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Two additional midline barriers function with midline *lefty1* expression to maintain asymmetric Nodal signaling during left-right axis specification in zebrafish

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SUMMARY

Left-right (L/R) patterning is crucial for the proper development of all vertebrates and requires asymmetric expression of *nodal* in the lateral plate mesoderm (LPM). The mechanisms governing asymmetric initiation of *nodal* have been studied extensively, but because Nodal is a potent activator of its own transcription, it is also crucial to understand the regulation required to maintain this asymmetry once it is established. The 'midline barrier', consisting of *lefty1* expression, is a conserved mechanism for restricting Nodal activity to the left. However, the anterior and posterior extremes of the LPM are competent to respond to Nodal signals yet are not adjacent to this barrier, suggesting that *lefty1* is not the only mechanism preventing ectopic Nodal activation. Here, we demonstrate the existence of two additional midline barriers. The first is a 'posterior barrier' mediated by Bmp signaling that prevents *nodal* propagation through the posterior LPM. In contrast to previous reports, we find that Bmp represses Nodal signaling independently of *lefty1* expression and through the activity of a ligand other than Bmp4. The 'anterior barrier' is mediated by *lefty2* expression in the left cardiac field and prevents Nodal activation from traveling across the anterior limit of the notochord and propagating down the right LPM. Both barriers appear to be conserved across model systems and are thus likely to be present in all vertebrates.

KEY WORDS: *nodal*, *southpaw*, Zebrafish, Bmp, Left-right asymmetry, Midline barrier, *lefty*

INTRODUCTION

L/R patterning is crucially important for the proper development of all vertebrates. Left-restricted signaling through the Nodal pathway plays a conserved role in this process by establishing the initial molecular differences between left and right that are essential for the later asymmetric morphogenesis and positioning of visceral organs (Burdine and Schier, 2000; Raya and Izpisua Belmonte, 2006). Defects in the initiation or maintenance of left-sided Nodal activity result in organ abnormalities that are often fatal (Bisgrove et al., 2003; Burdine and Schier, 2000; Ramsdell and Yost, 1999; Sutherland and Ware, 2009).

Initiation of Nodal signaling in the left LPM is thought to be generated by cilia motility and asymmetric fluid flow in 'organs of asymmetry', including the node in mouse and Kupffer's vesicle (KV) in zebrafish (Raya and Izpisua Belmonte, 2006). Once present in the LPM, the Nodal ligand positively regulates its own transcription, leading to propagation of *nodal* throughout the left side of the embryo. However, because Nodal ligands propagate their own expression, and the right LPM is competent to respond to Nodal signals, the asymmetric initiation of *nodal* is not sufficient to maintain left-restriction (Nakamura et al., 2006; Wang and Yost, 2008).

The Nodal targets and antagonists *lefty1* and *lefty2* are crucial to prevent ectopic *nodal* induction after initiation. *lefty1* at the midline acts as a 'molecular midline barrier', preventing Nodal propagation from left to right LPM. Mouse embryos with a mutation in *Lefty1* or zebrafish injected with *lefty1* morpholino both exhibit proper left initiation of *nodal*, but display later bilateral activation of Nodal targets (Meno et al., 1998; Nakamura et al., 2006; Wang and Yost, 2008). Although establishment of this canonical midline barrier is essential to restrict Nodal activity, cells at the anterior and posterior extremes of developing embryos are beyond the range of Lefty1 antagonism (see e.g. Furtado et al., 2008; Meno et al., 1998) and, yet, are competent to respond to Nodal signals as they express the Nodal co-factor *one-eyed pinhead* and the transcription factor FoxH1 (Pogoda et al., 2000; Sirotkin et al., 2000; Zhang et al., 1998). Therefore, other mechanisms must exist to prevent ectopic *nodal* propagation into these tissues.

Here, we describe two additional midline barriers that function to restrict Nodal signaling to the left LPM. The first is a previously unidentified 'posterior barrier' mediated by Bmp signaling that prevents propagation of the zebrafish Nodal *southpaw* (*spaw*) from left to right LPM through the ventral mesoderm underlying the tail bud. Bmp4 has been widely implicated as the primary Bmp signal required during L/R patterning, both for the induction of midline *lefty1* and the later establishment of organ asymmetries (Chen et al., 1997; Chocron et al., 2007; Schilling et al., 1999). Surprisingly, our analysis of a new putative null allele of *bmp4* indicates that Bmp4 is not the ligand necessary to establish the posterior barrier or for correct organ laterality in zebrafish. Additionally, we describe an 'anterior barrier' in the embryo, where *lefty2* in the left cardiac field prevents *spaw* expression from propagating around the anterior of the notochord and back down the right LPM. Both 'midline barriers' we describe appear to be conserved across model systems, although the location of barrier activity might have been modified in a species-specific manner to compensate for unique embryo architecture and development.

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MATERIALS AND METHODS

Zebrafish strains and genotyping

The *laf*^{tm110b} mutants and genotyping strategy have been described previously (Mintzer et al., 2001).

Generation and verification of *bmp4* TILLING mutants

TILLING mutants were generated as previously described (Moens et al., 2008). A 987-base pair (bp) fragment containing exon 2 of *bmp4* was amplified using primers IRD700 (CACCCCTGCTCTCAACTATCAA) and IRD800 (GTGTCCACGTGTGGATGTTTTT) and screened for mutations. To verify the presence of single genetic lesions within the coding sequences of the *bmp4* gene in each TILLING mutant, the complete coding region was sequenced.

Genotyping strategy for the new *bmp4* alleles

The mutation in *bmp4*^{Y180*} eliminates an *Nde*I site. For genotyping, the 68-bp PCR product from *bmp4tillf2* (GGTTTGCATCGGATAAACACATA) and *bmp4till-r2* (GAGCTGCGTGATGAGCTGTC) was digested with *Nde*I resulting in 18- and 50-bp bands in wild type. The mutation in *bmp4*^{S355*} creates an *Fsp*BI/*Mae*I site. For genotyping, the 150-bp PCR product from *bmp4tillf1* (CATGGAGAGTGTCCCTTTC) and *bmp4till-r1* (GTCCAGGT-AAAGCATGGAG) was digested with *Fsp*BI/*Mae*I resulting in 75-bp bands in the mutant. The mutation in *bmp4*^{C365S} creates a *Pst*I site. For genotyping, the 150-bp PCR product from *bmp4tillf1* and *bmp4till-r1* was digested with *Pst*I resulting in 44- and 106-bp bands in the mutant background.

RNA in situ hybridizations

In situ hybridizations were per the standard protocol (Thisse and Thisse, 2008) using *spaw* (Long et al., 2003) and *lefty1* (Bisgrove et al., 1999) probes.

RNA and morpholino injections

RNAs and morpholinos were injected using standard protocols. Constructs used to generate RNA include: zfbmp4wt (BL485), zfbmp4Y180 (BL482), zfbmp4S355 (BL486) and zfbmp4Cs365S (BL484), generated by amplification of the full-length cDNA from wild-type and MZ*bmp4* mutant cDNAs using primers *bmp4f2* (CCGCTCGAGatgATTCCTGGTAAT) and *bmp4r2* (CCGTCTAGAttaGCGGCAGCCACA). *lefty1* MO (2 ng) or *lefty2* MO (3 ng) were used (Agathon et al., 2001).

RESULTS AND DISCUSSION

Bmp signaling generates a posterior midline barrier distinct from *lefty1* in the notochord

spaw expression is initially visible in two small, symmetric domains on either side of Kupffer's vesicle at 6-8 somites (S) (Fig. 1A) (Long et al., 2003). Asymmetric activation of *spaw* in the left LPM is evident in most embryos by 10S, when the Nodal antagonist *lefty1* is expressed in the notochord and is believed to act as a molecular midline barrier, preventing left-initiated *spaw*

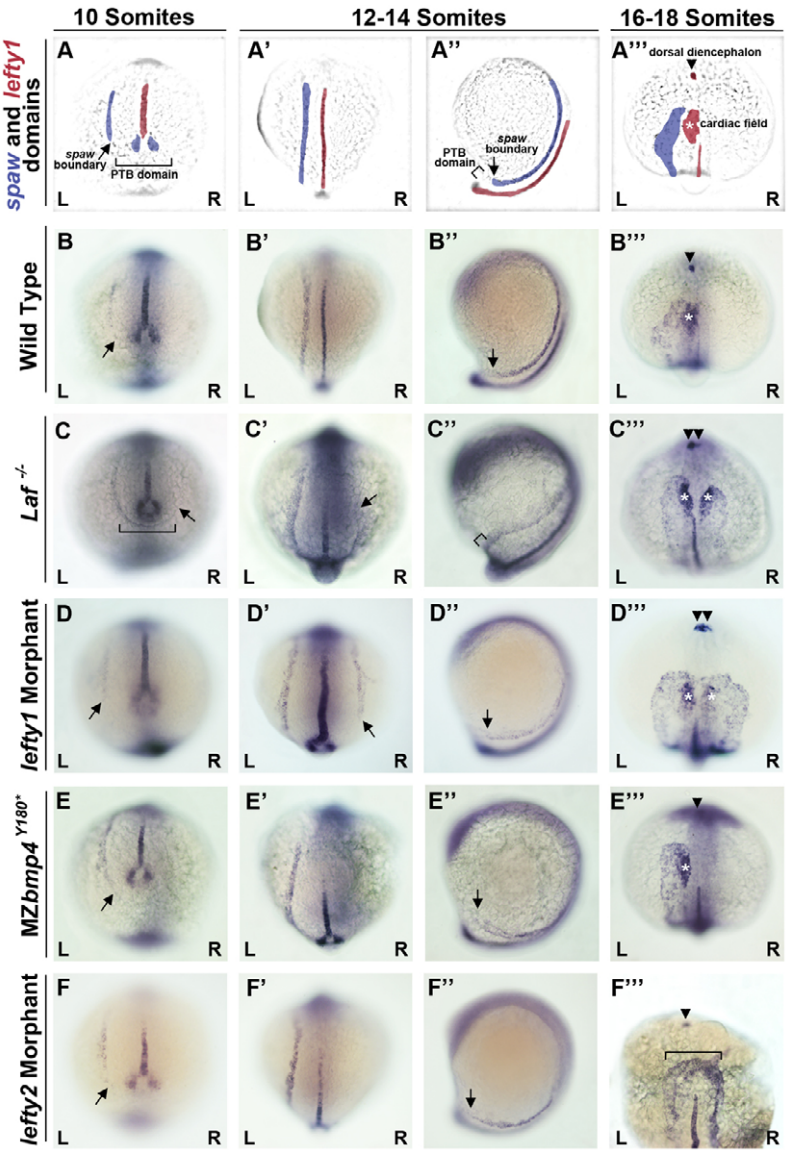

Fig. 1. *spaw* and *lefty1* expression phenotypes. (**A-F‴**)Time course of single color, double in situ hybridizations for *spaw* and *lefty1* from 10-18 somites in posterior (A-F), mid-LPM (A′-F′), left lateral (A″-F″) and anterior LPM (A‴-F‴) views. (A-A‴) False-colored images of zebrafish embryos from B-B‴ depicting the domains of *spaw* and *lefty1*. In situ hybridizations of *spaw* or *lefty1* alone confirm the reported phenotypes. Arrows, boundary of detectable *spaw* expression; brackets, ectopic *spaw* across the midline; arrowheads, *lefty1* in the diencephalon; asterisks, *lefty1* in the cardiac mesoderm. L, left; R, right.

from inducing its own expression in the right LPM (Fig. 1A,B; Fig. 3C). The asymmetry in *spaw* expression is self-propagated throughout the left LPM, and by 18-20S *spaw* has reached the anterior and activated expression of *lefty1* and *lefty2* in the heart field (Fig. 1A′-A‴,B-B‴; Fig. 3C′-C‴).

To understand the mechanisms involved in restricting *nodal* expression to the left, we focused on Bmp signaling. In zebrafish, overexpression of *bmp2b* eliminates *spaw* in the LPM, whereas complete inhibition of Bmp signaling leads to bilateral expression of *spaw* by 18S (Chocron et al., 2007). Bmp signaling in the mouse LPM prevents ectopic *Nodal* expression by limiting the availability of Smad4 (Furtado et al., 2008). Moreover, the Bmp pathway is reported to be required for activation of midline *lefty1* in mouse and zebrafish (Chocron et al., 2007; Kishigami et al., 2004; Monteiro et al., 2008).

To analyze the timing and effect of Bmp signaling on the initiation of *spaw* in the LPM, we utilized the *lost-a-fin* (*laf*) mutation in the type I receptor Alk8 (Acvr1 – Zebrafish Information Network). These mutants display defects in visceral L/R patterning (Bauer et al., 2001; Chocron et al., 2007; Mintzer et al., 2001) but the effect on early asymmetric gene expression has not been reported. In contrast to previous analyses, we find that *lefty1* expression is present in all *laf* mutants at 10S and is maintained throughout L/R axis specification, similar to wild type (WT), suggesting that Bmp signaling through Alk8 is not required for midline *lefty1* induction (Fig. 1C-C‴; Fig. 3D-D‴; Table 1). LPM expression of *spaw* is correctly initiated on the left in all *laf* embryos by 10S (Fig. 1C; Fig. 3D; Table 1). However, these embryos also exhibit ectopic propagation of LPM *spaw* into the ventral mesoderm posterior to the tailbud (hereafter referred to as the posterior tailbud domain, or PTB) (Fig. 1C, bracket; Fig. 3D; Table 1). Although the PTB tissue expresses components of the Nodal pathway necessary for auto-induction, *spaw* RNA is never observed in this region in WT embryos (Fig. 1B-B‴; Table 1). Consequently, Nodal pathway activation in the PTB domain of *laf* mutants strongly suggests that Bmp signaling through Alk8 is required to prevent LPM *spaw* from ectopically propagating through this tissue. By 12-14S, bilateral expression of *spaw* is observed in the LPM in all *laf* embryos, 97% of which maintain *spaw* expression in the PTB (Fig. 1C′-C″, bracket; Fig. 3D′; Table 1). This bilateral expression is maintained throughout later somite stages, as is ectopic *spaw* surrounding the tail bud (Fig. 1C‴; Fig. 3D″; Table 1). Taken together, these data suggest that the bilateral expression of *spaw* in *laf* mutants results from inappropriate propagation of Nodal signaling from the left LPM, through the PTB domain to the right.

The ‘posterior repressor’ is distinct from the *lefty1* midline barrier. We see asymmetric initiation upon knockdown of *lefty1*, with subsequent bilateral expression of *spaw* in the LPM, but the bilateral phenotype does not arise through ectopic Nodal activation in the PTB domain (Fig. 1D-D‴; Fig. 3E; Table 1). Instead, we observe consistent induction of *spaw* in the right LPM of *lefty1* morphants anterior to the PTB, by 12-14S (Fig. 1D1; Fig. 3E1). This suggests that right-sided *spaw* is induced in *lefty1* morphants by diffusion of left-derived Spaw directly across the embryonic midline, rather than through the PTB domain as in *laf* mutants. Thus, both midline and posterior molecular barriers are required for maintenance of asymmetric Nodal activation.

bmp4 is not required for Bmp-mediated restriction of asymmetric Nodal

We hypothesized that Bmp4 might be the ligand responsible for mediating posterior repression because Bmp4 has been implicated in zebrafish L/R patterning in several reports (Chen et al., 1997; Chocron et al., 2007; Schilling et al., 1999). However, embryos homozygous for a putative null *bmp4* mutation do not exhibit L/R defects (Stickney et al., 2007), though these mutants also display incompletely penetrant dorsal-ventral (D/V) phenotypes, making it difficult to determine the function of Bmp4 in L/R patterning from these embryos.

Table 1. Midline *lefty1* and asymmetric *spaw* expression phenotypes in MZ*bmp4*Y180*, *Laf*$^{-/-}$, *lefty1* morphants and *lefty2* morphants

			spaw						
Genotype	Stage	Midline *lefty1* (%)	Left (%)	Right (%)	Bilateral (%)	Absent (%)	Ectopic tail bud[¶] (%)	Ectopic anterior[†] (%)	*n*
Wild type	10S	100	63	0	0	37	0	0	54
	12-14S	100	100	0	0	0	0	0	87
	16-18S	100	100	0	0	0	0	0	106
MZ*bmp4* Y180*	10S	100	100	0	0	0	0	0	26
	12-14S	100	85	3	12	0	3	0	26
	16-18S	100	91	2	7	0	2	0	96
Laf siblings	10S	100	67	0	0	33	0	0	172
Laf$^{-/-}$	10S	100	100	0	0	0	100	0	60
	12-14S	100	0	0	100	0	97	0	29
	16-18S	100	0	0	100	0	100	0	24
lefty1 morphants[‡]	10S	100	70	0	23	7	0	0	74
	12-14S	100	32	0	68	0	2	0	199
	16-18S	100	13	0	87	0	0	0	74
lefty2 morphants[§]	10S	100	70	0	2	28	0	0	50
	12-14S	100	70	0	30	0	6	0	96
	16-18S	100	67	4	26	3	0	70	73

[†]Embryos exhibiting ectopic *spaw* within the cardiac field. 16% of these embryos show propagation of *spaw* down the right LPM from the anterior. These embryos probably have a more complete knockdown of *lefty2*.
[‡]*lefty1* expression is retained in *lefty1* morphants, as morpholinos do not disrupt RNA transcription.
[§]*lefty2* morphants display low levels of early defects in *spaw* expression, probably as a consequence of disrupting the early requirement for Nodal signaling in midline development (Weng and Stemple, 2003).
[¶]Propagation of *spaw* across the midline through the PTB domain.

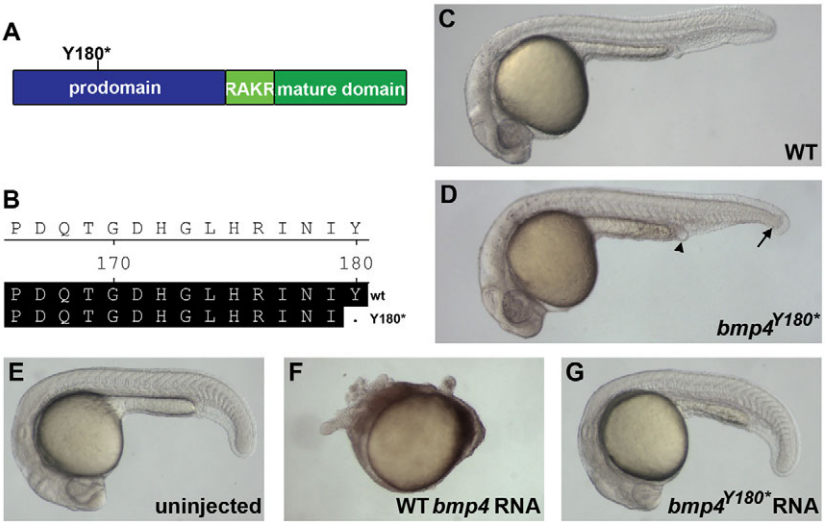

Fig. 2. Characterization of the *bmp4*^{Y180}* allele. (**A**) Bmp4 protein with location of the *Y180** mutation indicated. RAKR, cleavage sequence. (**B**) Sequence comparisons of cDNA from wild type (wt) and *bmp4*^{Y180}* mutants. (**C-G**) Zebrafish embryos at 24-36 hours post-fertilization (hpf). (C) WT embryo. (D) *bmp4*^{Y180}* mutant with absent ventral fin (arrow) and cloaca defects (arrowhead). (E) Uninjected embryo. (F) Embryo injected with *bmp4* RNA exhibiting V4 ventralization. (G) Embryo injected with *bmp4*^{Y180}* RNA lacking morphological defects.

To analyze Bmp4 in L/R patterning, we used new alleles of *bmp4* generated through TILLING (Fig. 2B; see Fig. S1C,E in the supplementary material). Characterization of two alleles, *bmp4*^{S355}* and *bmp4*^{C365S}, revealed erratically penetrant ventral patterning defects present at non-Mendelian ratios (see Table S1 in the supplementary material). Additional analysis confirmed that both mutations have partially penetrant dominant-negative effects (see Fig. S1I,J in the supplementary material; see Table S3 in the supplementary material) and were not studied further. By contrast, the *bmp4*^{Y180}* allele, has a stop codon early in the prodomain, that truncates the Bmp4 protein prior to the carboxy-terminal active signaling molecule (Fig. 2A,B). Whereas overexpression of *bmp4* mRNA produces severe ventralization as reported (Neave et al., 1997; Weber et al., 2008) (Fig. 2F; see Table S2 in the supplementary material), overexpression of *bmp4*^{Y180}* has no phenotypic effect (Fig. 2E,G; see Table S3 in the supplementary material), further suggesting that this allele represents a true loss of function. Zygotic *bmp4*^{Y180}* mutants do not display obvious D/V defects early in development and display a low penetrance of later D/V phenotypes (Fig. 2D; see Table S1 in the supplementary material). In addition, L/R patterning of the visceral organs is unaffected (see Table S3 in the supplementary material).

To determine whether maternal Bmp4 (Hwang et al., 1997) compensates for zygotic loss during D/V and L/R patterning, we generated maternal-zygotic (MZ) *bmp4*^{Y180}* mutants. Although MZ embryos do not display early D/V phenotypes, all of the MZ*bmp4*^{Y180}* mutants lack the ventral fin and 23% display defects in cloaca development (Fig. 2D; see Table S1 in the supplementary material). These data suggest that the *bmp4*^{Y180}* mutation is a true null and that the presence of maternal *bmp4* can partially compensate for zygotic loss of Bmp4 activity.

Given their significant and consistent phenotypes, MZ*bmp4*^{Y180}* embryos provide the ideal system to address the role of Bmp4 during L/R patterning. Interestingly, we find that organ laterality is properly established in 89% of MZ mutants (see Table S3 in the supplementary material), and *spaw* expression is initiated and maintained correctly in the majority of these embryos (Fig. 1E-E‴; Table 1). Furthermore, we do not observe loss of midline *lefty1* in any MZ mutant embryos (Fig. 1E-E‴; Table 1). This indicates that Bmp4 is not necessary for expression of *lefty1* at the midline as was previously reported, and that Bmp4 is not the primary ligand required for posterior repression. As *bmp2b* is strongly expressed in the ventral mesoderm and epidermis posterior to KV (Thisse and Thisse, 2004), Nodal inhibition in the PTB domain might be mediated by this Bmp ligand. However, as loss of Bmp2b severely disrupts formation of ventral posterior tissues (Kishimoto et al., 1997), confirmation of a role for this ligand in the PTB domain will require the development of methods for spatial and temporal specificity in gene knockdown.

Bmp4 and Alk8 limit Nodal responsiveness in the LPM

Bmp signaling in the mouse LPM helps maintain *Nodal* asymmetry by dampening the ability of Nodal to activate downstream targets on both the left and right (Furtado et al., 2008). Our analysis of *laf* and MZ*bmp4*^{Y180}* mutants suggests a similar role for Bmp signaling in zebrafish. At 10S, only 63% of WT embryos and 67% of *laf* siblings express weak to barely detectable *spaw* asymmetrically in the LPM (Table 1). We find that *spaw* expression in the LPM is consistently apparent in WT embryos only by the 12S stage. By contrast, all *laf* and MZ*bmp4*^{Y180}* mutants display strong expression of *spaw* in the left LPM at 10S (Table 1). This robust *spaw* expression is consistent with phenotypes reported for mouse *Smad1* mutants, which exhibit precocious expression of *Nodal* in the LPM (Furtado et al., 2008). Although we do not observe *spaw* in the LPM of *laf* and MZ*bmp4*^{Y180}* mutants prior to 10S, the strong and consistent left initiation exhibited by these embryos at 10S suggests that the Nodal pathway is more robustly activated in the absence of Alk8 and Bmp4.

The later defects in *spaw* expression in MZ*bmp4*^{Y180}* mutants might also support a role for Bmp4 in limiting Nodal activity in the LPM. We note that 12% of MZ*bmp4*^{Y180}* mutants exhibit bilateral *spaw* by 12-14S (Table 1), which is likely to be due to a continued requirement for Bmp4 in limiting the responsiveness of LPM cells to Nodal signals. Thus, our evidence suggests that Bmp signaling sets a threshold for Nodal activation in LPM cells that cannot be overcome by low concentrations of Spaw.

This role for the Bmp pathway is consistent with the weak expression of *spaw* we observe in the right posterior LPM in most WT embryos by 18S (R.D.B., unpublished) (Gourronc et al., 2007) This right-sided *spaw* expression does not propagate in the LPM, but does suggest that the right LPM is exposed to Spaw protein. In WT embryos, the low concentration of Spaw reaching the right side would be dampened by Bmp signaling, preventing *spaw*

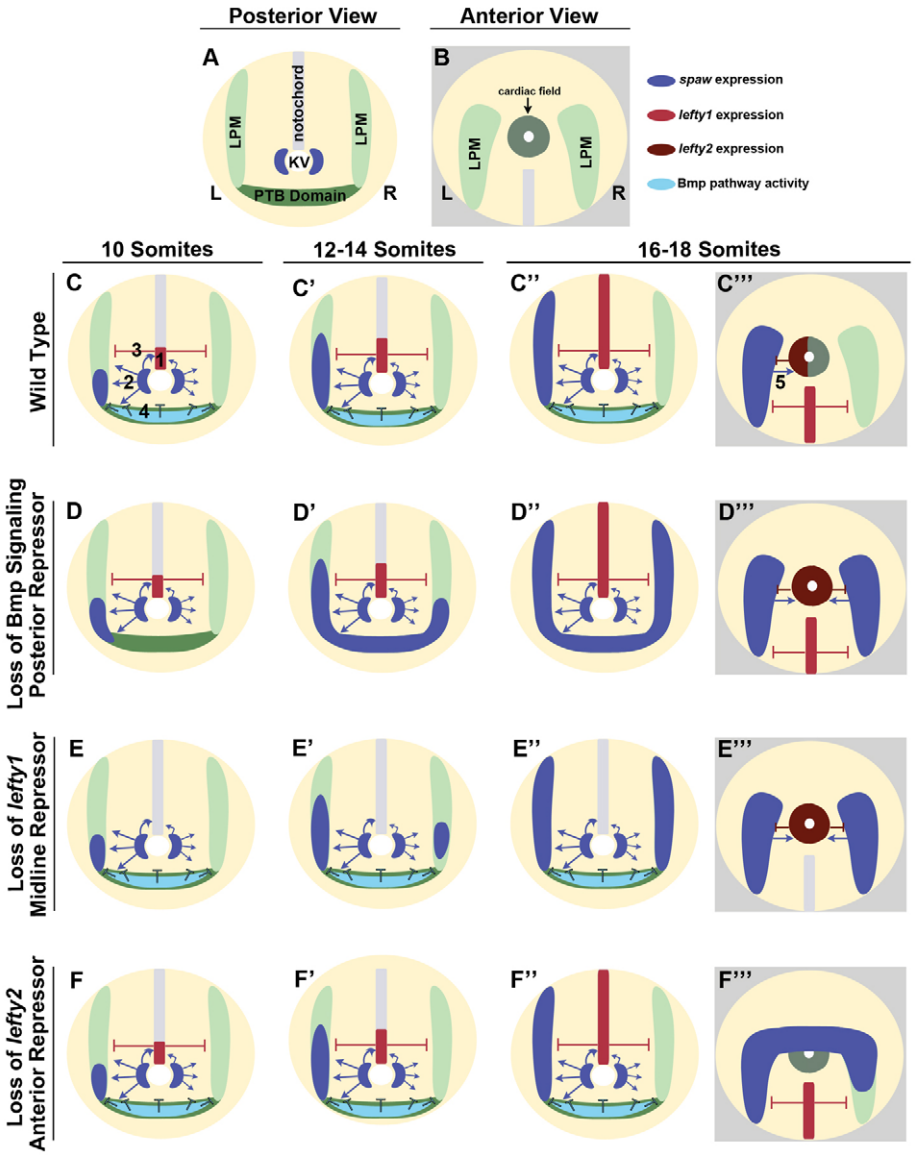

Fig. 3. Model of molecular midline barriers. (**A**,**B**) Posterior and anterior views of a zebrafish embryo. LPM, lateral plate mesoderm; KV, Kupffer's vesicle; PTB, posterior tail bud; L, left; R, right. (**C-C‴**) (1) KV Spaw activates *lefty1* expression in the posterior notochord. (2) Unknown events in KV increase Nodal signaling on the left which initiates *spaw* expression in the left LPM. (3) Lefty1 from the notochord represses Spaw in the LPM but is overcome on the left by increased Spaw signaling. (4) Bmp signaling in the PTB domain prevents Nodal activation in this tissue. (5) At 18S, anterior Spaw activates expression of *lefty2* in the left cardiac field. *lefty2* inhibits Nodal activation in the cardiac LPM. (**D-D‴**) In *laf* mutants, *lefty1* is still maintained in the notochord but absence of Bmp signaling in the PTB domain relieves repression of Nodal activation in this tissue. Consequently, *spaw* propagates through the PTB domain and into the right LPM. *spaw* then propagates bilaterally to the anterior, activating *lefty1* and *lefty2* transcription bilaterally in the heart field. (**E-E‴**) In the absence of *lefty1*, *spaw* is still induced asymmetrically in the left LPM. Although *spaw* is restricted from the PTB domain, the lack of the *lefty1* midline barrier permits Spaw to diffuse across the midline and activate the Nodal pathway in the right LPM. *spaw* then propagates bilaterally towards the anterior and activates expression of *lefty1* and *lefty2* in the left and right of the cardiac field. (**F-F‴**) In the absence of *lefty2*, early initiation and subsequent propagation of *spaw* in the left LPM is not disrupted. However, when *spaw* reaches the anterior, loss of Nodal antagonism by *lefty2* on the left of the heart allows *spaw* expression to propagate into the cardiac field and, in some embryos, down the right LPM from anterior to posterior.

propagation in the right LPM. In MZ*bmp4*^{Y180*} mutants, however, diminished Bmp signaling decreases the threshold level of Spaw required for pathway activation. As a consequence, the low concentration of Spaw diffusing to the right LPM might be sufficient in some embryos to induce *spaw* expression earlier than normal, at a time when anterior propagation is still possible (Long et al., 2003). Given the apparent high level of conservation between mouse and zebrafish concerning this regulation, it will be interesting to see whether similar requirements for Bmp signaling are uncovered in other vertebrates.

lefty2 in the cardiac field provides a third molecular midline barrier in the anterior

spaw in the LPM extends beyond the anterior boundary of the notochord and midline barrier activity of *lefty1*. Because the cardiac field and right LPM are competent to respond to Nodal signals, an additional molecular barrier must exist in the anterior to prevent ectopic *spaw* propagation across the midline. Unlike other vertebrates, which express *lefty2* throughout the left LPM, zebrafish *lefty1* and *lefty2* are restricted to the cardiac field (Thisse and Thisse, 1999). Thus, we determined whether cardiac *lefty2* expression serves as an anterior molecular barrier to ectopic *spaw* propagation.

At 16-18S, we find that 70% of *lefty2* morphants display ectopic activation of *spaw* across the heart field to the midline (Fig. 1F‴; Fig. 3F‴; Table 1). In 16% of these embryos, *spaw* passes above the anterior notochord and propagates back down the right LPM from anterior to posterior (Fig. 1F‴; Fig. 3F‴). This phenotype is not the result of bilateral Nodal propagation from the posterior, because *spaw* in these embryos is restricted to the anterior LPM on the right and *lefty1* is induced only in the left diencephalon (Fig. 1F‴, arrowhead). As midline *lefty1* expression is present in all *lefty2* morphants, this suggests that the Nodal antagonism provided by *lefty2* functions as a distinct molecular barrier (Table 1). Furthermore, ectopic anterior *spaw* expression is never observed in *lefty1* morphants, indicating that Lefty2 provides the crucial anterior barrier function (Table 1).

Although zebrafish do not express *lefty1* or *lefty2* in the majority of the LPM, retention of *lefty2* within the cardiac mesoderm is necessary to maintain anterior asymmetric restriction of Nodal

activity. It is possible that, owing to the architecture of the zebrafish embryo, induction of Nodal antagonists throughout the LPM would block anterior propagation of *spaw*, whereas in other vertebrates, more significant overlap between *nodal* and *lefty2* is necessary to restrict Nodal activation to the left. Interestingly, loss of *Lefty2* in mouse also leads to bilateral Nodal pathway activation through ectopic propagation of *Nodal* across the midline from left to right, suggesting that cardiac *lefty2* in zebrafish and LPM *Lefty2* in mouse act from different tissues to perform the same Nodal-regulatory role (Meno et al., 1998). Together, these phenotypes highlight what appears to be a recurring theme in left-right patterning: regulatory signals and mechanisms required to limit the activity of the Nodal pathway are conserved across vertebrates but with species-specific modifications in the timing and location of these genetic programs.

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Competing interests statement
The authors declare no competing financial interests.

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Table S1. D/V patterning defects in Z and MZ *bmp4* mutants

Genotype	Wild type (%)	Ventral fin defect (%)	Ventral fin and cloaca defect (%)	*n*
Z *bmp4* Y180*/+ IX	91	8	1	772
Z *bmp4* S355*/+ IX	71	25	4	284
Z *bmp4* C365S/+ IX	80	19	1	718
MZ *bmp4* Y180*–/–	0	77	23	426
MZ *bmp4* S355*–/–	61	35	4	344
MZ *bmp4* C365S–/–	38	59	3	281

Presence and morphology of the ventral fin and correct development of the cloaca (Esterberg et al., 2008; Stickney et al., 2007) were scored in Z and MZ *bmp4* mutants. Percentages for zygotic in-crosses contain both mutant and sibling populations. Morphological defects are not completely penetrant in mutants and are partially dominant in the *S355** and *C365S* alleles.

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Table S2. Effects of mutant and WT *bmp4* mRNA injections

			Phenotype[†]					
			Dorsalized (%)			Ventralized (%)		
RNA	Concentration (pg)	Normal (%)	C1	C2-C3	C4	V3	V4	*n*
Uninjected	0	100	0	0	0	0	0	30
bmp4 WT	50	0	0	0	0	2	98	87
bmp4 Y180*	50	100	0	0	0	0	0	180
bmp4 S355*	50	96	1	3	0	0	0	97
bmp4 C365S	50	72	13	13	2	0	0	208
bmp4 WT	100	0	0	0	0	0	100	84
bmp4 Y180*	100	100	0	0	0	0	0	101
bmp4 S355*	100	86	2	8	4	0	0	183
bmp4 C365S	100	59	16	16	9	0	0	74

To test whether mutant *bmp4* RNAs would retain activity we carried out overexpression assays. D/V patterning defects were scored in wild-type (WT) embryos injected with WT and mutant *bmp4* RNAs.

[†]Phenotypes are classified according to previously determined designations for degrees of dorsalization and ventralization (Kishimoto et al., 1997; Neave et al., 1997; Weber et al., 2008). C1, only the ventral tail fin is reduced; C2-C3, curled and progressive loss of tail; C4, head structure visible on yolk; C5, most extreme class with no posterior or ventral structures; V3, tissue at either end of the yolk; V4, most extreme class with disrupted epiboly.

Table S3. Organ laterality phenotypes in Z and MZ *bmp4* mutants

Genotype	Situs solitus[†] (%)	Situs inversus[‡] (%)	Heterotaxia[§] (%)	*n*
Z *bmp4* Y180*/+ IX	99	0	1	162
Z *bmp4* S355*/+ IX	99	0	1	83
Z *bmp4* C365S/+ IX	98	2	0	352
MZ *bmp4* Y180*–/–	89	7	4	152
MZ *bmp4* S355*–/–	99	1	0	148
MZ *bmp4* C365S–/–	100	0	0	94

Positions of the heart, liver and pancreas were determined for Z and MZ *bmp4* mutants at 48 hpf by RNA in situ hybridization. Because zygotic mutants cannot be consistently identified by morphology, percentages for zygotic in-crosses contain both mutant and phenotypically wild-type siblings.

[†]Embryos with correct organ placement.

[‡]Embryos with complete reversals in organ placement.

[§]Embryos with random positioning of organs about the L/R axis.