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

Kari F. Lenhart1, Shin-Yi Lin1, Tom A. Titus2, John H. Postlethwait2 and Rebecca D. Burdine1,*

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., 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.

© 2011. Published by The Company of Biologists Ltd

1Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA.
2Institute of Neuroscience, University of Oregon, Eugene, OR 97403-1254, USA.
*Author for correspondence (rburdine@princeton.edu)

Accepted 12 August 2011
MATERIALS AND METHODS

**Zebrafish strains and genotyping**

The *lfp* 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 (GTGTCACAGTGTGAGATTTT) 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* eliminates an NdeI site. For genotyping, the 68-bp PCR product from *bmp4tillf2* (GGTTTGCATCGGATAAACACATA) and *bmp4till-r2* (GAGCTGCGTGATGAGCTGTC) was digested with NdeI resulting in 18- and 50-bp bands in wild type. The mutation in *bmp4Y180* creates an FspBI/MaeI site. For genotyping, the 150-bp PCR product from *bmp4tillf1* (CATGGAGAGTGTCCCTTTC) and *bmp4till-r1* (GTCCAGGTAAGGCTGGCA) was digested with FspBI/MaeI resulting in 75-bp bands in the mutant. The mutation in *bmp4C365S* creates a PstI site. For genotyping, the 150-bp PCR product from *bmp4tillf1* and *bmp4till-r1* was digested with PstI 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 zfbmp4C365S (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* expression in the diencephalon; asterisks, *lefty1* in the cardiac mesoderm. L, left; R, right.

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 from B–R 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* expression across the midline; arrowheads, *lefty1* in the diencephalon; 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 (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 MZbmp4Y180*, Laf–/–, 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>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10S</td>
<td>100</td>
<td>63</td>
<td>0</td>
<td>0</td>
<td>37</td>
<td>0</td>
<td>0</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>12-14S</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>16-18S</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td><strong>MZbmp4 Y180</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10S</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>12-14S</td>
<td>100</td>
<td>85</td>
<td>3</td>
<td>12</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>16-18S</td>
<td>100</td>
<td>91</td>
<td>2</td>
<td>7</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td><strong>Laf siblings</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10S</td>
<td>100</td>
<td>67</td>
<td>0</td>
<td>0</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>172</td>
<td></td>
</tr>
<tr>
<td><strong>Laf–/–</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10S</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>12-14S</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>97</td>
<td>0</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>16-18S</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td><strong>lefty1 morphants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10S</td>
<td>100</td>
<td>70</td>
<td>0</td>
<td>23</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>12-14S</td>
<td>100</td>
<td>32</td>
<td>0</td>
<td>68</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>199</td>
<td></td>
</tr>
<tr>
<td>16-18S</td>
<td>100</td>
<td>13</td>
<td>0</td>
<td>87</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td><strong>lefty2 morphants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10S</td>
<td>100</td>
<td>70</td>
<td>0</td>
<td>2</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>12-14S</td>
<td>100</td>
<td>70</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>16-18S</td>
<td>100</td>
<td>67</td>
<td>4</td>
<td>26</td>
<td>3</td>
<td>0</td>
<td>70</td>
<td>73</td>
<td></td>
</tr>
</tbody>
</table>

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, bmp4<sup>Y180*</sup> and bmp4<sup>C365S</sup>, 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. S1J in the supplementary material; see Table S3 in the supplementary material). These data suggest that the bmp4<sup>Y180*</sup> 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<sup>Y180*</sup> 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<sup>Y180*</sup> mutants do not display obvious D/V defects early in development and display a low penetration 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<sup>Y180*</sup> mutants. Although MZ embryos do not display early D/V phenotypes, all of the MZbmp4<sup>Y180*</sup> 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<sup>Y180*</sup> 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, MZbmp4<sup>Y180*</sup> 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 (Thissie and Thissie, 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 MZbmp4<sup>Y180*</sup> 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 MZbmp4<sup>Y180*</sup> 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 MZbmp4<sup>Y180*</sup> 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 MZbmp4<sup>Y180*</sup> mutants might also support a role for Bmp4 in limiting Nodal activity in the LPM. We note that 12% of MZbmp4<sup>Y180*</sup> 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 MZbmp4<sup>Y180H</sup> 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 expression in the left 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 (Thissen and Thissen, 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.

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 Eggerschwiler, Andrew Mi 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 HD022486 to I.H.P.; and NIH R01 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

References