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.
MATERIALS AND METHODS
Zebrafish strains and genotyping
The \textit{laf}m110b mutants and genotyping strategy have been described previously (Mintzer et al., 2001).

Generation and verification of \textit{bmp4} TILLING mutants
TILLING mutants were generated as previously described (Moens et al., 2008). A 987-base pair (bp) fragment containing exon 2 of \textit{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 \textit{bmp4} gene in each TILLING mutant, the complete coding region was sequenced.

Genotyping strategy for the new \textit{bmp4} alleles
The mutation in \textit{bmp4}Y180* eliminates an \textit{Nde}I site. For genotyping, the 68-bp PCR product from \textit{bmp4}tillf2 (GGTTTGCATCGGATAAACACATA) and \textit{bmp4}till-r2 (GAGCTGCGTGATGAGCTGTC) was digested with \textit{Nde}I resulting in 18- and 50-bp bands in wild type. The mutation in \textit{bmp4}S355* creates an \textit{Fsp}BI/MaeI site. For genotyping, the 150-bp PCR product from \textit{bmp4}tillf1 (CATGGAGAGTGTCCCTTTC) and \textit{bmp4}till-r1 (GTCCAGGTAAAGCATGGAG) was digested with \textit{Fsp}BI/MaeI resulting in 75-bp bands in the mutant. The mutation in \textit{bmp4}C365S creates a \textit{Pst}I site. For genotyping, the 150-bp PCR product from \textit{bmp4}tillf1 and \textit{bmp4}till-r1 was digested with \textit{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 \textit{spaw} (Long et al., 2003) and \textit{lefty1} (Bisgrove et al., 1999) probes.

RESULTS AND DISCUSSION
Bmp signaling generates a posterior midline barrier distinct from \textit{lefty1} in the notochord
\textit{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 \textit{spaw} in the left LPM is evident in most embryos by 10S, when the Nodal antagonist \textit{lefty1} is expressed in the notochord and is believed to act as a molecular midline barrier, preventing left-initiated \textit{spaw} expression.

RNA and morpholino injections
RNAs and morpholinos were injected using standard protocols. Constructs used to generate RNA include: \textit{zbmp4}wt (BL485), \textit{zbmp4}Y180 (BL482), \textit{zbmp4}S355 (BL486) and \textit{zbmp4}C365S (BL484), generated by amplification of the full-length cDNA from wild-type and \textit{M2bmp4} mutant cDNAs using primers \textit{bmp4}f2 (CCGCTCGAGatgATTCCTGGTAAT) and \textit{bmp4}r2 (CCGTCATAGAttaGCGGCAGCCACA). \textit{lefty1} MO (2 ng) or \textit{lefty2} MO (3 ng) were used (Agathon et al., 2001).

Fig. 1. \textit{spaw} and \textit{lefty1} expression phenotypes. (A-F) Time course of single color, double in situ hybridizations for \textit{spaw} and \textit{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 \textit{spaw} and \textit{lefty1}. In situ hybridizations of \textit{spaw} or \textit{lefty1} alone confirm the reported phenotypes. Arrows, boundary of detectable \textit{spaw} expression; brackets, ectopic \textit{spaw} across the midline; arrowheads, \textit{lefty1} in the diencephalon; asterisks, \textit{lefty1} in the cardiac mesoderm. L, left; R, right.
Table 1. Midline lefty1 and asymmetric spaw expression phenotypes in MZbmp4Y180*; Lf+/−, lefty1 morphants and lefty2 morphants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Stage</th>
<th>Midline lefty1 (%)</th>
<th>Left (%)</th>
<th>Right (%)</th>
<th>Bilateral (%)</th>
<th>Absent (%)</th>
<th>Ectopic tail bud (%)</th>
<th>Ectopic anterior (%)</th>
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<td>0</td>
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<td>0</td>
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<tr>
<td></td>
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<td>70</td>
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1Embryos 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.

2lefty1 expression is retained in lefty1 morphants, as morpholinos do not disrupt RNA transcription.

3lefty2 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).

4Propagation of spaw across the midline through the PTB domain.
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, bmp4Y180* and bmp4C365*, 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) and were not studied further. By contrast, the bmp4Y180* 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 bmp4Y180* 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 bmp4Y180* 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) bmp4Y180* mutants. Although MZ embryos do not display early D/V phenotypes, all of the MZbmp4Y180* 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 bmp4Y180* 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, MZbmp4Y180* 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 MZbmp4Y180* 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 MZbmp4Y180* 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 MZbmp4Y180* 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 MZbmp4Y180* mutants might also support a role for Bmp4 in limiting Nodal activity in the LPM. We note that 12% of MZbmp4Y180* 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...
propagation in the right LPM. In MZbmp4Y180* 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 required for pathway activation. As a consequence, the low diminished Bmp signaling decreases the threshold level of Spaw 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.

**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

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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.

Acknowledgements
We thank John Willoughby, Joy Murphy, Amber Starks and Cecilia Moens for help with the TILLING screen; Derrick Bosco for zebrafish care; and Jonathan Eggenschwiler, Andrew Miri and members of the Burdine laboratory for discussions and comments on the manuscript.

Funding
This work was supported by the following: Award #10PRE4180027 from the American Heart Association to K.F.L.; NIH R01 HG002995 and NIH P01 HD048584 to R.D.B. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.071092/-/DC1

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