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Multiple pathways in the midline regulate concordant brain, heart and gut left-right asymmetry

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SUMMARY

The embryonic midline in vertebrates has been implicated in left-right development, but the mechanisms by which it regulates left-right asymmetric gene expression and organ morphogenesis are unknown. Zebrafish embryos have three domains of left-right asymmetric gene expression that are useful predictors of organ situs. *cyclops (nodal), lefty1* and *pitx2* are expressed in the left diencephalon; *cyclops, lefty2 and pitx2* are expressed in the left heart field; and *cyclops* and *pitx2* are expressed in the left gut primordium. Distinct alterations of these expression patterns in zebrafish midline mutants identify four phenotypic classes that have different degrees of discordance among the brain, heart and gut. These classes help identify two midline domains and several genetic pathways that regulate left-right development. A *cyclops*-dependent midline domain, associated with the prechordal plate, regulates brain asymmetry but is dispensable for normal heart and gut left-right development. A second midline domain, associated with the anterior notochord, is dependent on *no tail, floating head* and *momo* function and is essential for restricting asymmetric gene expression to the left side. Mutants in *spadetail* or *chordino* give discordant gene expression among the brain, heart and gut. *one-eyed pinhead* and *schmalspur* are necessary for asymmetric gene expression and may mediate signaling from midline domains to lateral tissues. The different phenotypic classes help clarify the apparent disparity of mechanisms proposed to explain left-right development in different vertebrates.

Key words: Zebrafish, Left-right asymmetry, Embryonic midline, Concordance, Heterotaxia, *cyclops*, *nodal*, *lefty1*, *lefty2*, *pitx2*

INTRODUCTION

Through the generation of an embryonic midline, the vertebrate embryo develops left and right halves that are bilaterally symmetric for most external structures and left-right asymmetric for most internal organs. For example, the heart, stomach and spleen lie on the left side, the liver on the right, and the large intestine coils from right to left. A considerable amount is known about left-right development of the heart (reviewed in Goldstein et al., 1998; Yost, 1998a,b) and studies of gut left-right development are currently emerging (Logan et al., 1998; Campione et al., 1999; Schilling et al., 1999; Yan et al., 1999; Essner et al., 2000). Abnormal left-right development of any of these organs with respect to the others is called discordance or heterotaxia, and often has severe pathology (Bowers et al., 1996).

In addition to well-defined heart and gut asymmetries, there are morphological left-right asymmetries in the brain, which are highly conserved, and functional left-right asymmetries, which are essential for normal behavior (Schlaug et al., 1995; Yost, 1998c). Many vertebrates have morphological left-right asymmetry in the habenula nucleus of the diencephalon, but the function of this region is poorly understood (Engbretson et al., 1981; Gurusinghe and Ehrlich, 1985; Morgan, 1991; Gugliemotti and Fiorino, 1998). The discovery of three asymmetrically expressed genes in the zebrafish diencephalon, *cyclops* (Rebagliati et al., 1998a,b; Sampath et al., 1998), *lefty1* (or *antivin*) (Bisgrove et al., 1999; Thisse and Thisse, 1999) and *pitx2* (Essner et al., 2000) should facilitate studies of left-right brain asymmetry.

It is clear from work in several vertebrates that left-right asymmetry, at least in the heart and gut, is dependent on development of a normal embryonic midline. Midline tissues, including notochord and floorplate, arise from the embryonic node and are not morphologically asymmetric (reviewed in Yost, 1998d). In mice and chick the first apparent break in bilateral symmetry occurs at or near the node during gastrulation. Perhaps in response to the unidirectional rotation of nodal cilia (reviewed by Vogan and Tabin, 1999), TGFβ signaling molecules including *lefty* and *nodal* become localized in small asymmetric domains around the node and in the developing midline (reviewed in Harvey, 1998; Ramsdell and Yost, 1998; King and Brown, 1999). During somitogenesis in birds, mice, frogs and zebrafish, homologs of *nodal* (reviewed in Yost, 1999) and *lefty* (Meno et al., 1996, 1997; Bisgrove et al., 1999; Rodriguez Esteban et al., 1999; Cheng et al., 2000) are expressed in the left lateral plate mesoderm. The interaction of *lefty* and *nodal* appears to regulate

expression of the transcription factor *pitx2*, which is thought to directly influence aspects of internal asymmetry such as heart looping, lung asymmetry and gut rotation (reviewed in Harvey, 1998; Yost, 1999).

The midline may function as a physical barrier to prevent the contralateral spread of asymmetric signals. Studies in *Xenopus* have showed that decreased dorsoanterior development of midline structures is correlated with increased incidence of cardiac reversals (Danos and Yost, 1995). Extirpation of presumptive notochord and floorplate during open neural plate stages also causes cardiac reversals and bilateral expression of *nodal* in lateral plate mesoderm (Danos and Yost, 1996; Lohr et al., 1997). Studies of conjoined twins suggest that signals on one side of the embryo cannot cross over the midline, but are able to influence a neighboring embryo if there is not an intervening midline (Levin et al., 1996; Nascone and Mercola, 1997). In mice homozygous for the *no turning* mutation, midline structures including the notochord and floorplate degenerate, embryonic rotation is altered, cardiac looping is randomized and *nodal* and *lefty2* are expressed bilaterally in the lateral plate mesoderm (Melloy et al., 1998). Similarly in zebrafish, mutations in at least 21 genes that affect development of the embryonic midline also perturb left-right asymmetry of the heart (Danos and Yost, 1996; Chen et al., 1997).

The midline barrier may be molecular as well as physical in nature. In mice, *lefty1* is expressed asymmetrically in the developing floorplate (Meno et al., 1997, 1998). In homozygotes for a null mutation of *lefty1*, both *nodal* and *lefty2* are expressed bilaterally in lateral plate mesoderm. This suggests that midline expression of *lefty1* acts as a molecular barrier, preventing left-sided factors that induce *nodal* and *lefty2* from acting on the right side of the embryo (Meno et al., 1998).

While a growing number of genes and cellular processes have been implicated in left-right development, little is known about the genetic pathways that control the concordant left-right asymmetry of the heart and gut, and nothing is known about the genetic regulation of left-right development in the brain (Yost, 1998c). Currently, none of the proposed mechanisms by which midline cells regulate left-right development are capable of explaining the diverse laterality defects that are seen in humans and in model organisms. In order to assess the roles of the midline in concordant development of the brain, heart and gut, a comprehensive Discordance Assay was developed using a panel of asymmetric markers that are predictive of left-right development. This assay was applied to nine zebrafish mutants previously shown to be defective in both midline development and heart left-right development (Chen et al., 1997). These analyses identify four distinct classes of laterality defects, which exhibit different degrees of discordance among the brain, heart and gut, and suggest that the left-right orientation of each organ can be regulated independently. Furthermore, these analyses identify defects within the prechordal plate and anterior notochord that are predictive of the classes of laterality defects. We propose that there are distinct genetic pathways expressed in specific domains along the anterior-posterior axis of the embryonic midline, and that each domain-specific pathway regulates distinct steps in left-right development of the brain, heart and gut.

MATERIALS AND METHODS

Embryo culture and zebrafish stocks

Zebrafish, *Danio rerio*, were maintained at 28.5°C on a 14 hour:10 hour light:dark cycle. Embryos were collected from natural spawnings, cultured and staged by developmental time and morphological criteria (Westerfield, 1995). Wild-type embryos were descendants of outbred stocks obtained from Ekkwill Breeders (Gibsonton, Fl, USA). Zebrafish carrying mutations were obtained from stocks originally produced at the University of Oregon (*cyc*^{b229}, *ntl*^{b160}, *ntl*^{b195}, *spt*^{b104}), MGH/Harvard (*oep*^{m134}), Newcastle (*flh*^{n1}) or the Max-Planck-Institut Fur Entwicklungsbiologie (*cyc*^{tf219}, *din*^{tm84}, *sur*^{ty686}, *mom*^{tl211}).

RNA in situ localization

For in situ hybridizations, embryos were fixed in 4% paraformaldehyde in sucrose buffer (Westerfield, 1995), rinsed in PBS, dehydrated into absolute methanol and stored at –20°C. Riboprobes were synthesized from linearized DNA templates using T3 or T7 polymerases and digoxigenin or fluorescein labeling mixes (BMB). In situ hybridizations were carried out as previously described (Stachel et al., 1993; Hauptmann and Gerster, 1994). Probes used include: *lefty1*, *lefty2* and *pitx2* (Bisgrove et al., 1999); *cyclops* (*ndr2*, Rebagliati et al., 1998a); *preproinsulin* (Milewski et al., 1998); *pax2* (*pax(zf-b)*, Krauss et al., 1991); *axial* (Strahle et al., 1993); *goosecoid* (Stachel et al., 1993), *shh* (Krauss et al., 1993) and *no tail* (Schulte-Merker et al., 1994). Embryos were cleared in 70% glycerol/PBS and photographed with a Leica MZ12 dissecting microscope or Leica DMR compound microscope. Images on Kodak T160 film were scanned and processed using Adobe Photoshop.

RESULTS

Asymmetric gene expression domains in the diencephalon and heart field

Several genes are asymmetrically expressed in the heart field and dorsal diencephalon of zebrafish embryos. The homeobox gene *nkx2.5* marks the position of cardiac precursors (Chen and Fishman, 1996; Danos and Yost, 1996). The left side of its expression domain extends more posteriorly than the right at 20-24 somites (Schilling et al., 1999). *bmp4* is expressed uniformly in the bilateral heart tubes just prior to heart tube fusion at the 20-somite stage (Chin et al., 1997). At the 22-somite stage, just prior to jogging, *bmp4* expression becomes markedly asymmetric, with more on the left than on the right side of the heart tube (Chen et al., 1997). The TGFβ signaling proteins *cyclops* (Rebagliati et al., 1998a,b; Sampath et al., 1998), *lefty1*/*antivin* and *lefty2* (Bisgrove et al., 1999; Thisse and Thisse, 1999) are expressed asymmetrically in the left lateral plate mesoderm and in the left dorsal diencephalon, beginning at the 19- to 20-somite stage. The transcription factor *pitx2* is also expressed in the left diencephalon, heart field and gut, with the asymmetric expression domains evident at 22 somites (Campione et al., 1999; Essner et al., 2000).

Two-color double in situ hybridization analyses with *lefty2* (*lft2*) and each of the other genes indicate that expression domains in the heart field have regions of overlap and regions of exclusive expression. *lft2* is expressed in the left heart field in a domain that extends beyond cardiac precursor lineages, lateral to the expression of *nkx2.5* (Fig. 1A). *bmp4* is expressed broadly across the lateral plate mesoderm at the level of the heart field, with a crescent of strong expression in cardiac

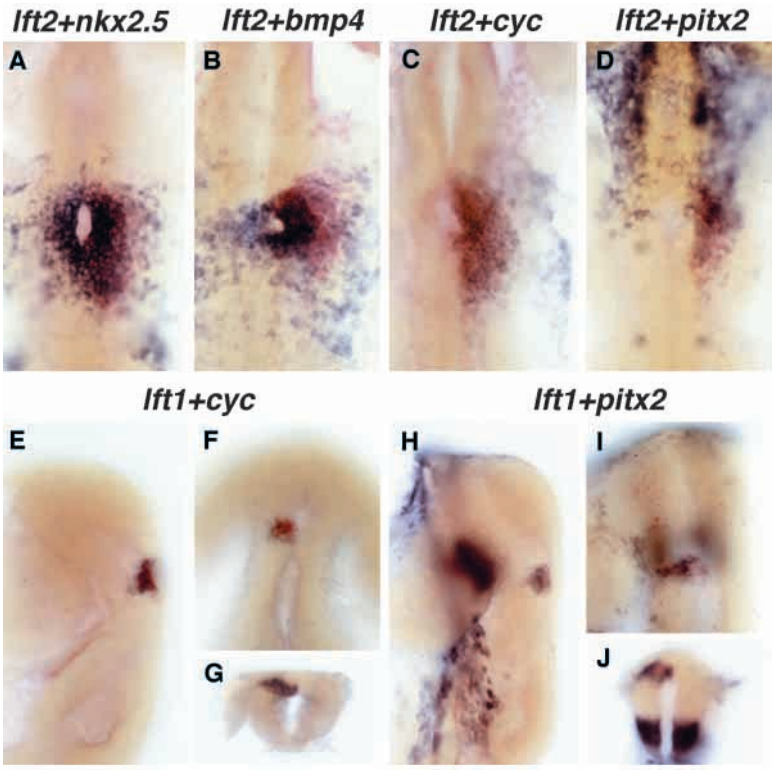

Fig. 1. Two-color double in situ hybridization identifies overlapping asymmetric gene expression patterns in the heart field (A-D) and diencephalon (E-J) of 22- to 24-somite stage zebrafish embryos. Ventral views, anterior at top, of *lft2* expression (in red) and *nkx2.5* (A), *bmp4* (B), *cyc* (C) or *pitx2* (D) expression (in purple), in the heart field. (E-J) Coexpression of *lft1* (in red) with *cyc* (E-G, in purple) and *pitx2* (H-J, in purple) in the left diencephalon (E, H, lateral views, dorsal at right; F, I, dorsal views, anterior at top; G, J, transverse sections, dorsal at top).

progenitors lying within the *lft2* expression domain (Fig. 1B). *cyclops* (*cyc*) expression overlaps with *lft2* expression in the heart field (Fig. 1C). In addition *cyc* extends further anterior, lateral and posterior to the *lft2* domain, encompassing much of the left lateral plate mesoderm. *pitx2* expression, which is extensive in 22- to 24-somite embryos, includes a predominantly bilateral domain in the anterior lateral plate mesoderm. The left side of this domain extends further posteriorly and laterally than does the right side, and overlaps with the anterior half of the *lft2* expression domain in the heart field (Fig. 1D).

In contrast to non-contiguous expression domains in the heart field, the asymmetric expression domains of *lft1*, *cyc* and *pitx2* in the left dorsal diencephalon overlap completely (Fig. 1E-J). Expression levels of *cyc* and *lft1* are uniform throughout the domain (Fig. 1E-G), while *pitx2* expression is strongest in one or two rows of cells on the lateral edges of the domain and is weaker in cells in the center of the domain (Fig. 1H-J). A summary diagram (Fig. 2) depicts the gene expression domains in the heart field and dorsal diencephalon.

Table 1. Altered patterns of asymmetric gene expression in 22- to 25-somite stage zebrafish midline mutant embryos

	Laterality phenotype														
	Diencephalon (%)					Heart field (%)					Gut (%)				
	n	L	R	B	A	*n*	L	R	B	A	*n*	L	R	B	A
Class I (bilateral)															
*ntl**	98	2	2	88	8	172	7	6	85	2	23	0	0	91	9
wt sibs	228	77	3	8	12	444	80	11	7	2	63	92	0	6	2
flh^{n1}	106	2	1	77	20	177	6	5	79	10	43	2	0	91	7
wt sibs	283	84	3	2	11	473	87	4	4	5	134	97	2	1	0
mom^{tl211}	45	0	2	27	71	45	0	0	98	2	22	0	0	100	0
wt sibs	195	78	4	17	1	195	82	4	12	2	97	92	4	4	0
Class II (random)															
spt^{b104}	117	31	25	35	9	185	33	22	43	2	54	19	7	61	13
wt sibs	369	95	1	1	3	553	95	1	3	1	184	98	0	1	1
din^{tm84}	114	41	14	12	33	114	47	20	21	12	49	59	18	4	19
wt sibs	396	77	7	14	2	396	77	6	16	1	182	93	3	3	1
Class III (bilateral, normal)															
cyc^{tf219}	86	3	0	16	81	44	100	0	0	0	42	98	0	2	0
wt sibs	319	96	1	1	2	195	98	1	0	1	124	98	0	0	2
cyc^{b229}	131	4	6	12	78	117	13	20	17	50	19	5	5	16	74
wt sibs	368	74	5	12	9	336	81	5	13	1	50	72	6	16	6
cyc^{b229}/*cyc*^{tf219}	94	1	0	37	62	50	86	0	14	0	44	82	0	18	0
wt sibs	301	85	4	10	1	161	91	4	5	0	140	79	7	14	0
Class IV (absent)															
oep^{m134}	92	0	0	0	100	92	0	0	0	100	35	0	0	0	100
wt sibs	298	88	1	10	1	298	90	2	8	0	135	80	1	19	0
sur^{ty68b}	44	0	0	0	100	44	0	0	0	100	21	0	0	0	100
wt sibs	140	79	7	7	7	140	82	11	6	1	69	81	9	3	7

Diencephalon expression=*lft1* expression data+*pitx2* expression data; heart field expression=*lft2* expression data+*pitx2* expression data (except for *cyc* alleles; *lft2* expression only as *pitx2* domain is severely reduced and difficult to score); gut expression=*pitx2* expression data.

L, left-sided expression; R, right-sided expression; B, bilateral expression; A, absence of expression; wt, wild type.

* *ntl* data includes analysis of *ntl*^{b160} and *ntl*^{b195}.

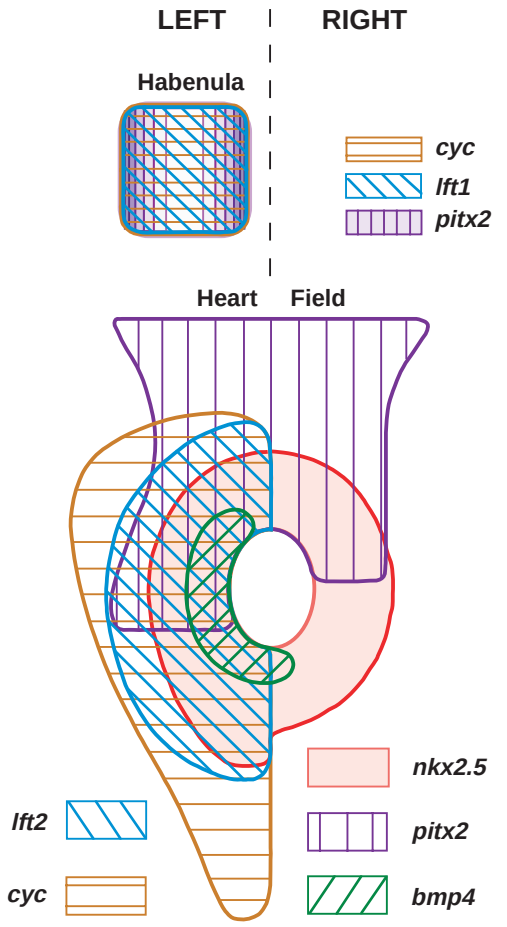

Fig. 2. In 22- to 24-somite stage embryos *lft1*, *cyc* and *pitx2* are coexpressed in the left habenula of the diencephalon, and *lft2*, *nkx2.5*, *bmp4*, *cyc* and *pitx2* are coexpressed within the heart field in the lateral plate mesoderm. The drawing depicts a dorsal view, anterior at top.

Patterns of asymmetric gene expression define distinct laterality classes

It is unknown whether all midline perturbations alter left-right development through similar disruptions of patterning information or whether diverse mechanisms are involved. Of the zebrafish mutants that have midline defects and altered cardiac left-right development (Danos and Yost, 1996; Chen et al., 1997), nine mutants were selected for study, based on the diversity of midline defects. In *no tail* (*ntl*) mutants notochord precursors are present but fail to differentiate, resulting in embryos that lack a notochord but have a relatively normal floorplate (Halpern et al., 1993). *floating head* (*flh*) and *momo* (*mom*) mutants also exhibit a loss of notochord, develop only patches of floorplate and have somites that are fused across the midline (Halpern et al., 1995; Odenthal et al., 1996). Embryos lacking *cyc*, *one-eyed pinhead* (*oep*) or *schmalspur* (*sur*) function show varying degrees of loss of prechordal plate mesendoderm and median fore-, mid- and hindbrain tissue. Although the notochord is largely intact in these mutants, the floorplate is absent (Hatta et al., 1991; Yan et al., 1995; Brand et al., 1996; Schier et al., 1997). *spadetail* (*spt*) mutants exhibit convergence/extension defects during gastrulation and have trunk mesoderm deficiencies at late somite stages, but have relatively normal notochord development (Kimmel et al., 1989). Mutants in *chordino* (*din*) are ventralized. The tail is expanded at the expense of the head and anterior trunk and although the anterior notochord is largely normal, the posterior notochord is reduced or absent (Hammerschmidt et al., 1996a,b).

The midline mutants had distinct patterns of *lft1*, *lft2* and *pitx2* expression in the diencephalon, heart and gut (Fig. 3 and data not shown). For each mutant, alterations in the patterns of *lft1*, *lft2* and *pitx2* expression were coordinated within each asymmetric expression domain. Four classes of laterality mutants were defined, based on alterations in asymmetric gene expression patterns (Table 1). These results indicate that different midline mutants have distinct alterations in left-right development of the diencephalon, heart and gut.

Two mutants in Class I, *ntl* and *flh*, displayed predominantly bilateral expression of *lft1*, *lft2* and *pitx2* in the dorsal diencephalon, heart field and gut (Fig. 3B). The *ntl* alleles ntl^{b160} and ntl^{b195} had the same phenotype and have been combined in the data set. In addition to being bilaterally expressed, *lft1* and *lft2* often appear to be expressed at higher levels in mutant embryos than in their wild-type siblings, suggesting that these mutations result in the loss of a factor that normally inhibits *lefty* expression. In *mom*, asymmetric genes are expressed bilaterally in the lateral plate mesoderm but often fail to be expressed in the diencephalon (Fig. 3D). *ntl* and *flh*

Table 2. Correlation of *lft2* expression in the lateral plate mesoderm of 22- to 25-somite stage zebrafish midline mutant embryos with the direction of heart looping in siblings 48 hours post-fertilization

	Lateral plate expression (%)					Predicted heart looping (%)		Observed heart looping (%)		
Mutant	*n*	L	R	B	A	R	L	*n*	R	L
spt^{b104}	47	32	21	47	0	56	44	43	56	44
wt sibs	160	98	1	1	0	98	2	145	99	1
ntl^{b160}	69	0	0	100	0	50	50	71	62	38
wt sibs	206	82	9	7	2	86	14	202	88	12
flh^{n1}	63	0	3	94	3	48	52	59	61	39
wt sibs	178	91	6	2	1	92	8	163	90	10
cyc^{b229}	54	13	26	20	41	44	56	50	54	46
wt sibs	132	95	3	2	0	96	4	123	99	1
cyc^{tf219}	25	100	0	0	0	100	0	29	100	0
wt sibs	131	98	1	1	0	98	2	103	100	0

L, left-sided expression; R, right-sided expression; B, bilateral expression; A, absence of expression; wt, wild type.
Predicted right heart looping=left-sided *lft2*+1/2 bilateral *lft2*+1/2 absence of *lft2*.
Predicted left heart looping=right-sided *lft2*+1/2 bilateral *lft2*+1/2 absence of *lft2*.

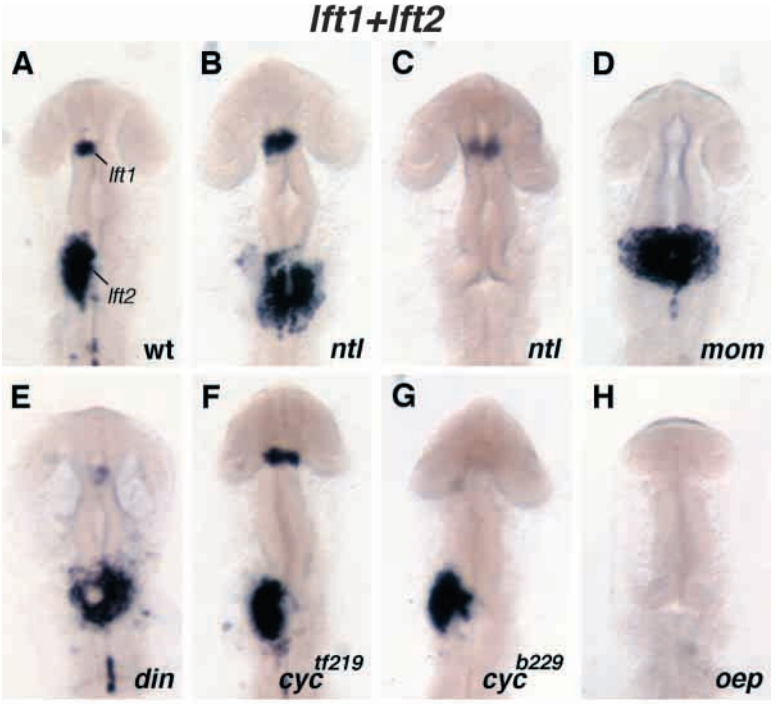

Fig. 3. Examples of altered patterns of asymmetric *lft1* and *lft2* expression in zebrafish midline mutant embryos. In situ hybridization of *lft1* and *lft2* in the diencephalon and heart field of 22- to 24-somite stage wild-type and mutant zebrafish embryos. Embryos, shown in dorsal view, anterior at top, have been removed from the yolk cell. (A) Wild-type, (B, C) ntl^{b160}, (D) *mom*, (E) *din*, (F) cyc^{tf219}, (G) cyc^{b229}, (H) *oep*.

mutants have been reported to have bilateral expression of *cyc* in the diencephalon and lateral plate mesoderm (Rebagliati et al., 1998a; Sampath et al., 1998).

Asymmetric gene expression is randomized in Class II mutants, represented by *spt* and *din* (Fig. 3E). Within a population of embryos, there was a high incidence of each of the four possible expression phenotypes (left-sided, right-sided, bilateral or absence of expression) in the diencephalon, heart and gut. Absence of expression was more common in *din* than *spt*, particularly in the diencephalon.

The Class III laterality phenotype of *cyc* is unique among the mutants examined. In cyc^{tf219}, *lft1* and *pitx2* expression was largely absent in the diencephalon with a low incidence of weak bilateral gene expression. In striking contrast to the altered expression in the diencephalon, expression of *lft2* in the heart field (Fig. 3F) and *pitx2* in the gut was normal. Mutants of the other allele, cyc^{b229}, usually lacked asymmetric gene expression in the diencephalon (Fig. 3G), often had random *lft2* expression in the heart field and showed an absence of *pitx2* expression in the gut. The distinctions between these *cyc* alleles were explored further. cyc^{tf219} is an ENU-induced mutation, which changes the methionine initiation codon to an isoleucine. The truncated protein product lacks an effective signal sequence and is not correctly processed (Rebagliati et al., 1998b). cyc^{b229}, on the other hand, lies within a large gamma-ray-induced deletion that might include other loci (Talbot et al., 1998). As the molecular lesions responsible for the two *cyc* alleles are considerably different, we examined asymmetric gene expression in mutants derived from an intercross of heterozygous cyc^{tf219}, and cyc^{b229} carriers. The trans-allelic combination of cyc^{tf219} and cyc^{b229} had an asymmetric gene expression phenotype similar to that of cyc^{tf219}, suggesting that the phenotype due specifically to mutation of the *cyc* gene is reflected most accurately by the cyc^{tf219} allele.

Class IV includes *oep* and *sur*, both of which lack expression of *lft1*, *lft2* and *pitx2* in the asymmetric domains of the diencephalon, heart field and gut (Fig. 3H). In *oep*, virtually all *lft1*, *lft2* and *pitx2* expression is abolished, coincident with the loss of most mesendodermal tissue (Schier et al., 1997). Interestingly, in *sur*, expression of *lft1* in the notochord and bilateral expression domains of *pitx2* expression are not altered, indicating that loss of

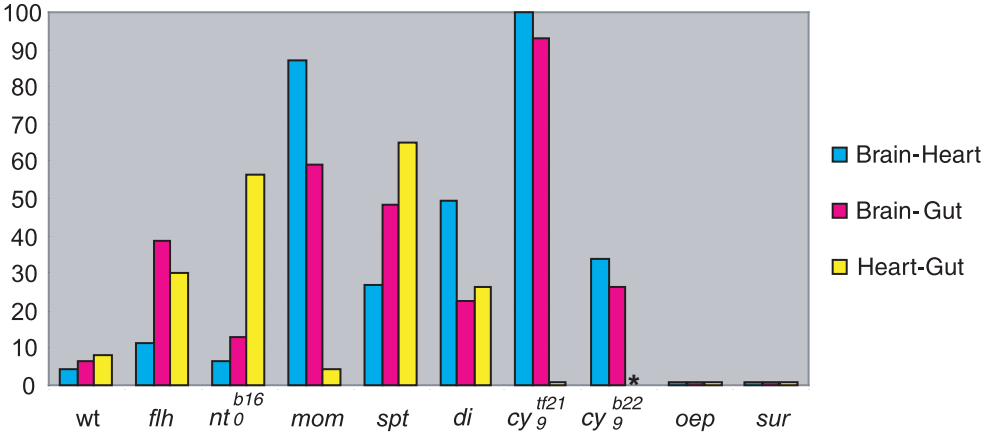

Fig. 4. Discordance Index of asymmetric gene expression patterns in midline/laterality mutants. Percentage discordance is shown for wild-type and each mutant for the pairwise comparisons of brain and heart (blue), brain and gut (red), and heart and gut (yellow) gene expression patterns. * No data available. Wild-type data were combined from wild-type siblings of all homozygous recessive mutants and includes both heterozygous and homozygous wild-type.

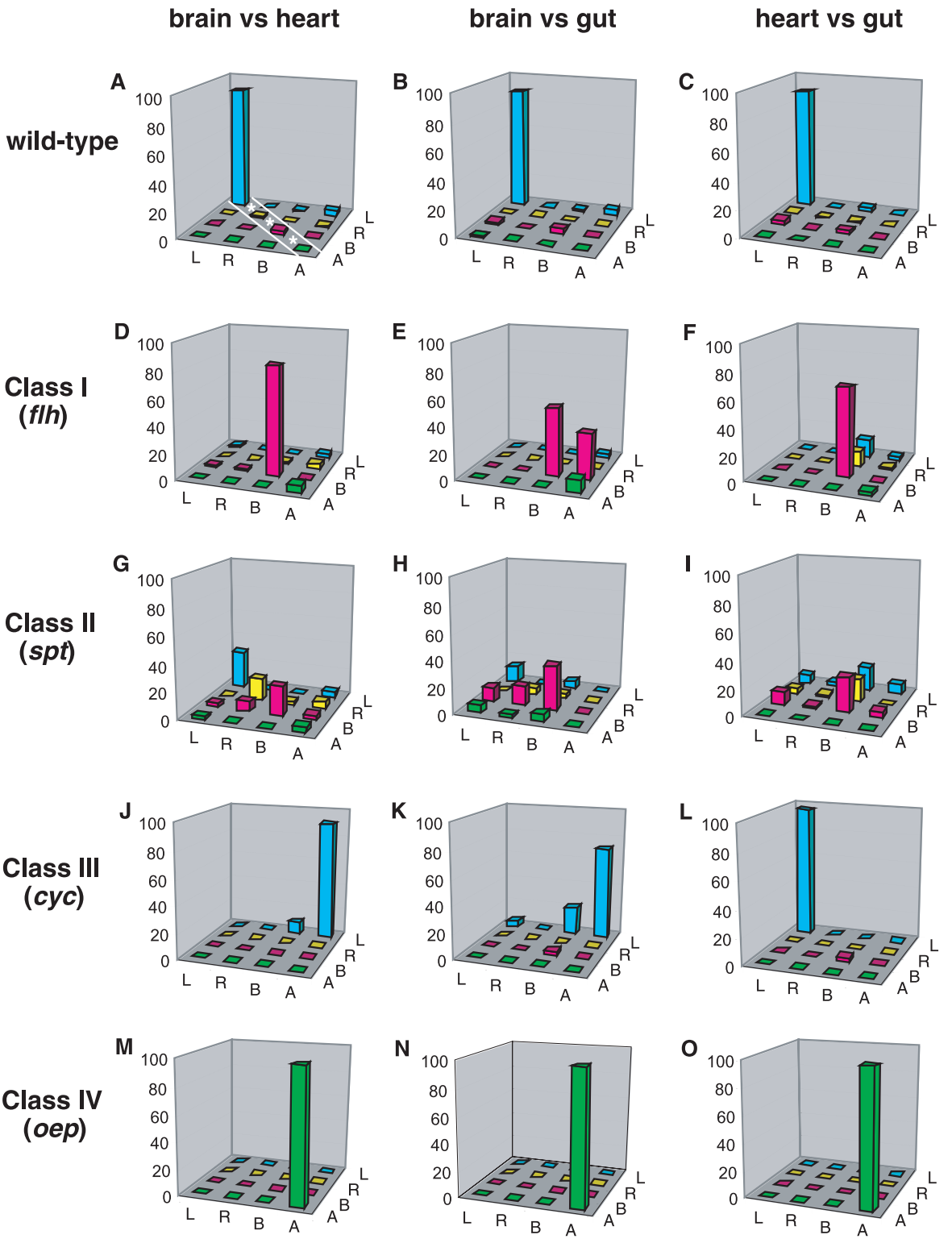

Fig. 5. Concordance/discordance plots showing the distribution of asymmetric gene expression patterns in wild type (A-C) and representative mutants from laterality phenotypic Class I (D-F), Class II (G-I), Class III (J-L) and Class IV (M-O). The pairwise comparisons of brain versus heart, brain versus gut and heart versus gut are depicted on the *x* versus *z* axes, respectively. As depicted by white asterisks in A, values lying along the diagonal extending from the top left to bottom right corner of each graph indicate the percentage of embryos having concordant gene expression between the domains being compared. Values lying outside this diagonal indicate the percentage of embryos having discordant expression between the domains. Numbers on the ordinate are percentage concordance, letters on the *x* and *z* axes indicate: L, left; R, right; B, bilateral expression; A, absence of expression. Wild-type data were combined from wild-type siblings of homozygous recessive *flh*, *spt*, *cyc*^{tf219} and *oep* mutants and include both heterozygous and homozygous wild-type.

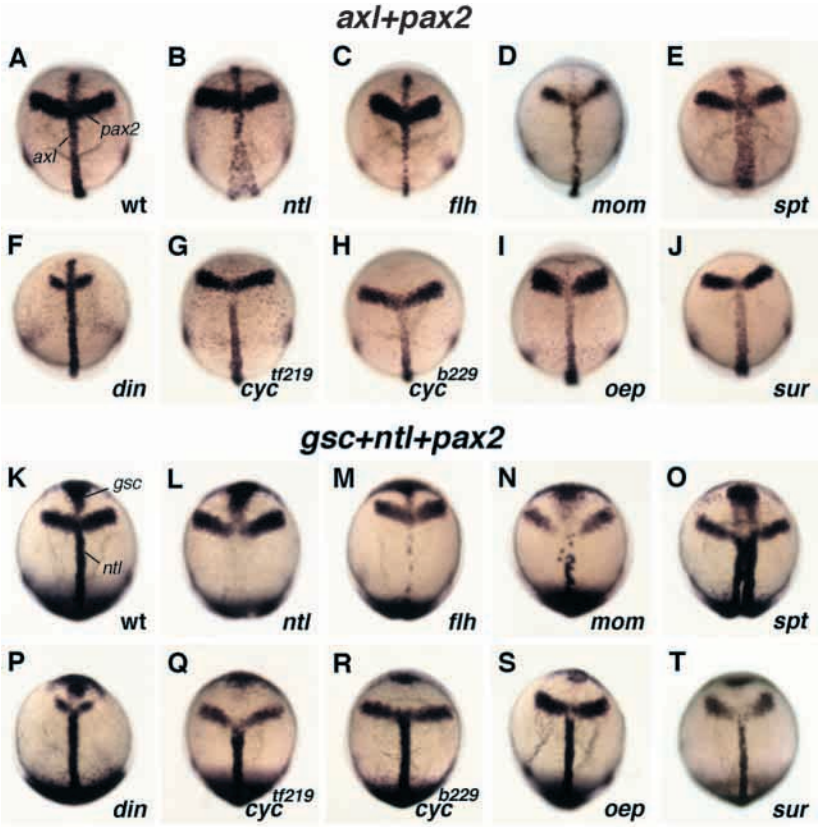

Fig. 6. Expression patterns of midline mesendodermal marker genes are altered in zebrafish midline mutant embryos. Dorsal views, anterior at top, of tailbud-stage embryos, probed with *axl* (A-J) and *gsc* + *ntl* (K-T). All embryos were also probed with *pax2*, which is expressed in stripes that extend laterally from the midline, as a reference of anteroposterior position. (A,K) wild-type. (B-D, L-N) Class I: (B,L) *ntl*^{b160}, (C,M) *flh*, (D, N) *mom*. (E,F,O,P) Class II: (E,O) *spt*, (F,P) *din*. (G,H,Q,R) Class III: (G,Q) *cyc*^{tf219}, (H,R) *cyc*^{b229}. (I,J,S,T) Class IV: (I,S) *oep* and (J,T) *sur*.

asymmetric expression domains is not due merely to large-scale loss of cell types.

Expression patterns correlate with the direction of heart looping

The earliest morphological evidence of internal asymmetry in vertebrate development is the rotation and rightward looping of the midline heart tube. To assess whether asymmetric gene expression is correlated with internal situs, we compared the pattern of *lft2* expression in the heart field of 22- to 25-somite stage embryos with the direction of heart looping in their siblings at 48 hours post-fertilization (Table 2). Embryos expressing *lft2* on the left side were predicted to have normal rightward looping hearts, those with *lft2* expression on the right side, leftward looping hearts. Embryos with bilateral expression or no *lft2* expression were predicted to have hearts that looped right or left at random and with equal frequency. In mutant and phenotypically wild-type embryos derived from intercrosses of *spt*, *ntl*, *flh* and *cyc* heterozygotes, there was a strong correlation between the direction of heart looping predicted from *lft2* expression in the lateral plate mesoderm and the direction of heart looping observed in live sibling embryos (Table 2). In addition, wild-type and mutant embryos double-labeled with *lft1* and *cyc* or *pitx2* antisense probes, or with *lft2* and *cyc* or *pitx2* antisense probes, showed that, in almost all cases, *lft1* and *lft2* were expressed concomitantly with *cyc* and *pitx2* in the diencephalon and lateral plate mesoderm (data not shown). These results indicate that the expression domains of these genes are useful predictors of left-right axis specification in mutant and wild-type embryos.

Discordance analyses of brain, heart and gut left-right development

An important measure of laterality is the coordinated development of left-right asymmetries in the brain, heart and gut. We have designed a Discordance Assay that scores pairwise comparisons of brain, heart and gut gene expression patterns in individual embryos. There are three possible pairwise comparisons for discordance (heart versus gut, heart versus brain, brain versus gut), and for each pairwise comparison, there are 16 possible categories of gene expression patterns (left, right, bilateral or absent). Thus, for discordance analysis of three organ fields, there are 48 possible categories (3×16), arguing that reliable analysis of the genetic regulation of concordance can only be done in a vertebrate model that yields large numbers of genetically defined embryos. Here, we examined asymmetric gene expression patterns in over 3500 embryos from nine mutant lines. In order to analyze whether gene expression patterns among organ primordia are concordant in a mutant, we devised a numerical measure, the Discordance Index (DI), defined as: DI = Sum of the number of embryos in each of 12 discordant categories × 100 / Total embryos scored for this genotype, where a discordant category in the pairwise comparison of heart and brain is, for example, brain left-heart right. Note that the remaining four categories (of the 16 total possibilities) are concordant categories: heart left-brain left, heart right-brain right, both bilateral, and both absent. The discordance index was plotted for each pairwise comparison for each mutant, allowing comparison of the results of the concordance analyses within each mutant and among the mutant lines (Fig. 4).

Three-dimensional plots of the concordance/discordance between gene expression patterns in the diencephalon and heart field, the diencephalon and gut, and the heart field and gut, allow quantification of the percentage of embryos assigned to each of 48 categories. Plots are shown for representative mutants from each of the four laterality classes (Fig. 5A-O). In these plots, values on the diagonal (white asterisks in Fig. 5A), extending from the back left corner to the bottom right corner, represent the percentage of embryos with concordant gene expression, while values lying outside this diagonal represent the percentage of embryos with discordant gene expression.

Wild-type siblings of mutant embryos showed concordant left-sided expression in the brain, heart and gut (Figs 4, 5A-C). Class I embryos exhibited predominantly concordant bilateral expression of *lft1, lft2* and *pitx2* in the brain, heart and gut (Fig. 5D-F). Within this class, *flh* embryos had concordant

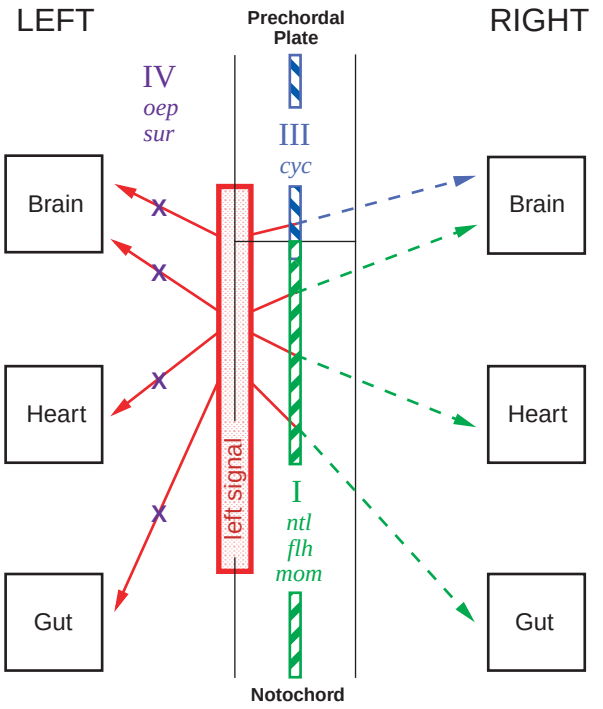

Fig. 7. Model of potential signaling pathways perturbed in different phenotypic classes of zebrafish midline mutants. Classes I and III mutants lack differentiated anterior notochord or prechordal plate, respectively. These tissues are postulated to act as physical and/or molecular barriers that prevent left-sided signals from crossing the midline. In class IV mutants asymmetric gene expression in lateral tissues is abolished due to a loss of signal propagation from midline to lateral tissues. This may arise from the loss of a left-sided signaling center or a loss of response in lateral tissues. Class II mutants (not illustrated), which give randomized expression of asymmetric markers, may affect establishment of the asymmetric signal through its transfer or reception.

anterior gene expression (between brain and heart), but posterior expression patterns (heart and gut) were less tightly coupled. In *ntl* mutants expression in the heart and gut were also discordant. *mom* embryos had high discordance between brain expression (largely absent) and the predominantly bilateral and mutually concordant expression in the heart and gut. A high percentage of Class II embryos (*spt* and *din*) exhibited discordant gene expression. Gene expression patterns within the brain, heart and gut were random within each domain and among the domains (Figs 4, 5G-I). This suggests that asymmetric gene expression patterns in each domain are independently determined in these mutants. In cyc^{tf219} embryos (Class III), there was discordance between brain (absent or bilateral expression) and either heart or gut expression. In contrast, the heart and gut exhibited concordant left-sided expression (Figs 4, 5J-L). Class IV embryos (*oep* and *sur*) exhibit complete concordance among all three domains, by virtue of the fact that there is no asymmetric gene expression (Figs 4, 5M-O).

Discordance of cardiac and visceral situs

Our Discordance Analysis of asymmetric gene expression predicts that the situs of internal organs in mutants with discordant or bilateral asymmetric gene expression, which predicts stochastic orientation of organ primordia, will exhibit heterotaxia. To assess whether mutants with altered asymmetric gene expression patterns exhibited altered organ situs, we separated 52 hpf mutant embryos into groups with normal and reversed heart looping. The embryos were then fixed and the position of the pancreas assessed by in situ hybridization with a *preproinsulin* probe (Milewski et al., 1998). The results of this analysis (Table 3) indicate that mutants from laterality Class I, II and IV exhibit cardiac and visceral heterotaxia. Class III mutants, which have normal asymmetric gene expression in the heart-field and gut, have normal cardiac and visceral situs.

Distinct perturbation of midline gene expression patterns in different laterality classes

It is clear from the above analysis that different classes of midline mutants have distinct effects on concordant left-right development. To assess whether the laterality classes are correlated with specific perturbations of midline gene expression, tailbud-stage mutant embryos and their wild-type siblings were analyzed by in situ hybridization with antisense probes to *axial* (*axl*, Strahle et al., 1993) or *no tail* (*ntl*, Schulte-Merker et al., 1994) and *goosecoid* (*gsc*, Stachel et al., 1993). These markers have previously been shown to be useful in assessing axial mesendoderm phenotypes in some of the mutants we examined (Halpern et al., 1993; Thisse et al., 1994; Strahle et al., 1996; Schier et al., 1997). Embryos were also hybridized with an antisense probe to *pax2* (Krauss et al.,

Table 3. Correlation of heart and pancreas asymmetry in midline mutant embryos and wild-type siblings 52 hours post-fertilization

			Organ symmetry		
Mutant	Laterality class	*n*	Normal (%)	Situs inversus (%)	Heterotaxia (%)
ntl^{b195}	I	117	20	11	69
wt sibs		296	88	2	10
din^{tm84}	II	68	32	15	53
wt sibs		195	95	1	4
cyc^{tf219}	III	71	89	3	8
wt sibs		176	92	2	6
sur^{cy686}	IV	50	26	14	60
wt sibs		176	96	1	3

Normal, embryos with rightward heart looping and right-sided pancreas; situs inversus, embryos with leftward heart looping and left-sided pancreas; heterotaxia, embryos with rightward heart looping and left-sided or midline pancreas or embryos with leftward heart looping and right-sided or midline pancreas; wt, wild type.

1991), which is expressed in the presumptive midbrain, to mark position along the anteroposterior axis. Midline mesendoderm posterior to *pax2* expression gives rise to notochord and hypochord while mesendoderm anterior to *pax2* gives rise to the prechordal plate.

By the end of gastrulation, *axl* expression in the midline mesendoderm extends from the diencephalon to the tailbud (Fig. 6A). At this time *axl* expression is also initiated in the floorplate precursors overlying the notochord (Strahle et al., 1993). In a dorsal view of *ntl* mutants, the posterior *axl* domain was expanded laterally (Fig. 6B). When viewed laterally it was evident that *axl* expression was restricted to floorplate precursors (not shown). In *flh* mutants, the posterior *axl* domain was narrower and discontinuous (Fig. 6C). Similar to *ntl* mutants, *axl* expression was absent from the notochord and restricted to floorplate precursors. Embryos derived from *mom* carriers showed variable phenotypes. Typically embryos had small gaps in *axl* expression in both anterior and posterior domains (Fig. 6D), with many embryos having almost wild-type expression. Within laterality Class II, *spt* mutant embryos displayed lateral expansion of *axl* expression. This expanded domain extended along the entire anteroposterior axis, and posterior to *pax2*, included both notochord and flooplate precursors (Fig. 6E). In *din* embryos, the anterior *axl* domain was shortened slightly, otherwise expression was largely wild-type (Fig. 6F). Members of laterality Class III, both *cyc* alleles (Fig. 6G,H), and Class IV, *oep* and *sur* (Fig. 6I,J), had similar midline expression phenotypes, i.e. loss of *axl* expression anterior to *pax2* in the prechordal plate, and expression in the posterior domain was restricted to notochord precursors.

At the end of gastrulation *ntl* is expressed in cells within the germ ring at the vegetal pole and within notochord precursors that extend anteriorly to the *pax2* expression domain (Schulte-Merker et al., 1994). *gsc* expression extends from the animal pole through the prechordal plate mesendoderm and overlying ectoderm (Stachel et al., 1993), ending posteriorly at the level of *pax2* expression and coincident with the anterior boundary of *ntl* expression in the notochord (Fig. 6K).

All mutants in laterality Class I displayed a loss of *ntl* expression from notochord precursors. *ntl* expression was absent from the midline of *ntl* embryos (Fig. 6L) and was present in only a few cells in *flh* embryos (Fig. 6M). Most *mom* mutants displayed a loss of *ntl* expression at the anterior extent of the notochord (Fig. 6N), while in the most severely affected embryos the phenotype resembled that of *flh* mutants. *gsc* expression was normal in *ntl* and *flh* but reduced in *mom*. Within Class II mutants, alterations in *ntl* and *gsc* expression were similar to those seen for *axl* expression. *spt* embryos displayed a lateral expansion of both *ntl* and *gsc* expression domains (Fig. 6O). In *din* mutants, the *ntl* domain was wild-type and the *gsc* domain showed slight anteroposterior shortening within the prechordal plate (Fig. 6P). In Class III (*cyc*) and Class IV (*oep*, *sur*) embryos, *gsc* expression was absent from the prechordal plate but maintained in the polster (Fig. 6Q-T). *ntl* expression was approximately wild-type in these mutants.

DISCUSSION

We have begun to examine the genetic pathways that link development of the embryonic midline and specification of the left-right axis. From our analysis it is apparent that separate genetic pathways functioning in discrete anteroposterior midline domains have distinct roles in left-right development. We have identified four classes of laterality defects, based on asymmetric gene expression patterns associated with the brain, heart and gut, and on discordance in gene expression patterns in these domains. As asymmetric gene expression in the brain has not been described in other vertebrate embryos, we examined whether brain asymmetry is controlled by the same mechanisms as visceral asymmetry, and found that left-right brain development is controlled by some of the same genetic pathways that regulate cardiac and gut left-right development. The relationship between the midline and laterality phenotypes of each class is discussed, and models linking the roles of specific midline domains and the establishment of the left-right axis are proposed (Fig. 7).

Class I: the anterior notochord as a midline barrier

Class I mutants (*ntl*, *flh*, and *mom*) express *lft1*, *lft2* and *pitx2* bilaterally in the diencephalon, heart field and gut. In *ntl* and *flh* mutant embryos *cyc* is also expressed bilaterally (Rebagliati et al., 1998a; Sampath et al., 1998). Class I mutants share the common feature of loss of *axl* and *ntl* expression in the anterior notochord during late gastrulation (Fig. 6B-D, L-N). *ntl* is a mutation in the zebrafish homolog of the T-box transcription factor *Brachyury* (Schulte-Merker et al., 1994). In *ntl* embryos notochord precursors are present but fail to differentiate, resulting in loss of notochord and alterations in floorplate (Halpern et al., 1993). *flh* is a mutation in an Xnot homeobox transcription factor homolog (Talbot et al., 1995) that causes loss of notochord, hypochord and floorplate (Halpern et al., 1995). In *mom*, a mutation in an unidentified gene leads to a variable phenotype that includes loss of notochord from the trunk, fused somites, patchy floorplate and head defects such as small eyes (Odenthal et al., 1996).

The midline phenotype of Class I mutants is consistent with a barrier model in which differentiated anterior notochord cells are required to prevent the bilateral spread of a left-right signaling cascade. Although the floorplate is affected in some of these mutants, it does not seem to play a critical role in this laterality phenotype, since it is also absent in *cyc* mutants, which have normal asymmetric gene expression in the heart and gut. In mice, *lefty* expression in the midline has been proposed to act as a molecular barrier to prevent left-sided signals from crossing the midline (Meno et al., 1998). *lefty1* might also play this role in zebrafish, as it is consistently absent in the anterior notochord of 22- to 24-somite *ntl* and *flh* embryos and discontinuous in the anterior notochord of *mom* embryos.

This phenotypic class is well represented in other systems. Frog embryos in which the presumptive floorplate and notochord are extirpated during open-neural plate stages have randomized cardiac orientation and bilateral gene expression (Danos and Yost, 1996; Lohr et al., 1997). Mice with spontaneous mutations at the *Fused toes* (van der Hoeven et al., 1994; Heymer et al., 1997) and *no turning* (Melloy et al., 1998) loci also have defective notochord development, randomized cardiac orientation and bilateral gene expression patterns. Targeted mutations of several genes implicated in laterality establishment also give Class I mutant phenotypes.

These include the kinesin superfamily proteins *KIF3A* (Takeda et al., 1999) and *KIF3B* (Nonaka et al., 1998), *Lefty-1* (Meno et al., 1998), *Shh* (Tsukui et al., 1999) and *SIL* (Izraeli et al., 1999), a protein thought to act in the Shh signaling pathway. The observation that KIF3A and KIF3B mutants have Class I gene expression patterns suggests that the primary defect in these mice is in the generation of a midline barrier, not in the generation of left-right signals at the node, as previously suggested (Nonaka et al., 1998). Interestingly, in chick, asymmetric expression domains are present prior to notochord differentiation (reviewed by Levin, 1998). This suggests that some of the genes that give Class I phenotypes might be involved in forming an initial barrier within the node.

Class II: randomized and discordant asymmetric gene expression

In Class II mutants (*spt* and *din*), asymmetric gene expression is randomized, with individual expression domains in a given embryo having left, right, bilateral or no gene expression. In this class, midline gene expression domains of *axl*, *gsc* and *ntl* are present and only slightly altered in shape (Fig. 6E,F,O,P). *spt* encodes a T-box transcription factor related to *ntl* (Griffin et al., 1998; Ruvinsky et al., 1998). The mutant phenotype includes convergence/extension defects (Kimmel et al., 1989; Ho and Kane, 1990) and failure to express paraxial mesoderm markers such as *MyoD* (Weinberg et al., 1996), but in contrast to Class I mutants, notochord development is relatively normal (Kimmel et al., 1989). *din* encodes *chordin* (Miller-Bertoglio et al., 1997; Schulte-Merker et al., 1997), a *bmp4* antagonist (Sasai et al., 1995; Piccolo et al., 1996). Mutants exhibit a ventralized phenotype; the tail is expanded at the expense of the head and anterior trunk, and a disrupted notochord is present anteriorly but is reduced posteriorly (Hammerschmidt et al., 1996a,c). *chordin* has been implicated in proper axial versus paraxial development as well as in dorsoventral development (Miller-Bertoglio et al., 1997).

Since both *spt* and *din* are expressed in midline and lateral tissues during gastrulation (Miller-Bertoglio et al., 1997; Schulte-Merker et al., 1997; Griffin et al., 1998; Ruvinsky et al., 1998), we postulate that randomization of asymmetric gene expression may be a result of impaired signaling in either of these tissues. Altered gene expression patterns could arise if the generation of a midline signal occurred at random with respect to the midline. Alternatively, impaired transmission of a signal to lateral tissues might result in lateral tissues independently establishing their own asymmetry, regardless of any midline signals. Interestingly *spt* embryos fail to maintain *chordin* expression in the midline (Miller-Bertoglio et al., 1997), suggesting that *spt* may be upstream of *din*. Midline and transfer signals have been identified in chick. *shh* acts as the midline signal (Pagan-Westphal and Tabin, 1998) and *caronte* participates in the transfer of information to lateral tissues including the cephalic mesenchyme and lateral plate mesoderm (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999).

Similar to Class II zebrafish mutants, mouse *situs inversus* (*iv*) mutants show randomized expression of *nodal*, *lefty* and *pitx2* across a population of embryos (Lowe et al., 1996; Meno et al., 1996, 1997; Supp et al., 1997; Campione et al., 1999). Individual embryos have been reported to have visceral isomerism and discordance between some of the large veins of the thorax or abdomen with the rest of the viscera (Hummel and Chapman, 1959; Layton, 1976; Seo et al., 1992).

Class III: isolated brain laterality defects and a distinct anterior midline barrier

The genetic pathways that regulate brain left-right development are unknown. In cyc^{tf219} mutants (Class III), gene expression in the diencephalon is bilateral or absent while heart field and gut gene expression are wild-type (left-sided). In general, mutant embryos with the strongest bilateral expression have the weakest cyclopic phenotype (not shown). This suggests that the expression pattern in the diencephalon is most accurately defined as bilateral. The absence of gene expression may reflect a requirement for *cyc* in the maintenance of *lft1* and *pitx2* expression or may be a secondary consequence of loss of ventral brain tissue. *cyc* expression has also been reported to be bilateral in the brain of cyc^{m294} and cyc^{tf219} mutants (Rebagliati et al., 1998b; Sampath et al., 1998). *cyc* is a mutation in a zebrafish *nodal* homolog. Mutant embryos are deficient in anterior midline mesendoderm and ventral neuraxis (Hatta et al., 1991; Yan et al., 1995; Feldman et al., 1998) as evident from the loss of *axl* and *gsc* expression anterior to the *pax2* domain (Fig. 6G,H,Q,R). In contrast to Class I mutants, expression of *ntl* in the anterior notochord appears normal. Although *nodal* (*cyc*) has been implicated in the regulation of *lefty* and *pitx2*, and in the establishment of cardiac laterality (reviewed in Yost, 1999), cyc^{tf219} mutants express *lft1* and *lft2* in the heart field (Fig. 3F) and *pitx2* in the gut, and do not have cardiac laterality defects (Table 1). This suggests that other genes may provide redundant functions for the induction of *lefty* and *pitx2* and for cardiac left-right development in zebrafish.

From comparison of Class I and Class III midline phenotypes, we propose a model in which the midline has multiple barriers along the anterior-posterior axis. In Class I, loss of the midline barrier associated with the anterior notochord results in bilateral gene expression in the anterior-posterior domains associated with the brain, heart and gut. In Class III, loss of the midline barrier associated with the prechordal plate mesendoderm and overlying ventral neural tissue alters asymmetric gene expression only in the diencephalon. Thus, there are at least two midline domains along the anteroposterior axis that may act as physical and/or molecular barriers, and these domains are dependent on distinct genetic pathways: *ntl*, *flh* and *mom* in the notochord and *cyc* in the prechordal plate (Fig. 7).

Class IV: *oep* and *sur* are required for asymmetric gene expression

Class IV mutants (*oep* and *sur*) lack asymmetric expression of *lft1*, *lft2* and *pitx2* in the brain, heart and gut. *oep* mutants lack an EGF-CFC protein that mediates *cyc* signaling (Zhang et al., 1998) and is required to promote formation of prechordal plate mesendoderm and floorplate (Schier et al., 1997). *sur* mutants show a loss of posterior prechordal plate mesendoderm, median mid- and hindbrain tissue and floorplate (Brand et al., 1996). In mice, mutations in the EGF-CFC protein *Cryptic* also cause Class IV phenotypes of randomized organ situs and an absence of asymmetric gene expression in lateral plate mesoderm (Yan et al., 1999).

Strikingly, the midline defects are similar in Class IV and Class III, as indicated by loss of anterior *axl* and *gsc* expression (Fig. 6G-J, Q-T). In addition, midline expression domains of *lft1*, *lft2* and *pitx2* are also absent in both *oep* (Class IV) and *cyc* (Class III) mutants at late gastrula (Bisgrove et al., 1999; Essner et al., 2000). How are the distinct laterality phenotypes of these two classes generated in the presence of similar defects in midline gene expression patterns? The absence of asymmetric gene expression in Class IV mutants suggests that *oep* and *sur* are required to mediate the propagation of asymmetric signals in lateral tissues. These genes may be required in or near the midline for the generation of an asymmetric signal, and in lateral tissues to provide competence to respond to these signals. Interestingly, the midline defect in maternal/zygotic *oep* mutants can be rescued by *oep* RNA injection, but asymmetric gene expression cannot be restored (Yan et al., 1999).

A laterality class not regulated by the midline

Full inversion of the left-right axis (situs inversus totalis) is rare in humans. Concordant inversion of asymmetric gene expression in the heart and gut is seen in only two model systems: in mouse *inversion of turning* (*inv*) mutants (Yokoyama et al., 1993; Collignon et al., 1996; Lowe et al., 1996; Meno et al., 1996, 1997) and in *Xenopus* embryos in which mature Vg1 protein is expressed in right lateral cells at the 16-cell stage (Hyatt and Yost, 1998). In both of these cases, midline development appears normal. Although the timing and embryonic location of *inv* function are unknown, Vg1 ectopic expression in *Xenopus* is most effective in regions distant from the locus of midline formation. Full inversion of asymmetric gene expression or organ primordia was not observed in the nine zebrafish midline mutants examined here, suggesting that the steps in the left-right pathway that must be perturbed to give full inversion precede the steps in which the affected genes of these midline mutants participate.

Asymmetric gene expression is coordinated and correlated with cardiac looping

Wild-type and mutant embryos double-labeled with antisense probes to either *lft1* or *lft2* and *cyc* or *pitx2* show a high coincidence (>99%, data not shown) of expression of these genes in the diencephalon and heart field (Fig. 1), suggesting that the regulation of these genes is tightly coordinated within each organ primordium. Asymmetric expression of *cyc*, *lft1* and *lft2* in the heart field is apparent at the 19-somite stage (Rebagliati et al., 1998a; Sampath et al., 1998; Bisgrove et al., 1999; Thisse and Thisse, 1999), preceding the asymmetric expression of *bmp4* at 22 somites (Chen et al., 1997), (Figs 1, 2). As such, it is possible that *cyc*/*lft* signaling may be responsible for mediating asymmetric expression of *bmp4* and cardiac laterality. In general, the asymmetric patterns of *bmp4* expression in the heart field (Chen et al., 1997) correlate well with the patterns of asymmetric *lft2* and *pitx2* expression. *bmp4* is predominantly symmetric (bilateral) in *ntl*, *flh* and *mom* embryos (Class I), random in *din* (Class II) and left-sided in cyc^{tf219} (Class III). *bmp4* expression is also symmetric in *sur*, a mutant with no expression of *lft2* or *pitx2* in the heart field (Class IV). Consistent with the hypothesis that *lft2* and *pitx2* provide instructive signals for asymmetric *bmp4* localization, in the absence of *lft2* or *pitx2* signals, *bmp4* expression is predicted to remain symmetric. One discrepancy between the *bmp4* expression data (Chen et al., 1997) and our *lft2* and *pitx2* expression data is for *spt*, which showed 88% bilateral expression of *bmp4* and random expression of *lft2, pitx2* (43% bilateral, Table 2). The significance of this is unclear, although it may simply reflect a sample size bias (n=24 for *bmp4* versus n=185 for the *lft2/pitx2* data).

Gene expression discordance and organ heterotaxia

Two phenotypic classes show concordant alterations of gene expression patterns in the brain, heart and gut, with either symmetric (Class I) or absent (Class IV) expression, suggesting a global perturbation of the laterality pathway. The other two classes of zebrafish mutants show highly discordant patterns of gene expression among all (Class II) or subsets (Class III) of the asymmetric expression domains, indicating that the domains along the anterior-posterior axis can be regulated independently. It is predicted that high discordance of asymmetric gene expression patterns would lead to heterotaxia among the asymmetric organs, and that left versus right-sided expression would drive organ laterality in the normal or reversed direction, respectively. While organ situs cannot be predicted in cases with bilaterally symmetric or absent signaling information, it is likely that individual organ primordia orient stochastically. In this case, mutants with concordant global perturbations (Class I and Class IV) are also predicted to exhibit heterotaxia among the brain, heart and gut. We have found that heterotaxia between cardiac looping and the position of the pancreas is present in members of classes I, II and IV of laterality mutants (Table 3). Heterotaxia between cardiac looping and the position of the pancreas in rescued maternal/zygotic *oep* mutant embryos has also been reported (Yan et al., 1999), and heterotaxia between cardiac and hepatic diverticulum situs occurs in *ntl*, *flh*, *spt* and cyc^{b16} mutant embryos (Schilling et al., 1999).

In humans, it is becoming clear that midline defects are linked with laterality defects (Casey, 1998; Goldstein et al., 1998; Yost, 1998b; Morelli et al., 1999). The present results demonstrate that separate genetic pathways expressed in distinct regions of the midline have specific roles in left-right development. We predict that as laterality defects in humans are more carefully examined, separate syndromes that involve defects in a limited region of the midline and corresponding subsets of laterality defects in the heart, gut and brain will be recognized. The classes defined by genetic mutants in this study provide candidate genetic pathways for analysis of familial midline/laterality defect syndromes. Aside from minor differences, vertebrates share an important and conserved role for the embryonic midline in the generation of asymmetric gene expression of *nodal*, *lefty* and *pitx2*, and the establishment of left-right asymmetry. Genetic analysis in zebrafish provides an important tool for defining the roles of the midline in the genetic interactions that control left-right development.

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