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Boundary swapping in the *Drosophila* Bithorax complex

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Although the boundary elements of the *Drosophila* Bithorax complex (BX-C) have properties similar to chromatin insulators, genetic substitution experiments have demonstrated that these elements do more than simply insulate adjacent cis-regulatory domains. Many BX-C boundaries lie between enhancers and their target promoter, and must modulate their activity to allow distal enhancers to communicate with their target promoter. Given this complex function, it is surprising that the numerous BX-C boundaries share little sequence identity. To determine the extent of the similarity between these elements, we tested whether different BX-C boundary elements can functionally substitute for one another. Using gene conversion, we exchanged the *Fab-7* and *Fab-8* boundaries within the BX-C. Although the *Fab-8* boundary can only partially substitute for the *Fab-7* boundary, we find that the *Fab-7* boundary can almost completely replace the *Fab-8* boundary. Our results suggest that although boundary elements are not completely interchangeable, there is a commonality to the mechanism by which boundaries function. This commonality allows different DNA-binding proteins to create functional boundaries.

KEY WORDS: Bithorax, Chromatin, Boundaries, Insulator

INTRODUCTION

The large cis-regulatory region of the *BX-C* is divided into nine parasegment-specific chromatin domains that control the expression of the three BX-C homeotic genes along the anteroposterior (AP) axis (*Ubx*, *abd-A* and *Abd-B*) (for reviews, see Duncan, 1987; Maeda and Karch, 2006). The precise parasegment-specific expression pattern of these genes determines the segmental identity of each of the segments of the posterior two-thirds of the fly. Each domain is kept separate and autonomous by specialized elements known as domain boundaries (Barges et al., 2000; Gyurkovics et al., 1990; Karch et al., 1994; Mihaly et al., 1997). In transgenic constructs, these boundary elements behave as insulators, blocking enhancer activity when placed between the enhancer and its target promoter (Barges et al., 2000; Gruzdeva et al., 2005; Hagstrom et al., 1996; Zhou et al., 1996). However, within their native context, they are often found between an enhancer and its target promoter. How BX-C enhancers bypass intervening boundaries is still a topic of contention.

Boundary deletions indicate that these elements are required to provide functional autonomy to the enhancers and silencers within the large cis-regulatory region. The *Fab-7* boundary element, for example, is normally found separating the *iab-6* and *iab-7* cis-regulatory domains (see Fig. 1A). The *iab-6* enhancer region controls the level of *Abd-B* expression in parasegment 11 (PS11) and determines the identity of segment A6. The *iab-7* region, however, controls the level of *Abd-B* expression in PS12 and determines the identity of segment A7 (Celniker et al., 1990; Galloni et al., 1993; Mihaly et al., 2006; Sanchez-Herrero, 1991). When *Fab-7* is deleted, the *iab-6* and *iab-7* domains become fused into a single domain, allowing both the *iab-6* and *iab-7* enhancers or silencers to become active in PS11 and PS12. In most cells in PS11, the *iab-7* enhancers are activated by *iab-6* initiation elements, resulting in a homeotic transformation of PS11/A6 into PS12/A7. However, in other cells of PS11, the *iab-6* initiators fail to activate the fused domain before *iab-7* Polycomb Response Elements (PRE) silence the domain, causing these cells to take on a PS10/A5 identity (Galloni et al., 1993; Gyurkovics et al., 1990; Mihaly et al., 1997).

Previously, we have shown that insulators such as *gypsy* (Geyer and Corces, 1992) or *scs* (Kellum and Schedl, 1992) cannot substitute for *Fab-7* within the *BX-C* (Hogga et al., 2001). Both of these insulators block interactions between the distal *Abd-B* enhancers and the *Abd-B* promoter. To test whether the boundaries of the *BX-C* can functionally replace each other, we used gene conversion to exchange the *Fab-7* and *Fab-8* boundaries within the *BX-C*. Although these two boundaries perform similar functions, they share little sequence identity. Surprisingly, we find that the *Fab-7* boundary is almost completely capable of replacing the *Fab-8* boundary, indicating that there is a similarity in the mechanism of boundary function that cannot be predicted through modern sequence analysis.

MATERIALS AND METHODS

Fab-7 replacement by *Fab-8*

The *Fab-8* boundary element is an *Alu*I-*Msc*I 659 bp fragment (3R:12745503-12744844) cloned into an *Nsi*I site of a P-CaSpER-based plasmid containing the genomic region surrounding *Fab7*^{2} (Hogga and Karch, 1995). This construct was injected into *white*^{1118} flies. Third chromosome inserts were recombined with the bluetail insertion (Galloni et al., 1993). Convertants were obtained and verified as described previously (Hogga and Karch, 2001).

Fab-8 replacement by *Fab-7*

The genomic region surrounding the *Fab-8*^{305}deletion (3R:12745801-12744797) was generated by PCR using Pfu polymerase (Promega) and the following primers: 5′-TCTAGAGCTCCACTTGCTCGGGGG-3′ and 5′-CTCGAGTTCGGATTTCTGCTTTCTGAGC-3′ for the proximal region, and 5′-TCTAGACATAAAGGGAAGCGGAGGC-3′ and 5′-CTCGAGGTTCTTCATTATTGTGCCTTC-3′ for the distal region. The *Fab-7* boundary (a 0.8 kb fragment) was generated by PCR using 5′-CTCGAGGCAGCAAAAATCGTAAAAAAG-3′ and 5′-CTCGAGGCAGAAACAAAGGCCGACG-3′, and was inserted between the two break points of the *Fab-8*^{305} deletion. Transgenic flies were made as above and recombined onto a chromosome carrying the *fs(3)5649* P-element insertion. In trans to this chromosome, we placed the Df(3R)R59 chromosome

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carrying the Δ2-3 transposase and a Tp(3;1)bxd^{111} duplication to rescue the sterility of these dysgenic males. Putative conversion events were screened as above.

Antibody staining

Embryos were stained as previously described (Karch et al., 1990), using mouse monoclonal antibodies against Abd-B obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA.

Preparation of abdominal cuticles

Abdominal cuticles were prepared as described previously (Mihaly et al., 1997).

RESULTS AND DISCUSSION

The *Fab-7* boundary can substitute for the *Fab-8* boundary

Three reasons dictated our choice in converting *Fab-8* to *Fab-7* (F8→F7). First, *Fab-7* and *Fab-8* perform similar functions yet share almost no sequence similarity. Second, we wanted to test whether a *BX-C* cis-regulatory domain could interact with the *Abd-B* promoter over a boundary element that it generally never encounters. As *Fab-7* is located on the promoter distal side of *iab-7*, *iab-7* enhancers are never faced with the challenge of bypassing the *Fab-7* boundary (see Fig. 1A). And third, recent data have suggested that *BX-C* boundaries are regulated along the AP axis (Cléard et al., 2006). From these data, it seems that boundaries interact with the *Abd-B* promoter until the neighboring (probably more posterior) domain becomes active (see also Maeda and Karch, 2007). If this regulated association is responsible for boundary function and the association is controlled by the boundary element itself, then a substitution of *Fab-8* by *Fab-7* should result in the inactivation of the boundary one parasegment too anterior. The expected phenotype resulting from such an event would be a homeotic transformation of A7 to A8 (much like a boundary deletion).

In the gene conversion, the *Fab-8* region was removed and replaced by a minimal *Fab-7* boundary element (Chen et al., 2005), inserted, in separate constructs, in each orientations. In order to completely remove the *Fab-8* boundary without removing potentially important *iab-7* or *iab-8* sequences, we deleted the region around *Fab-8* that is removed in the *Fab-8^{305}* deletion. The *Fab-8^{305}* deletion is the smallest characterized *Fab-8* deletion that displays a complete *Fab-8* phenotype; homozygous adult females are sterile and the A7 segment disappears due to an A7 to A8 transformation (Fig. 2B). Both the *iab-8PRE* (Barges et al., 2000) and most of the promoter targeting sequence 7 (PTS7) element (Zhou and Levine, 1999) are left intact in the *Fab-8^{305}* deletion. As convertants for both orientations display identical phenotypes, we will simply call them F8→F7.

Given the simple nature of the experiment, we expected one of three outcomes: that the *Fab-7* boundary would act as a simple insulator and block *iab-7* from interacting with the *Abd-B* promoter (like an *iab-7* deletion); that *Fab-7* would not be functional in replacing *Fab-8* and behave as an *Fab-8* deletion mutation; or that *Fab-7* would substitute for *Fab-8*. Scoring females homozygous for either F8→F7 conversion showed that *Fab-7* can almost completely substitute for *Fab-8*. Almost all F8→F7 flies are wild-type appearance and are fertile (Fig. 2). In rare cases, we do observe homozygous flies displaying evidence of slight *Abd-B* misexpression. Patches of cells in A7 occasionally take on an A6 or A8 identity. To characterize this phenotype more carefully, we looked at F8→F7 hemizygous flies. F8→F7/Df(3R)P9 flies have

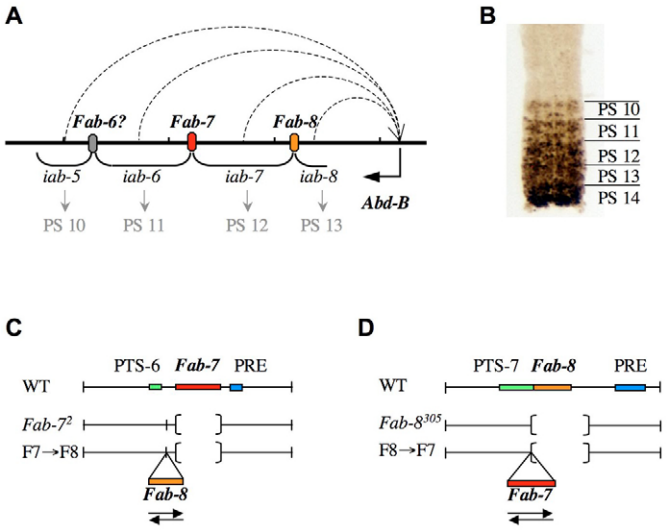

Fig. 1. The cis-regulatory region of Abd-B. (**A**) The *Abd-B* cis-regulatory region. The arrows are a graphical illustration of the targeting of each cis-regulatory domain to the *Abd-Bm* promoter (Boulet et al., 1991). The *iab-6*, *iab-7* and *iab-8* cis-regulatory domains interact with the *Abd-Bm* promoter in PS11/A6, PS12/A7 and PS13/A8, respectively. (**B**) The wild-type expression pattern of *Abd-B* in the embryonic CNS is characterized by an anterior-to-posterior increasing step gradient in the level of Abd-B. (**C**,**D**) Molecular maps of the *Fab-7* and *Fab-8* region (upper line). The boundary deletions mimicked are shown on the middle line and the final conversion products are shown on the bottom line.

features reminiscent of *Fab-8* homozygotes (Fig. 3), indicating that although *Fab-8* can mostly substitute for *Fab-7*, the boundary system in F8→F7 flies is less robust, occasionally allowing the iab-7 domain to be influenced by neighboring cis-regulatory domains. However, in a non-sensitized background, this effect is quite mild, affecting <5% of the flies scored.

Abd-B antibody staining confirms our results. In the embryonic CNS of wild-type flies, *Abd-B* is expressed in a step gradient pattern that noticeably increases parasegmentally from PS10 to PS13 (Fig. 2E). In *Fab-8* deletion mutants, that pattern changes such that PS12 expression levels mimic those found in PS13. Meanwhile in *iab-7* mutants, PS12 expression drops to the level of PS11. In F8→F7 conversion lines, we observe a staining pattern that is similar to that found in wild-type embryos.

This result was quite surprising. The fact that *Fab-7* can substitute for *Fab-8* means that everything required to restore *Fab-8* function is present in the *Fab-7* fragment inserted. However, at the DNA sequence level, the *Fab-7* and *Fab-8* boundaries share almost no similarity. A detailed analysis of the two sequences using dot-plot and Markov analysis found little in common between the two elements other than GAGA factor-binding sites (six in *Fab-7* and two in *Fab-8*). The GAGA factor binding sites have previously been shown to be important for *Fab-7* enhancer blocking activity in transgenic contexts (Schweinsberg et al., 2004). However, the role of the GAGA factor in *Fab-8* enhancer blocking activity is still unknown. Thus far, the only factor shown to be important for *Fab-8* function is the dCTCF factor. Previously, it has been shown that deleting the dCTCF-binding sites in *Fab-8* impaired its insulator function in transgenic insulator assays (Moon et al., 2005). Moreover, dCTCF mutants display phenotypes reminiscent of *Fab-8*

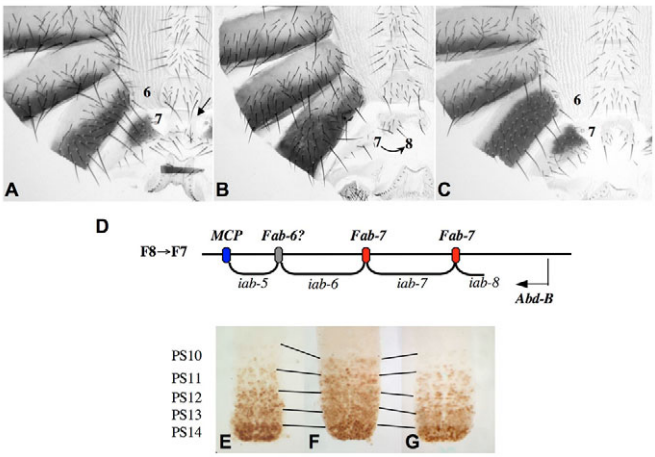

Fig. 2. Fab-7 is able to substitute for Fab-8. (**A-D**) Cuticles were prepared from adult homozygous females of the indicated genotypes [see Mihaly et al. (Mihaly et al., 1997) for a detailed description of cuticular phenotypes]. (A) Wild-type: A8 does not contribute to any visible tergite or sternite structure in the adult female. The A7 sternite (ventral) displays a characteristic shape with large bristles pointing towards the posterior (arrow). (B) *Fab-8*^{305}: the A7 to A8 transformation leads to the absence or reduction of the tergite (dorsal) and sternite (ventral). (C) F8→F7 homozygous females display a wild-type A7 cuticle. (D) Diagram of F8→F7 substitution. (**E-G**) Abd-B expression in the embryonic CNS. (E) Wild-type: Abd-B is expressed in a step gradient pattern from PS10 to PS14. (F) *Fab-8*^{305}: the Abd-B expression level in PS12 increases to the level normally observed in PS13. (G) F8→F7 embryos show a restoration of the wild-type expression pattern of Abd-B.

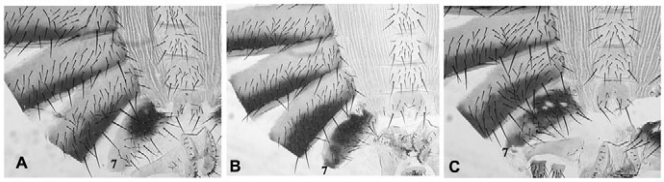

Fig. 3. Abd-B misregulation in A7 of F8→F7 hemizygous females. Although F8→F7 homozygotes look essentially wild type, the hemiyzgous females F8→F7/Df(3R)P9 harbor occasionally features reminiscent of Fab-8 homozygotes. (**A**) +/Df(3R)P9 hemizygous female. Note the narrow shape of the sternite on A7 with bristles pointing towards the posterior. (**B**,**C**) Two examples of F8→F7/Df(3R)P9 females harboring opposite homeotic transformations. The cuticle in B has a wider seventh sternite with bristles oriented towards the lateral side similar to the sternite found on A6 (an *iab-7* loss-of-function phenotype). The cuticle shown in C has spots of naked cuticle on the seventh tergite. Careful examination reveals the absence of trichomes in those spots, indicating a transformation towards A8. These opposing phenotypes are reminiscent of *Fab-8* mutations in which A7 can be a mixture of A6 and A8 identity (Barges et al., 2000), suggesting that boundary function is slightly impaired in F8→F7.

mutants (Mohan et al., 2007). As *Fab-7* was shown to be one of the few BX-C boundaries to which dCTCF does not bind (Holohan et al., 2007), our results show that dCTCF is not absolutely required for *Fab-8*-like function.

One explanation for this substitution could be that there are different ways to make a functional boundary. It has been reported that the GAGA factor, which binds to both boundaries, is a protein involved in nucleosome remodelling (Tsukiyama et al., 1994). Given that each of the BX-C boundaries has been isolated as a DNase hypersensitive site (Karch et al., 1994; Barges et al., 2000) and as a site of intense histone H3.3 replacement (Mito et al., 2007), what could be important for boundary function is the chromatin structure of the locus. It is easy to imagine that different combinations of proteins might be able to induce a similar chromatin structure, dCTCF and GAGA being two of them.

The *Fab-8* boundary cannot fully substitute for the *Fab-7* boundary

In the F8→F7 flies, the *iab-7* enhancers are able to bypass *Fab-7* even if, in the wild-type situation, they are never faced with the challenge of bypassing it. Because *Fab-7* could substitute for *Fab-8*, we wondered whether all *BX-C* boundaries are capable of substituting for each other. We, therefore, decided to replace the *Fab-7* boundary with the *Fab-8* boundary.

For this gene conversion, we replaced the *Fab-7* boundary with a minimal *Fab-8* boundary element (in both orientations) (Fig. 1C). To do this, we removed a *Fab-7* fragment identical to that deleted in the *Fab-7*^{2} deletion. The *Fab-7*^{2} deletion is the smallest characterized deletion that completely removes *Fab-7* boundary function; *Fab-7*^{2} homozygous adult flies primarily show an A6 towards A7 transformation (Fig. 4) (Galloni et al., 1993; Mihaly et al., 1997). Previous genetic and molecular analysis indicates that the nearby *iab-7PRE* (Mihaly et al., 1997) and PTS6 element (Chen et al., 2005) are left intact in the *Fab-7*^{2} deletion. Again, we isolated conversants for each *Fab-8* orientation. Although the two conversions differ slightly in their intensity, for the most part, they display similar phenotypes. Therefore, we will simply call the mutants F7→F8, indicating, when necessary, where the two orientations differ.

Although *Fab-8* can restore the autonomy of the *iab-7* domain (freeing it from ectopic activation by iab-6), surprisingly, F7→F8 homozygous flies show a transformation of A6 towards A5. This means that there is a loss of *Abd-B* activation by *iab-6* (Fig. 4). Abd-B antibody staining confirms these results (Fig. 4E-G). Instead of the normal stepwise gradient seen in the wild-type embryonic CNS, F7→F8 embryos display PS10-like Abd-B expression in PS11 (Fig. 4G). This phenotype is reminiscent of the phenotype obtained by substituting a minimal *scs* insulator for *Fab-7* (Hogga et al., 2001). For that substitution, it was believed that the loss of *iab-6* function was due to the blocking of *iab-6* by the intervening insulator. A second possible explanation for this phenotype is that *iab-6* is somehow being silenced in the F7→F8 substitution by the nearby *iab-7PRE*. This hypothesis is presented because *Fab-7* functions, not only to prevent the inappropriate activation of adjacent cis-regulatory domains, but also to prevent the inappropriate silencing of adjacent domains. In *Fab-7*^{2} mutants, for example, one sees a clonal mixture of both ectopic activation and ectopic silencing (Mihaly et al., 1997). The balance between these two clonal populations is sensitive to mutations in Polycomb group genes. We, therefore, crossed F7→F8 flies to the Polycomb-group mutant, *Pcl*. Because the phenotype of F7→F8 flies does not change upon the introduction of a *Pcl*/+ mutation (data not shown), we believe that *Fab-8* is acting like a short-range insulator at this locus, blocking *iab-6* enhancers from interacting with the *Abd-B* promoter.

As mentioned above, there is a slight orientation effect with the F7→F8 substitution. Lines with *Fab-8* placed in the wild-type orientation (F7→F8^{+}) (relative to the *Abd-B* gene) display a slightly

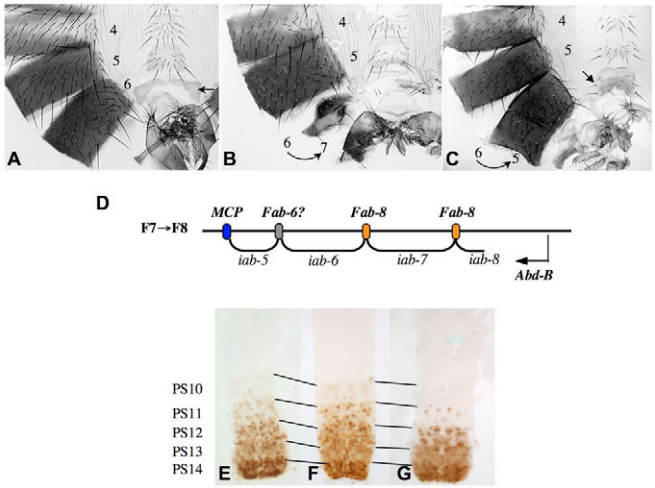

Fig. 4. Fab-8 is not able to substitute for Fab-7. (**A-C**) Cuticles were prepared from adult males (note that A7 does not contribute to any visible cuticle structures in the adult male). (A) Wild-type: the A6 sternite is recognizable by the absence of bristles (black arrow). (B) In *Fab-7*^{2} homozygote males, A6 is partially transformed into A7 as revealed by the reduction of the A6 tergite (Mihaly et al., 1997). (C) In F7→F8 males, the transformation of A6 to A5 is visible on the sixth sternite, which exhibits an A5 shape and has bristles (black arrow). (**D**) Diagram of the F7→F8 substitution. (**E-G**) Abd-B expression in the embryonic CNS. (E) Wild-type. (F) *Fab-7*^{2}: the Abd-B expression level in PS11 increases to the level normally observed in PS12. (G) F7→F8: the Abd-B expression level in PS11 decreases to a level similar to that seen in PS10.

less-severe transformation than lines with *Fab-8* placed in the opposite orientation (F7→F8^{-}). The difference in phenotype can be seen by looking at the trichome pattern in the transformed A6 segment. In F7→F8^{-} flies, trichomes cover most of the transformed segment (A5-like), whereas in F7→F8^{+} flies, trichomes primarily cover the ventral-anterior region of the transformed segment (more A6-like) (see Fig. S1 in the supplementary material). In all other assays, the two transformants behave identically (Fig. 4).

In the case of the F8→F7 conversion, we found that the *iab-7* cis-regulatory domain was capable of bypassing a boundary element that it never has to bypass but in the case of the F7→F8 conversion, we found that *iab-6* is partially blocked by a boundary element that it must normally bypass (*Fab-8* is located between *iab-6* and the *Abd-B* promoter). One possible explanation is discrepancy is that the *Fab-8* fragment inserted lacked a specific element required for insulator bypass. Although this is a possibility, we do not believe this to be the case. Both the *Fab-7* and *Fab-8* regions have been extensively scanned for elements allowing insulator bypass. In these attempts, elements called promoter-targeting sequences (PTSs) have been identified that allow enhancers to bypass insulator elements on reporter transgenes (Chen et al., 2005; Zhou and Levine, 1999). In our experiments, we replaced the smallest characterized boundary deletions with the smallest characterized insulator fragments. In both cases, molecular data suggest that the fragments we introduced were separated from any PTS-type activity, but were capable, in transgenic contexts, of being bypassed by known PTS elements. Conversely, the deletions we created were chosen to be clean boundary deletions; as much as possible, all known nearby elements, including PTS elements, were left intact. In the F7→F8 substitution, for example, the entire PTS-6 element that was capable of bypassing the identical *Fab-8* insulator fragment is still present.

Therefore, if no PTS-type elements were deleted, the main difference between the cases tested is context. For example, in the wild-type situation, *Fab-8* is located between the *iab-7* and *iab-8* cis-regulatory domains, whereas in F7→F8, *Fab-8* is placed between the *iab-6* and *iab-7* cis-regulatory domains. We have recently found that the *Fab-7* boundary seems to be regulated along AP axis (Cléard et al., 2006). If we assume that all boundaries behave in a similar manner, then *Fab-8* would also be regulated along the AP axis. As this regulation does not seem to come from the boundary element itself (see above), it must come through specific interactions with the nearby cis-regulatory domains. Previous work has pointed to PTS elements as the mediators of this function. However, based on our data and because PTS deletions have little phenotype when deleted, we believe that there must be something more that inactivates boundary elements (Mihaly et al., 2006; Zhou and Levine, 1999). For now, the identity of these elements remains a mystery.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/24/3983/DC1

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