *nt* is a recessive mutation affecting L/R and axial patterning in the mouse embryo. Embryos die by E11.5 and show a variety of developmental defects. They fail to undergo embryonic turning and exhibit caudal truncation of the body axis, notochordal degeneration, and ran-

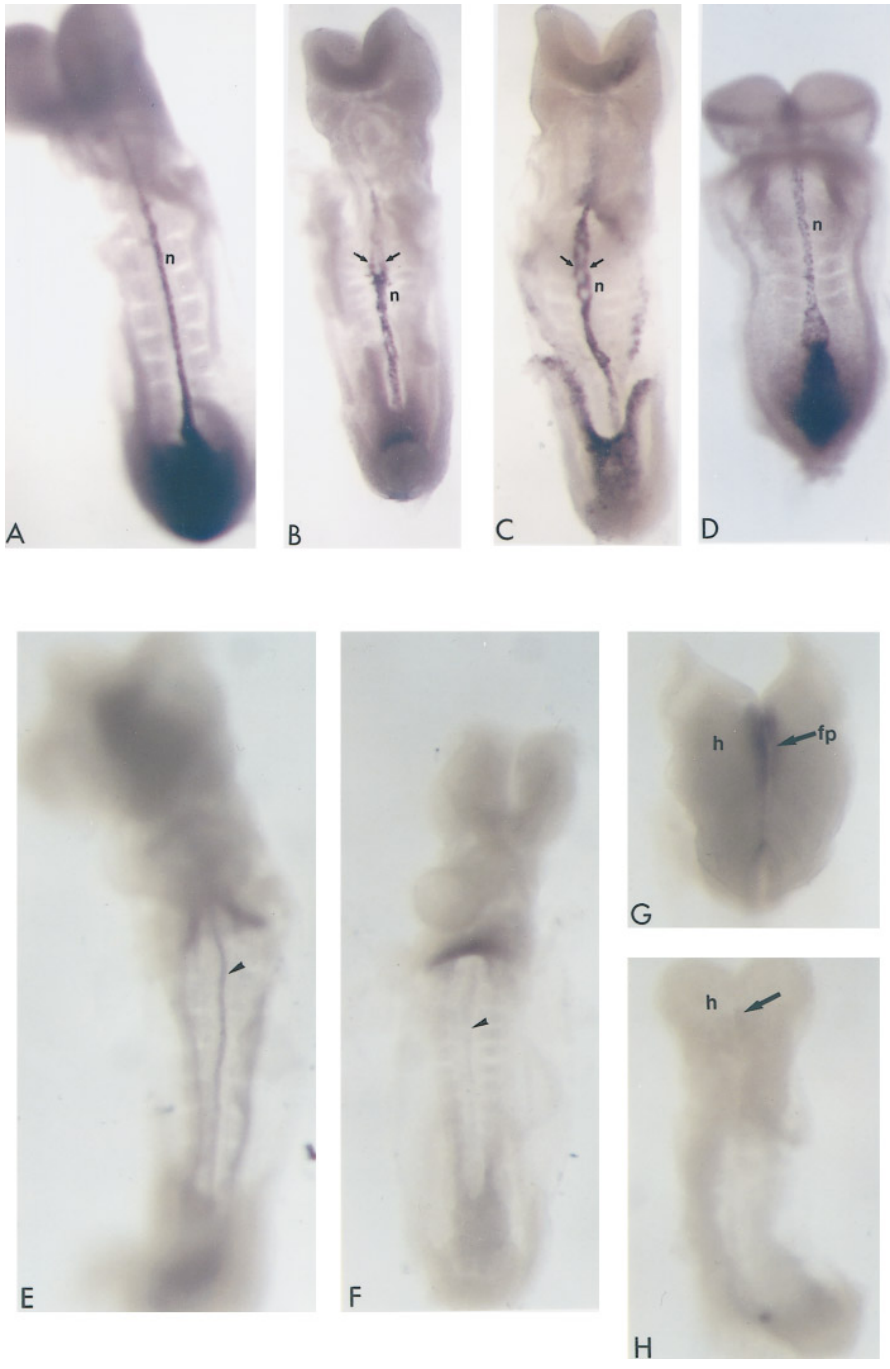

FIG. 6. Whole-mount *in situ* hybridization analysis of *Brachyury*, *shh*, and *HNF-3β* expression indicates notochordal defects in *nt* embryos. (A) In a normal E8.5 embryo, *Brachyury* expression is found in abundance in the notochord (n) and tail bud. (B) In an E8.5 *nt* embryo, the pattern of *Brachyury* expression indicates bifurcation and fragmentation of the notochord (n) (see arrows). (C) *shh* whole-mount *in situ* hybridization analysis of an *nt* embryo showed a similar pattern of expression to that seen with *Brachyury*, that is bifurcation of the notochord (n) (see arrows). (D) An E8.0 *nt* embryo shows a continuous line of *Brachyury* expression along the axial midline, thus indicating an intact notochord (n) at this earlier stage of development. (E–F) *HNF-3β* expression in an E8.5 normal and *nt* embryo. Ventral view of a normal embryo (E) show *HNF-3β* expression in the notochord (see arrowhead), while an *nt* embryo (F) at the same somite stage of development showed little or no detectable *HNF-3β* expression (see region indicated with an arrowhead). (G, H) Dorsal view of the hindbrain (h) region of a normal embryo (G) (prior to completion of cranial neural fold closure at E8.0). Note *HNF-3β* expression in the presumptive floorplate (fp) (see arrow in G). In contrast, dorsal view of an *nt* embryo showed no detectable *HNF-3β* expression over the same region of the hindbrain (h) (see arrow in H). Scale bar, 250 mm. All images are of the same magnification.

domized heart looping. The laterality defect is accompanied by changes in the asymmetric pattern of *nodal* and *lefty* expression in the lateral plate mesoderm. In addition, expression pattern of *HNF-3β, shh, lefty,* and *Brachyury* exhibit changes indicative of notochordal defects. Although *nt* mutants cannot be unambiguously identified before E8.0, at earlier stages of development (E7.5–8.0), smaller or developmentally retarded embryos are noted in embryo collections obtained from the mating of presumed *nt* carriers. It is interesting to note that whole-mount *in situ* hybridization analysis of one of these small embryos in an E7.5–E8.0 *nt* litter exhibited bilateral *lefty* expression (Lo, unpublished observations).

At present only a few mouse mutations are known to affect L/R patterning, such as *iv, inv, legless, Mgat-1,* and *fused toes* (Hummel and Chapman, 1959; Layton, 1976; Singh *et al.,* 1991; Yokoyama, *et al.,* 1993; Metzler *et al.,* 1994; van der Hoeven *et al.,* 1994). Two of the more extensively characterized are the *iv* and *inv* mutants. *inv* mutants exhibit a reversal of heart looping, while *iv*/*iv* mice show randomization of heart looping (Hummel and Chapman, 1959; Layton, 1976; Yokoyama *et al.,* 1993). In *iv*/*iv* mutants, the pattern of *nodal* expression in the lateral plate mesoderm is similar to that found in *nt* mutants—unilateral, on both sides, or neither side (Lowe *et al.,* 1996). In contrast, in *inv* mutants, *nodal* expression is inverted (Meno *et al.,* 1996). However, it should be noted that the *nt* mutation is much more pleiotropic in its effects than *iv*. *iv/iv* mice are viable and otherwise develop normally outside of the laterality defects (Hummel and Chapman 1959; Icardo and Sanchez de Vega 1991; Layton, 1976). There are also marked differences in *lefty* expression in *nt* vs *iv* (and *inv*) mutants. In *nt, lefty* was either not expressed in the lateral plate mesoderm, or it was expressed bilaterally. In contrast, half of the *iv/iv* mutants and presumably all of the *inv/inv* mutants showed right-sided expression of *lefty* (Meno *et al.,* 1996). In addition, unlike *inv* and *iv* mutants, no floor plate expression of *lefty* was found in *nt* mutants. Given the difference in phenotype between *nt* and other mutations affecting L/R patterning, it seems unlikely that *nt* is an allele of these previously characterized mutations affecting laterality. However, ultimately only complementation analysis, or chromosome localization and positional cloning will confirm whether *nt* is truly a novel mutation/locus.

Abnormal Notochord Function in nt Mutants

It is striking that the phenotype of *nt* embryos shows overlap with mouse mutations exhibiting notochordal defects. For example, the gain of function *Brachyury* allele, T^{wis}, has a normal-appearing notochord at E8.0, which subsequently undergoes fragmentation between E8.5 and E9.5 (Conlon *et al.,* 1995). In some T^{wis} heterozygotes, the notochord becomes bifurcated in the posterior region, a phenotype reminiscent of that observed in the *nt* mice. *Brachyury* mutants also have been reported to have heart defects (see discussion below) and defects in the formation of the vasculature (Rennebeck *et al.,* 1995; Danos and Yost, 1996; Sumoy *et al.,* 1997). However, *nt* is distinct from *Brachyury* because heterozygous *nt* embryos do not show the tail defects typically associated with *T* mutations.

In *nt* mutants, initially a morphologically normal notochord is formed, complete with *Brachyury* and *shh* expression. Consistent with a functional notochord is the finding in *nt* embryos of *shh* expression associated with the presumptive floorplate, a structure dependent on inductive signals from the notochord (Jessell and Dodd, 1992; Placzek, 1995). However, because *lefty* expression never comes on in the presumptive floorplate, this would suggest that the notochord is functionally abnormal, even prior to any evidence of notochordal degeneration. Further consistent with this possibility is the fact that little or no *HNF-3β* expression is observed in the notochord and floorplate of *nt* embryos. Together these results suggest that *nt* is required for normal notochordal function, and that this may involve a role in the maintenance of *HNF-3β* expression in the notochord (Fig. 6). It should be noted that although *HNF-3β* expression in the early *nt* embryo has not been demonstrated (since we cannot identify *nt* embryos prior to E8.0), we expect that *HNF-3β* expression is initiated in a normal fashion because genes dependent on *HNF-3β,* such as *shh* and *Brachyury,* are expressed normally in *nt* embryos (Fig. 6). In the future, mapping of this mutation and identification of appropriate DNA markers will make it possible to unambiguously identify and study *nt* mutants at earlier stages of development.

Notochord and Laterality Specification

The importance of the notochord in the specification of heart laterality has been indicated by recent studies in *Xenopus* and zebrafish embryos. Randomized heart looping was found with notochordal extirpation in *Xenopus* embryos, and also in zebrafish mutants with notochordal defects (Danos and Yost, 1996). Also of interest is the observation from the study of conjoined twins in chick embryos which suggested that cell signaling molecules involved in L/R patterning, such as *shh* or *nodal,* can act over long distances, but may be prevented from doing so by a barrier in the midline of the embryo (Levin *et al.,* 1996). That the notochord may provide barrier function also has been suggested for the specification of laterality in the distribution of endothelial cells in avian embryos (Klessinger and Christ, 1996). It is significant that *nt* embryos possess an morphologically intact notochord during the time when laterality is established (at the neural plate stage; see Fujinaga and Baden, 1991). This would suggest that the notochord is not functioning simply as a passive physical barrier, but rather genes such as *nt* may provide the notochord with an activity or activities important in L/R and axial patterning. Studies using lateral plate mesoderm explant cultures suggest that one such activity may serve to inhibit *nodal* expression in the lateral plate mesoderm (Lohr *et al.,* 1997).

The importance of the notochord in L/R patterning suggests a linkage between laterality and patterning along the A/P and D/V axes. In the *nt* mutant, laterality defects are in fact accompanied by defects along the A/P and D/V axes. Such linkage is also suggested by the finding of laterality defects in *Xenopus* embryos in which dorsoanterior development was disrupted by treatment with UV irradiation (Danos and Yost, 1995). Such studies showed a quantitative correlation between laterality and dorsoanterior defects. In another study in which dorsoanterior development was perturbed with the ectopic expression of *Vgl*, a signaling molecule involved in early events in dorsoanterior patterning in *Xenopus* embryos, L/R patterning was not effected (Hyatt *et al.*, 1997). However, in such embryos, notochord development was found to be normal.

The Role of nt in Laterality Specification and Notochord and Floorplate Development

We propose that the role of *nt* in L/R patterning may arise from its modulation of notochordal expression of *HNF-3β* (and perhaps other genes) (Fig. 7). This possibility is consistent with the previous observation of laterality defects in double heterozygous *HNF-3β/nodal* knockout embryos (Collignon *et al.*, 1996). The latter study together with results from the analysis of the *nt* mutant would suggest that the level of *HNF-3β* expression in the notochord is of critical importance to L/R patterning. Notochordal expression of *HNF-3β* may, directly or indirectly, regulate or help establish the asymmetric expression of *nodal* and *lefty* in the lateral plate mesoderm. In future studies, it will be interesting to examine double heterozygous mutant combinations consisting of *nt* and the *HNF-3β* (or *nodal*) knockout alleles. The latter studies may provide further insights into the role of *nt* in the specification of laterality.

The floorplate defects in *nt* embryos may result secondarily from defects in the notochord. Alternatively, *nt* may have separable roles in notochord and floorplate development. It should be noted that the phenotype of the *nt* mutant would suggest *shh*, though necessary (Chiang *et al.*, 1996), is not sufficient for normal floorplate development. The fact that early *nt* embryos exhibited an intact notochord complete with *shh* and *Brachyury* expression would suggest that *nt* acts downstream of the initial events involved in the induction of the floorplate. Consistent with this possibility is the fact that histological analysis and the examination of *shh* expression demonstrated the presence of a presumptive floorplate in early but not late *nt* embryos. However, as *lefty* failed to be expressed in the presumptive floorplate of early *nt* mutants (prior to when *shh* expression is initiated in the floorplate), this would suggest that the floorplate in *nt* mutants is abnormal.

Cell and Matrix Adhesion and the nt Mutation

It is intriguing that *nt* mutants share some characteristics with embryos deficient in extracellular matrix or cell adhesion molecules. Thus mouse embryos lacking the α_4 integrin (Yang *et al.*, 1993) or the adhesion molecule VCAM-1 (Gurtner *et al.*, 1995; Kwee *et al.*, 1995) are defective in chorioallantoic membrane fusion, while those without α_5 integrin show leaky blood vessels, somite defects, and truncation of posterior structures (Yang *et al.*, 1995). Among other defects, mouse embryos lacking fibronectin have a truncated A/P axis, neural tube defects, and reduced head mesenchymal tissue (George *et al.*, 1993). These parallels, together with the rough appearance of early *nt* embryos and the presence of matrix deposits in the heart, suggest that the *nt* mutation could affect some aspect of cell/matrix adhesion. This possibility is particularly intriguing given previous studies showing that the perturbation of the extracellular matrix near the site of heart tube formation can cause randomization of looping (Yost, 1992). Moreover, two recent studies in chick have revealed asymmetric expression of several extracellular matrix molecules during heart

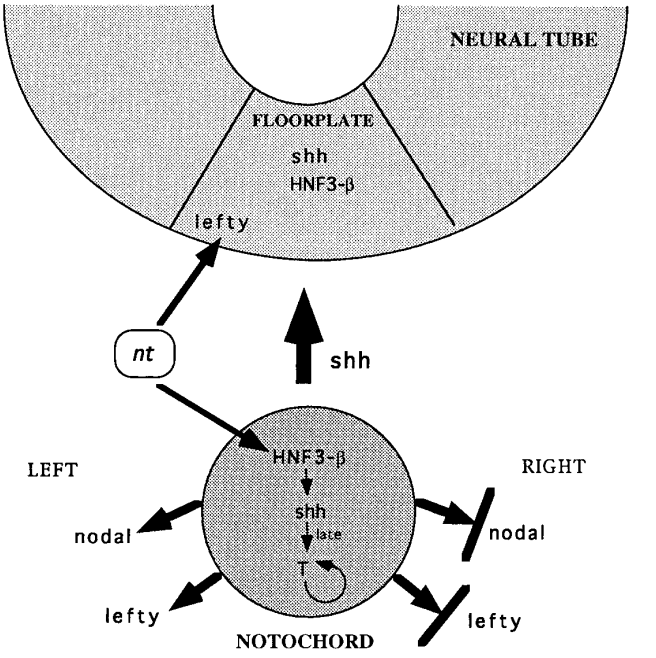

FIG. 7. A model showing the role of *nt* in notochord and floorplate development, and the specification of laterality. The requirement for *nt* in notochord development likely involves a role in the maintenance of *HNF-3β* expression. This modulation of notochordal expression of *HNF-3β* (and perhaps other genes) may underlie the role of *nt* in left/right patterning, a possibility suggested by the fact that mouse embryos double heterozygous for the *HNF-3β/nodal* knockout alleles exhibit laterality defects (Collignon *et al.*, 1996). The requirement for *nt* in floorplate development may involve steps downstream of the induction mediated by *shh*. This is indicated by the finding that *nt* embryos initially exhibit an intact notochord complete with *shh* and *Brachyury* expression. The fact that *nt* mutants fail to express *lefty* in the floorplate would suggest that *shh* is necessary but not sufficient for normal floorplate development.

morphogenesis (Tsuda *et al.*, 1996; Smith *et al.*, 1997). In this context, it is interesting to note that extracellular matrix abnormalities also have been reported in *Brachyury* mutants (Jacobs-Cohen *et al.*, 1983). Whether the matrix or cell adhesion perturbations in *nt* mutants are secondary or primary defects remains unknown and must await the mapping and positional cloning of this mutation. Such studies are time-consuming not only because *nt* is a recessive mutation, but also because *nt* was derived from mice of a heterogeneous genetic background; thus, mapping of *nt* mutation will first require breeding of *nt* into evolutionarily distant mice, such as the *Mus musculus castaneus* subspecies.

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REFERENCES

Ang, S.-L., and Rossant, J. (1994). *HNF-3β* is essential for node and notochord formation in mouse development. *Cell* **78,** 561–574.

Beddington, R., Rashbass, P., and Wilson, V. (1992). *Brachyury*—A gene affecting mouse gastrulation and early organogenesis. *Dev. Suppl.* 157–165.

Britz-Cunningham, S. H., Shah, M. M., Zuppan, C. W., and Fletcher, W. H. (1995). Mutations of the *connexin43* gap-junction gene in patients with heart malformations and defects of laterality. *N. Engl. J. Med.* **332,** 1323–1329.

Burgess, R., Cserjesi, P., Ligon, K. L., and Olson, E. N. (1995). Paraxis: A basic helix–loop–helix protein expressed in paraxial mesoderm and developing somites. *Dev. Biol.* **168,** 296–306.

Burn, J. (1991). Disturbance of morphological laterality in humans. *Ciba Found. Symp.* **162,** 282–296.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H., and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking *Sonic hedgehog* gene function. *Nature* **383,** 407–413.

Collignon, J., Variet, I., and Robertson, E. J. (1996). Relationship between asymmetric *nodal* expression and the direction of embryonic turning. *Nature* **381,** 155–158.

Conlon, F. L., Wright, C. V. E., and Robertson, E. J. (1995). Effects of the T^{Wis} mutation on notochord formation and mesodermal patterning. *Mech. Dev.* **49,** 201–209.

Danos, M. C., and Yost, H. J. (1995). Linkage of cardiac left–right asymmetry and dorsal-anterior development in *Xenopus*. *Development* **121,** 1467–1474.

Danos, M. C., and Yost, H. J. (1996). Role of the notochord in specification of cardiac left–right orientation in zebrafish and *Xenopus*. *Dev. Biol.* **177,** 96–103.

Echelard, U., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A., and McMahon, A. (1993). *Sonic hedgehog*, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75,** 1417–1430.

Fujinaga, M. (1997). Development of sidedness of asymmetric body structures in vertebrates. *Int. J. Dev. Biol.* **41,** 153–186.

Fujinaga, M., and Baden, J. M. (1991). Critical period of rat development when sidedness of body asymmetry is determined. *Teratology* **44,** 453–462.

George, E. L., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H., and Hynes, R. O. (1993). Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development* **119,** 1079–1091.

Gurtner, G. C., Davis, V., Li, H., McCoy, M. J., Sharpe, A., Cybulsky, M. I. (1995). Targeted disruption of the murine *VCAM1* gene: Essential role of VCAM-1 in chorioallantoic fusion and placentation. *Genes Dev.* **9,** 1–14.

Herrmann, B. G., and Kispert, A. (1994). The *T* genes in embryogenesis. *Trends Genet.* **10,** 280–286.

Hummel, K. P., and Chapman, D. B. (1959). Visceral inversion and associated anomalies in the mouse. *J. Hered.* **50,** 9–13.

Hyatt, B. A., Lohr, J. L., and Yost, H. J. (1996). Initiation of vertebrate left–right axis formation by maternal *Vgl*. *Nature* **384,** 62–65.

Icardo, J. M., and Sanchez de Vega, M. J. (1991). Spectrum of heart malformations in mice with *situs solitus*, *situs inversus*, and associated visceral heterotaxy. *Circulation* **84,** 2547–2558.

Jacobs-Cohen, R. J., Spiegelman, M., and Bennett, D. (1983). Abnormalities of cells and extracellular matrix of *T/T* embryos. *Differentiation* **25,** 48–55.

Jessell, T. M., and Dodd, J. (1992). Midline signals that control dorsal-ventral polarity of the neural tube. *Semin. Neurosci.* **4,** 317–332.

Kaufman, M. H. (1992). ''The Atlas of Mouse Development.'' Academic Press, London.

King, T., and Brown, N. A. (1997). Embryonic asymmetry: Left *TGFβ* at the right time? *Curr. Biol.* **7,** R212–215.

Klessinger, S., and Christ, B. (1996). Axial structures control laterality in the distribution pattern of endothelial cells. *Anat. Embryol.* **193,** 319–330.

Krauss, S., Concordet, J-P., and Ingham, P. W. (1993). A functionally conserved homolog of the *Drosophila* segment polarity gene *hedgehog* is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75,** 1431–1444.

Kwee, L., Baldwin, S., Stewart, C. W., Buck, C., and Labow, M. A. (1995). Defective development of the embryonic and extraembryonic circulatory systems in vascular cell adhesion molecule (VCAM-1) deficient mice. *Development* **121,** 489–503.

Layton, W. M. (1976). Random determination of a developmental process. *J. Hered.* **67,** 336–338.

Levin, M. (1997). Left–right asymmetry in vertebrate embryogenesis. *BioEssays* **19,** 287–296.

Levin, M., Johnson, R. L., Stern, C. D., Kuehn, M., and Tabin, C. (1995). A molecular pathway determining left/right asymmetry in chick embryogenesis. *Cell* **82,** 803–814.

Levin, M., Roberts, D. J., and Tabin, C. (1996). Laterality defects in conjoined twins. *Nature* **384,** 321.

Lohr, J. L., Danos, M. C., and Yost, H. J. (1997). Left-right asymmetry of a *nodal*-related gene is regulated by dorsoanterior midline structures during *Xenopus* development. *Development* **124,** 1465–1472.

Lowe, L. A., Supp, D. M., Sampath, K., Yokoyama, T., Wright, C. V. E., Potter, S. S., Overbeek, P., and Kuehn, M. R. (1996). Conserved left–right asymmetry of *nodal* expression and alterations in murine *situs inversus*. *Nature* **381,** 158–161.

Meno, C., Saijoh, Y., Fujii, H., Ikeda, M., Yokoyama, T., Yokoyama, M., Toyoda, Y., and Hamada, H. (1996). Left–right asymmetric expression of the *TGFβ*-family member *lefty* in mouse embryos. *Nature* **381,** 151–155.

Metzler, M., Gertz, A., Sarkar, M., Schachter, H., Schrader, J. W., and Marth, J. D. (1994). Complex asparagine-linked oligosaccharides are required for morphogenic events during post-implantation development. *EMBO J.* **13,** 2056–2065.

Placzek, M. (1995). The role of the notochord and floor plate in inductive interactions. *Curr. Opin. Genet. Dev.* **5,** 499–506.

Rennebeck, G. M., Lader, E., Chen, Q., Bohm, R. A., Cai, Z. S., Faust, C., Magnuson, T., Pease, L. R., and Artzt, K. (1995). Is there a *Brachyury the second?* Analysis of a transgenic mutation involved in notochord maintenance in mice. *Dev. Biol.* **172,** 206–217.

Riddle, R. D., Johnson, R. L., Laufer, E., and Tabin, C. (1993). Sonic hedghog mediates the polarizing activity of the ZPA. *Cell* **75,** 1401–1416.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., and Jessell, T. M. (1994). Floor plate and motor neuron induction by *vhh-1,* a vertebrate homolog of *hedgehog* expressed by the notochord. *Cell* **76,** 761–775.

Simeone, A., Acampora, D., Mallamaci, A. Stornaiuolo, A., D'Apice, M. R., Nigro, V., and Bonicelli, E. (1993). A vertebrate gene related to orthodenticle contains a homeodomain of the bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo. *EMBO J.* **12,** 2735–2747.

Simeone, A., Gulisano, M., Acampora, D., Stornaiuolo, A., and Boncinelli, E. (1992). Two vertebrate homeobox genes related to the *Drosophila* empty spiracles gene are expressed in the embryonic cerebral cortex. *EMBO J.* **11,** 2541–50.

Singh, G., Supp, D. M., Schreiner, C., McNeish, C., Merker, H. J., Copeland, N. G., Jenkins, N. A., Potter, S. S., and Scott, W. (1991). *Legless* insertional mutation: Morphological, molecular, and genetic characterization. *Genes Dev.* **5,** 2245–2255.

Smith, S. M., Dickman, E. D., Thompson, R. P., Sinning, A. R., Wunsch, A. M., and Markwald, R. R. (1997). Retinoic acid directs cardiac laterality and the expression of early markers of precardiac asymmetry. *Dev. Biol.* **182,** 162–171.

Sumoy, L., Keasey, J. B., Dittman, T. D., and Kimelman, D. 1997. A role for notochord in axial vascular development revealed by analysis of phenotype and the expression of VEGR-2 in zebrafish *flh* and *ntl* mutant embryos. *Mech. Dev.* **63,** 15–27.

Tsuda, T., Philp, N., Zile, M. H., and Linask, K. K. (1996). Left–right asymmetric localization of flectin in the extracellular matrix during heart looping. *Dev. Biol.* **173,** 39–50.

van der Hoeven, F., T. Schimmang, T., Volkmann, A., Mattei, M-G., Kyewski, B., and Ruther, U. (1994). Programmed cell death is affected in the novel mouse mutant *Fused toes* (*Ft*). *Development* **120,** 2601–2607.

Weinstein, D. C., Ruiz i Altaba, A., Chen, W. S., Hoodless, P., Prezioso, V. R., Jessell, T. M., and Darnell, J. E., Jr. (1994). The winged-helix transcription factor *HNF-3β* is required for notochord development in the mouse embryo. *Cell* **78,** 575–588.

Wood, G. O., and Blalock, A. (1940). *Situs inversus totalis* and disease of the biliary tract. *Arch. Surg.* **40,** 885–896.

Yang, J. T., Rayburn, H., and Hynes, R. O. (1993). Embryonic mesodermal defects in α_5 integrin mice. *Development* **119,** 1093–1105.

Yang, J. T., Rayburn, H., and Hynes, R. O. (1995). Cell adhesion eventsmediated by α4 integrins are essential in placental and cardiac development. *Development* **121,** 549–560.

Yokoyama, T., Copeland, N. G., Jenkins, N. A., Montgomery, C. A., Elder, F. F. B., and Overbeek, P. (1993). Reversal of left–right asymmetry: A *situs inversus* mutation. *Science* **260,** 679–682.

Yost, H. J. (1991). Development of the left–right axis in amphibians. *Ciba Found. Symp.* **162,** 165–181.

Yost, H. J. (1992). Regulation of vertebrate left-right asymmetries by extracellular matrix. *Nature* **357,** 158–161.

Zlotogora, J., and Elian, E. (1981). Asplenia and polysplenia syndromes with abnormalities of lateralization in a sibship. *J. Med. Genet.* **18,** 301–302.