Lineage-Independent Mosaic Expression and Regulation of the *Ciona multidom* Gene in the Ancestral Notochord

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The transcription factor *Ciona* Brachyury (Ci-Bra) plays an essential role in notochord development in the ascidian *Ciona intestinalis*. We characterized a putative Ci-Bra target gene, which we named *Ci-multidom*, and analyzed in detail its expression pattern in normal embryos and in embryos where Ci-Bra was misexpressed. *Ci-multidom* encodes a novel protein, which contains eight CCP domains and a partial VWFA domain. We show that an EGFP-multidom fusion protein localizes preferentially to the endoplasmic reticulum (ER), and is excluded from the nucleus. In situ hybridization experiments demonstrate that *Ci-multidom* is expressed in the notochord and in the anterior neural boundary (ANB). We found that the expression in the ANB is fully recapitulated by an enhancer element located upstream of *Ci-multidom*. By means of misexpression experiments, we provide evidence that Ci-Bra controls transcription of *Ci-multidom* in the notochord; however, while Ci-Bra is homogeneously expressed throughout this structure, *Ci-multidom* is transcribed at detectable levels only in a random subset of notochord cells. The number of notochord cells expressing *Ci-multidom* varies among different embryos and is independent of developmental stage, lineage, and position along the anterior–posterior axis. These results suggest that despite its morphological simplicity and invariant cell-lineage, the ancestral notochord is a mosaic of cells in which the gene cascade downstream of Brachyury is differentially modulated. *Developmental Dynamics* 236:1806–1819, 2007. © 2007 Wiley-Liss, Inc.

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INTRODUCTION

The notochord is a unifying character of the phylum Chordata and plays a major role in the organization of the body plan of all the animals grouped within this division. In vertebrates, the notochord is a transient embryonic structure that is replaced during development by the vertebral column. During early vertebrate development, in addition to providing structural support to the body, the notochord patterns the neural tube by inducing its ventral-most region, the floor plate (Clarke et al., 1991), and influences the formation of the paraxial mesoderm and its derivatives (Halpern et al., 1993), as well as the formation of heart and blood vessels (Goldstein and Fishman, 1998; Reese et al., 2004). Also the development of organs of endodermal origin, such as liver and pancreas, is profoundly affected by the notochord (Cleaver and Krieg, 2001).
The majority of the genes described so far in the notochord of different vertebrates are expressed quite homogeneously in all the notochord cells; however, heterogeneous expression along the anterior–posterior axis has been reported for a number of genes expressed in the chick notochord, including BMP2 as well as for a few genes expressed in the mouse notochord, including BMP2 and BMP7 (Cleaver and Krieg, 2001 and references therein). In the notochord of zebrafish, the Hox genes hoxb1, hoxb5, hoxc6, and hoxc8 are expressed in nested domains along the anterior–posterior axis (Prince et al., 1998).

A considerable body of knowledge on the evolutionary origins of the notochord and on the basic gene regulatory networks controlling its development comes from work on ascidian embryos (Passamaneck and Di Gregorio, 2005; Imai et al., 2006). In the center of their tail, ascidian larvae contain a single row of 40 notochord cells, which appear morphologically homogeneous. These 40 cells derive from two pairs of vegetal blastomeres, A4.1 and B4.1, of the 8-cell stage embryo. The A4.1 pair gives rise to the 32 anterior notochord cells (A-line, or primary notochord), while the 8 posterior-most notochord cells (B-line, or secondary notochord) originate from the B4.1 pair. Primary notochord cells never intercalate with secondary notochord cells during convergent extension (Satoh, 1994). Two distinct molecular mechanisms lead to the specification of the primary and secondary notochord lineage. Specification of primary notochord cells requires the FGF/MEK/ERK1/2 pathway (Imai et al., 2002; Hudson et al., 2003); specification of the secondary notochord requires, in addition to FGF signaling, a short-range Nodal signal emanating from the b6.5 blastomeres (Hudson and Yasuo, 2006). In response to FGF signaling, Nodal activates the Delta/Notch/Su(H) pathway and eventually Brachyury (Corbo et al., 1998; Yagi et al., 2004; Hudson and Yasuo, 2006). So far, there is no report indicating lineage-independent heterogeneity in gene expression levels among the 40 notochord cells.

In all chordate embryos studied so far, a major role in notochord formation is played by Brachyury. This gene encodes a DNA-binding transcription factor, which binds the regulatory regions of its target genes in a sequence–specific manner, thus controlling their expression (Kispert et al., 1995; Di Gregorio and Levine, 1999). In ascidian species as divergent as Ciona intestinalis (Order Enterogona) and Halocynthia roretzi (Order Pleurogona), notochord precursors start expressing Brachyury as soon as their fate is restricted to the notochord, and continue to specifically express this gene throughout the morphogenetic movements of convergent extension (Yasuo and Satoh, 1993, 1998; Corbo et al., 1998). When misexpressed, Ciona intestinalis Brachyury (Ci-Bra) is sufficient to induce endodermal and neural precursors to adopt a notochord-like phenotype (Takahashi et al., 1999); conversely, inactivation of ascidian Brachyury genes results in severe notochord defects (Satou et al., 2001; Di Gregorio et al., 2002). Taken together, these observations indicate that Brachyury is necessary and partly sufficient for ascidian notochord development. The misexpression of Ci-Bra allowed the identification of 38 bona fide Ci-Bra-downstream target genes via a subtractive screen (Takahashi et al., 1999). Nineteen of these genes have been thoroughly analyzed by in situ hybridization and found to be homogeneously expressed in all notochord cells (Hotta et al., 2000).

In this study, we analyze expression and transcriptional regulation of Ciona multiple domain protein (Ci-multidom), one of the 38 Ci-Bra-downstream genes identified so far, and the subcellular localization of its corresponding protein. We show that even though its expression in the notochord is controlled by the homogeneously distributed Ci-Bra, Ci-multidom is transcribed at detectable levels only in a subpopulation of notochord cells. The number and position of the notochord cells expressing Ci-multidom vary considerably among different embryos and are independent of their lineage, position along the anterior–posterior axis and developmental stage.

These results provide the first evidence of a molecular heterogeneity among adjacent notochord cells and suggest the existence of additional, yet uncharacterized molecular mechanisms by which the Brachyury gene hierarchy might be controlled.

RESULTS
Cloning and Domain Composition of Ci-multidom
The cDNA clone 204d was isolated as a bona fide Ci-Bra-downstream gene via a subtractive hybridization screen (Hotta et al., 1999; Takahashi et al., 1999). This cDNA clone contained an uninterrupted open reading frame (ORF) with no in-frame stop codons upstream of its putative first methionine. Hence, we carried out 5′RACE experiments (Froehman, 1993) to identify the 5′-end of this cDNA and an additional 1,047 bp were obtained, making the total cDNA 3,042 bp long (cDNA clone 204d-long; hereinafter 204d-L). Although additional putative initiation codons were found in this extended sequence, still no in-frame stop codons were identified. In an attempt to obtain additional sequence upstream of 204d-L, we repeated the 5′RACE using four different primer sets designed on the extended sequence, but we did not obtain any 5′-end sequence longer than that of 204d-L. Considering that the 204d-L sequence maps −1.1 kb downstream of the adjacent gene model (ci0100137181; Fig. 1A), we designated 204d-L as the bona fide full-length cDNA and started analyzing the domain composition and subcellular localization of the corresponding protein. Since 204d-L encodes a protein characterized by numerous domains with no direct homology to previously described proteins, we considered 204d-L as the cDNA clone of a novel gene, which we named Ciona multiple domain protein gene (Ci-multidom).

Figure 1A shows the genomic structure of Ci-multidom. This gene consists of 19 exons covering 2 JGI sequence scaffolds (scaffold 489 and scaffold 949 in Ciona intestinalis version 1.0; http://genome.jgi-psf.org/ciona4/ciona4.home.html), which have been recently mapped to chromosome 3p (Shoguchi et al., 2006). A tandem repeat of 2.3 kb of genomic sequence (shown as Repeat 1 and Repeat 2 in Fig. 1A), containing exons 7 to 11, was found in the region between 43 and 49.
Fig. 1. A: Genomic structure of Ci-multidom. The Ci-multidom locus spans ~18 kb and overlaps two separate sequence scaffolds (scaffolds 489 and 949 of the Ciona intestinalis genome version 1.0; http://genome.jgi-psf.org/ciona4/ciona4.home.html; see Supplemental Fig. 1 for alignment in version 2.0). These sequences have been recently mapped to chromosome 3p (Shoguchi et al., 2006). Boxes indicate exons and lines between the boxes indicate introns. The translated regions are colored in dark and light green. The 3’-untranslated region is indicated by an amber arrowhead. Duplicated exons (“Repeats”) are shown as light green boxes. The primers used for 5’RACE experiments are shown as small arrows mapped to their target sequences (ci-multil239 and ci-multil496). The pre-existing, computer-generated gene models encompassing the Ci-multidom locus are shown in the dashed box. Exons are shown as blue boxes. B: Domain composition of the predicted Ci-multidom protein. A blue box, yellow hexagons, and a larger red hexagon indicate the threonine-rich region, the CCP domains and the VWFA domain, respectively.

Fig. 2. The subcellular localization of the EGFP-multidom fusion protein in notochord cells, analyzed by confocal microscopy. A–H: Embryos electroporated with Ci-Bra->EGFP-multidom (A–D) or Ci-Bra->EGFP (E–H) and stained with rhodamin-phalloidin. Note that not all of the 40 notochord cells are fluorescent, due to mosaic incorporation of the electroporated plasmids. I–N: High-magnification views of notochord cells in embryos electroporated with either Ci-Bra->EGFP-multidom (I–K) or Ci-Bra->EGFP-multidom1-129 (L–N). J and M show optical sections across more peripheral regions of the notochord cells.

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The Ci-multidom cDNA clone that we have characterized in detail, 204d-L, contains only one set of exons 7–11, but we have also amplified less abundant, longer cDNA clones, which seemed to contain two sets of exons 7–11, possibly representing both Repeat 1 and Repeat 2. The cDNA clone 204d-L contains a putative ORF spanning 829 amino acids, distributed among eight complement control protein (CCP) domains, encompassing approximately 60 amino acid residues each (Fig. 1B) and a threonine-rich region, spanning 35 amino acid residues. Ci-multidom also contains a von Willebrand factor A (VWFA) domain in its C-terminal, comprising 130 amino acid residues (Fig. 1B). BLAST searches indicate that this predicted protein has sequence similarity to P-selectin, an adhesion molecule of the selectin family, which is characterized by an N-terminal lectin-like domain, an epidermal growth factor (EGF) repeat and a discrete number of CCP domains. However, differently from what is seen in the case of members of the selectin family, Ci-multidom lacks both a lectin-like domain and EGF repeats.

Within its sequence, Ci-multidom contains also putative phosphorylation sites for protein kinases such as PKC, CK2, and tyrosine kinase as well as putative myristylation sites (http://www.predictprotein.org; Rost et al., 2004) and several of its numerous cysteine residues (66 residues in total) are predicted to form disulfide bonds (http://disulfind.dsi.unifi.it/; Ceroni et al., 2006). In addition to that, Ci-multidom contains 7 NXS/T sequons (blue in Supplemental Fig. 1A, which can be viewed at www.interscience.wiley.com/jpages/1058-8388/suppmat), three of which might be glycosylated in vivo (red in Supplemental Fig. 1A) (http://www.cbs.dtu.dk/services/NetNGlyc/).

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Fig. 3. The EGFP-multidom fusion protein is predominantly localized to the ER. A–C: Embryos electroporated with Ci-BraEGFP-multidom and stained with TO-PRO-3 (see Experimental Procedures section). D–F: Embryos co-electroporated with Ci-Bra>EGFP-multidom and Ci-Bra>Nup50-mRFP. G–I: Embryos co-electroporated with Ci-Bra>EGFP-multidom and Ci-Bra>KDELR-mRFP. J–L: High-magnification views of the region indicated by white rectangles in G–I. Arrowheads indicate the subcellular regions in which only EGFP-multidom is detected. M–O: Embryos co-electroporated with Ci-Bra>EGFP-multidom and Ci-Bra>ST-mRFP.

Subcellular Localization of Ci-multidom

To analyze the subcellular localization of Ci-multidom, we electroporated fertilized Ciona eggs with the plasmid Ci-Bra>EGFP-multidom, encoding an EGFP-multidom fusion protein under the control of the notochord-specific Ci-Bra promoter, and observed the subcellular localization of EGFP-multidom in notochord cells of tailbud embryos by confocal microscopy (Figs. 2 and 3). As a control, we also analyzed the subcellular localization of EGFP in Ciona notochord cells by electroporating the plasmid Ci-Bra>EGFP into Ciona zygotes. In these experiments, the overexpression of EGFP or EGFP-multidom in notochord cells did not cause any obvious phenotype.
In embryos electroporated with Ci-Bra→EGFP, EGFP tends to localize preferentially to the nuclei of the notochord cells, possibly due to the presence of a cryptic nuclear localization signal within its sequence (Fig. 2A–D). In contrast, when we introduced Ci-Bra→EGFP-multidom into Ciona embryos, the fusion protein appeared to be excluded from the nucleus and was observed in interconnected tubular structures extending throughout the cytoplasm (Fig. 2E–H). These structures are reminiscent of the ER (Vedrenne and Hauri, 2006) and are clearly seen in close-ups of notochord cells, such as those shown in Figure 21–N. Interestingly, a truncated version of Ci-multidom containing only the first 129 amino acid residues from the N-terminal fused to EGFP showed the same localization pattern as EGFP-multidom (Fig. 2L–N), suggesting that the N-terminal region of Ci-multidom, in which no conserved domains are found, is sufficient to recapitulate the localization pattern of the full-length protein.

Figure 3A–C shows notochord cells electroporated with Ci-Bra→EGFP-multidom and subsequently stained with the nuclear marker TO-PRO-3, which labels both DNA and RNA, producing a strong blue fluorescent signal. In these embryos, the EGFP-multidom fusion was clearly detected only outside the nuclei. Figure 3D–F shows notochord cells that were co-electroporated with Ci-Bra→EGFP-multidom along with the Ci-Bra→Nup50-mRFP reporter construct, which expresses a component of the nuclear pore complex, the Ciona Nup50 protein, fused to mRFP. This fusion protein is localized exclusively to the nucleus (Fig. 3E,F). As shown in Figure 3D and F, the green fluorescent EGFP-multidom fusion protein is observed outside the nuclei, which are labeled in red by Nup50-mRFP. These results suggest that Ci-multidom is a cytoplasmic protein that is unable to enter the nuclei, either due to conformational constraints or due to an active mechanism of exclusion.

Since EGFP-multidom was observed in a structure resembling the ER, as a next step we compared the subcellular distribution of EGFP-multidom with that of fluorescent markers for the ER and the Golgi apparatus. To this end, we co-electroporated Ciona embryos with the Ci-Bra→EGFP-multidom plasmid along with either the Ci-Bra→KDEL-mRFP or the Ci-Bra→ST-mRFP reporter constructs (Fig. 3G–O). The Ci-Bra→KDEL-mRFP reporter construct expresses the KDEL receptor fused to mRFP in notochord cells. The KDEL receptor is known to cycle between the ER and the cis-side of the Golgi apparatus and is believed to function in the retrieval of ER resident proteins (Murshid and Presley, 2004). In notochord cells co-electroporated with Ci-Bra→EGFP-multidom and Ci-Bra→KDEL-mRFP, the red signal from the KDEL-mRFP fusion protein was observed in a mesh-like structure, similarly to the EGFP-multidom fusion protein (Fig. 3G–I). A close analysis of merged images from the red and the green channels shows that both KDEL-mRFP and EGFP-multidom are often, although not always, colocalized (Fig. 3J–L; white arrowheads indicate sub-compartments highlighted only by EGFP-multidom).

The Ci-Bra→ST-mRFP reporter construct expresses in notochord cells the amino terminal anchoring sequence from sialyltransferase fused to mRFP; a fusion protein containing this short peptide tag and EGFP was previously reported to localize to the Golgi apparatus in Ciona embryos (Zeller et al., 2006). In notochord cells co-electroporated with Ci-Bra→EGFP-multidom and Ci-Bra→ST-mRFP, the red signal from the ST-mRFP fusion protein was frequently observed in the form of patches surrounding the nucleus. From the analysis of merged images from the red and the green channels, we did not obtain clear evidence that this localization pattern is coincident with that of EGFP-multidom (Fig. 3M–O). These results suggest that EGFP-multidom is predominantly localized to the ER, which is labeled by KDEL-mRFP but not by ST-mRFP (compare Fig. 3H and N).

**Ci-Bra Is Able and Sufficient to Induce Ectopic Expression of Ci-multidom in Endoderm, Neural Tube, and Muscle Cells**

Since Ci-multidom was originally isolated in a subtractive hybridization screen aimed to identify potential Ci-Bra target genes (Hotta et al., 1999), we analyzed the expression of this gene in embryos misexpressing Ci-Bra (Fig. 4). In a first set of experiments, we employed the Ci-fkh→Ci-Bra construct, in which the 5’ cis-regulatory region from Ciona forkhead (Ci-fkh) was placed upstream of the Ci-Bra coding sequence. A 2.6-kb fragment from the Ci-fkh upstream region is capable of directing expression in cells of the trunk endoderm, endodermal strand, trunk epidermis and notochord, as well as in the ependymal cells of the neural tube (Di Gregorio et al., 2001). The Ci-fkh→Ci-Bra construct was previously used for the subtractive hybridization screen that led to the isolation of Ci-multidom (Takahashi et al., 1999). Larvae electroporated at the 1-cell stage with the Ci-fkh→Ci-Bra plasmid show a strong phenotype whereby a large number of cells, likely neural and endodermal precursors whose fate has been altered by Ci-Bra, are blocked in the tail, considerably affecting morphogenesis (Fig. 4F).

In addition to these experiments, to specifically target the misexpression of Ci-Bra to a single tissue, namely the more easily distinguishable muscle cells, we subcloned the Ci-Bra coding region downstream of the Ci-snail promoter, which directs strong expression in developing muscle cells (Erives et al., 1998), to create the Ci-snai→Ci-Bra plasmid. Larvae electroporated with Ci-snai→Ci-Bra show a distinct phenotype characterized by a short, flat tail in which the muscle cells have lost their typical polygonal shape as a result of the misexpression of Ci-Bra, and the notochord is distorted (Fig. 4G).

To quantify the effects of the misexpression of Ci-Bra on the transcription of Ci-multidom, we carried out semi-quantitative RT-PCR experiments aimed to compare the amount of Ci-multidom mRNA between control and transgenic embryos. As shown in Figure 4A and in the graph in Figure 4C, the intensity of the band detected for Ci-multidom increased 2.87 times in embryos electroporated with Ci-fkh→Ci-Bra, compared to the control embryos. In contrast, only a slight change in the intensity of the bands obtained for control housekeeping genes, elongation factor 2 (EF2) and
glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was detected (EF2 increased 0.86 times, GAPDH 1.17 times). Similarly, a significant increase in the intensity of the band corresponding to Ci-multidom was observed also in embryos electroporated with Ci-sna>Ci-Bra, while the relative intensity of the bands corresponding to EF2 and GAPDH remained approximately the same between transgenic and control embryos (Fig. 4B,D).

These results were confirmed by whole-mount in situ hybridization experiments (Fig. 4E–G). In late tailbud control embryos, expression of Ci-multidom is exclusively detected in the anterior neural boundary (ANB; black arrow in Fig. 4E–G). In embryos electroporated with Ci-fkh>Ci-Bra, strong ectopic expression of Ci-multidom was observed in endoderm, neural tube, and muscle cells. A–D: The levels of Ci-multidom transcripts in tailbud embryos electroporated with Ci-fkh>Ci-Bra (A) or Ci-sna>Ci-Bra (B) were compared by semi-quantitative RT-PCR. The levels of GAPDH and EF2 expression were also assessed as a control. The amount of Ci-multidom transcripts in control embryos and in embryos overexpressing Ci-Bra, as well as the amounts of transcripts relative to the control genes, were plotted using arbitrary units (graphs in C, D). E–G: Expression of Ci-multidom detected by whole-mount in situ hybridization in late tailbud control embryos and in embryos electroporated with Ci-fkh>Ci-Bra or Ci-sna>Ci-Bra. Note that by the late tailbud stage, Ci-multidom expression is no longer detectable in notochord cells. Arrows indicate expression of Ci-multidom in the ANB; small arrowheads indicate cells ectopically expressing Ci-multidom. The deformed notochord is indicated by a dotted line in G. Embryos in E and F are shown in lateral views. The embryo in G is shown in a dorsal view. Anterior is on the left.

Fig. 4. Comparison of Ci-multidom (A, C) and Ci-Bra (B, D) expression in mid-tailbud embryos. Embryos from the same batch were fixed in parallel and hybridized in situ with either antisense RNA probe. A,B: Twenty randomly picked embryos showing signal after hybridization with either the Ci-multidom probe (A) or the Ci-Bra probe (B). Arrowheads in A indicate three embryos containing a different number of positive notochord cells. C,D: High-magnification images of embryos in which either Ci-multidom (C) or Ci-Bra (D) expression was detected. Embryos are shown in lateral views, with anterior to the left. Scale bar = 100 μm.

Fig. 5. Misexpression of Ci-Bra induces ectopic expression of Ci-multidom in endoderm, neural tube, and muscle cells. A–D: The levels of Ci-multidom transcripts in tailbud embryos electroporated with Ci-fkh>Ci-Bra (A) or Ci-sna>Ci-Bra (B) were compared by semi-quantitative RT-PCR. The levels of GAPDH and EF2 expression were also assessed as a control. The amount of Ci-multidom transcripts in control embryos and in embryos overexpressing Ci-Bra, as well as the amounts of transcripts relative to the control genes, were plotted using arbitrary units (graphs in C, D).
dom was detected in the hump formed in the tail by the endodermal and neural cells perturbed by the ectopic expression of Ci-Bra (black arrowheads in Fig. 4F). In embryos electroporated with Ci-sna>Ci-Bra, expression of Ci-multidom was ectopically induced in the muscle lineage (black arrowheads in Fig. 4G). Taken together, these results suggest that Ci-Bra is able and sufficient to upregulate expression of Ci-multidom in virtually all the main larval tissues.

Expression Pattern of Ci-multidom During Embryogenesis

Whole-mount in situ hybridization experiments show that Ci-multidom is expressed in the notochord and in the ANB; expression in this latter region is detected starting around the late tailbud stage (Fig. 4E).

Expression of Ci-multidom in the notochord was detected beginning at neurulation and throughout the tailbud stages. During these developmental phases, Ci-multidom showed a peculiar expression pattern, being expressed at high levels only in what appeared to be a subset of notochord cells. To verify that these cells were part of the notochord, we compared the expression pattern of Ci-multidom to that of Ci-Bra, by hybridizing in parallel mid-tailbud embryos from a single batch, fixed at the same time, with the two respective probes. After the in situ hybridization was completed, we randomly picked 20 embryos showing positive signals for each probe (Fig. 5A,B).

While expression of Ci-Bra marked the entire population of notochord cells, highlighting a continuous rod-shaped structure in the center of the tail (Fig. 5B,D), Ci-multidom labeled only small, randomly distributed portions of this structure (Fig. 5C). We also noticed that the position and number of Ci-multidom-positive notochord cells varied considerably among different embryos at the same developmental stage (black arrowheads in Fig. 5A; see also Fig. 6). We observed at least 100 embryos at each of the stages when expression was detectable, including late neurula (Fig. 6A–D), early tailbud (Fig. 6E–T), mid-tailbud (Fig. 6U–X), and late tailbud stage (Fig. 6Y–AB), but we could not detect any preferential distribution of Ci-multidom-positive notochord cells. Ci-multidom expression was detected in both A-line and B-line notochord cells in the majority of the embryos (representative A-line and B-line notochord cells are indicated by a white arrow and by a black arrow, respectively, in Fig. 6Y).

Notochord morphogenesis in ascidians involves mediolateral intercalation, which results in convergent extension movements (Satoh, 1994). Since notochord cells from the left and the right side of the embryo mix randomly during this rearrangement, it seemed conceivable to hypothesize that the mosaic expression pattern of Ci-multidom expression represented the mosaic distribution of notochord cells originating from contralateral sides of the embryo. However, this is not likely to be happening, because the number of Ci-multidom-positive cells varies considerably among different embryos, despite the fact that the number of notochord cells deriving from each side is invariably 20 (black and white arrowheads in Fig. 6). In addition to that, Ci-multidom expression was detected in notochord cells on both the left and the right side at the neurula and early tailbud stages, when intercalation is underway (Fig. 6D,E,M; white arrowheads: cells on the left side; black arrowhead: cells on the right side).

Finally, to assess the contribution of cell division and cell migration to this unusual pattern, we analyzed Ci-multidom expression in embryos treated with cytochalasin-B at the 110-cell stage and fixed at the mid-tailbud stage (Fig. 7A,B). Treatment with cytochalasin-B arrests cytokinesis and cell migration. It has been previously shown that treatment with cytochalasin-B at the 110-cell stage does not affect the expression patterns of two other targets of ascidian Brachury, Ci-Noto1 (Hudson and Yasuo, 2006) and Cs-fibrinogen-like (Imai et al., 2002). Also in the case of Ci-multidom, while expression of Ci-Bra appeared uniform in most embryos (Fig. 7C), expression of Ci-multidom was still detected randomly in subsets of notochord precursors of both lineages (Fig. 7D–F).

We conclude that the mosaic expression pattern of Ci-multidom in the Ciona notochord is independent of developmental stage, position along the anterior–posterior axis, and lineage of the notochord cells. In addition to that, this peculiar expression pattern is not influenced by an arrest in cell division and migration of the notochord precursors.

Identification of a cis-Regulatory Module Directing Expression in the ANB

In an attempt to identify the cis-regulatory elements governing expression of Ci-multidom, we first compared the genomic sequence of the Ci-multidom locus to that from the sister species Ciona savignyi using the VISTA genome browser (http://genome.lbl.gov/vista/index.shtml). Even though the majority of the Ci-multidom exons appeared phylogenetically conserved, no significant conservation was seen in the non-coding regions, particularly in the 5′-flanking sequence (indicated by a red rectangle in Supplemental Fig. 1C). However, we identified and analyzed the following three genomic regions, which appeared most likely to harbor cis-regulatory elements: (1) A 2.7-kb genomic fragment (blue rectangle in Supplemental Fig. 1C), which contains a region weakly conserved in the C. savignyi genome (red arrowhead in Supplemental Fig. 1C) as well as a cluster of T-box sites whose arrangement is similar to that found in the notochord enhancer of Ci-tropomyosin (Ci-trop), a direct Ci-Bra target (Di Gregorio and Levine, 1999); (2) a 0.3-kb genomic fragment, which contains a small and compact cluster of T-box half-sites related to those found in the Ci-trop enhancer (purple rectangle in Supplemental Fig. 1C); (3) a 1.1-kb genomic fragment located 5′ of the Ci-multidom transcription unit and neighboring the adjacent gene model, cii0100137181 (Fig. 8A). Neither of the first two regions had any activity in notochord cells when cloned upstream of the Ci-fkh basal promoter (data not shown). Next, we cloned and tested in vivo the third genomic fragment, located 5′ of the Ci-multidom transcription unit (Fig. 8A). This 1.1-kb fragment contains the putative Ci-multidom promoter region, including a bona fide TATA-box (Fig. 8A). A construct containing the −1.1-kb Ci-multidi-
Fig. 6. Expression of Ci-multidom as detected by whole-mount in situ hybridization at different developmental stages. A–D: Neurula stage embryos, dorsal view. E–H: Four representative early tailbud embryos fixed 10 hr and 10 min after fertilization and incubation at 15°C. Dorsal (E–H) and lateral (I–L) views of each embryo are shown. M–P: Four representative early tailbud embryos fixed 10 hr and 30 min after fertilization and incubation at 15°C. Dorsal (M–P) and lateral (Q–T) views of each embryo are shown. U–X: Mid-tailbud embryos, lateral view. Y–AB: Late tailbud embryos, lateral view. Black and white arrowheads indicate positive cells on the right side and on the left side, respectively. A black arrow and a white arrow in Y indicate positive A-line cells and positive B-line cells, respectively.
Fig. 7. Expression of Ci-multidom is not affected by a block in cell division. A,B: Low-magnification view of a group of C. intestinalis embryos treated at the 110-cell stage with cytochalasin-B (see Experimental Procedures section) and hybridized in situ with the Ci-multidom antisense probe. C: Embryo treated in parallel with cytochalasin-B and hybridized in situ with the Ci-Bra antisense probe. D–F: Individual embryos hybridized with the Ci-multidom probe, showing mosaic expression in primary and secondary precursors. Red arrowheads indicate the primary (A-line) notochord precursors; pink arrowheads indicate the secondary (B-line) notochord precursors.
dom promoter fused to LacZ (construct 1 in Fig. 8B) is predominantly active in the ANB but does not show any activity in notochord cells. This staining pattern fully recapitulates the expression observed in the ANB by in situ hybridization (Fig. 8C–D’). When a slightly shorter fragment, which did not include the TATA-box, was placed 5′ of the heterologous Ci-fkh basal promoter (construct 2 in Fig. 8B), an ectopic staining in a small region of the sensory vesicle was detected in a fraction of the embryos, in addition to the staining in the ANB (Fig. 8G,G’). This ectopic activity derives, in all likelihood, from the Ci-fkh basal promoter, which has been previously reported to direct staining in this region (Harafuji et al., 2002). Also with construct 2, no staining was detected in notochord cells. Finally, when the Ci-Bra basal promoter was used (construct 3 in Fig. 8B), in addition to the staining in the ANB, which was observed in a smaller percentage of embryos as compared to the other constructs, a sporadic staining in notochord cells was detected in a small percentage of embryos (Fig. 8H,H’). This notochord staining might be due to the presence of a cluster of T-box half-sites (generic consensus: TNNCAC) within the 1.1-kb region (ovals in Fig. 8B; red ovals indicate half-sites that were previously shown to be bound in vitro by Ci-Bra) (Di Gregorio and Levine, 1999), which might act cooperatively with the Ci-Bra basal promoter to direct expression in the notochord.

**Discussion**

We have presented the first report of a gene expressed at different levels in the 40 cells that form the primitive notochord of *Ciona intestinalis*. This gene encodes a multiple domain protein, Ci-multidom, which is preferentially localized to the ER and is excluded from the nucleus. We have shown that the mosaic expression of *Ci-multidom* is independent of a cell’s lineage, position along the anterior–posterior axis, and developmental stage, and it is not affected by a block in cell division. We have identified a cis-regulatory element sufficient to recapitulate expression of *Ci-multidom* in the ANB, and we have shown that Ci-Bra is able to ectopically activate transcription of this gene in CNS, endoderm, and muscle cells.

**Structure and Subcellular Localization of Ci-multidom**

Ci-multidom contains a VWFA domain and eight CCP domains. CCP domains are known to be abundant in multiple domain proteins belonging to the regulators of complement activation (RCA) family (reviewed by Kirkitadze and Barlow, 2001) and most VWFA domains are found in cell adhesion and extracellular matrix proteins and mediate protein–protein interactions (Aquilina et al., 2002). However, the most ancient VWFA-containing proteins found in all eukaryotes are intracellular proteins involved in functions such as transcription, DNA repair, and membrane transport (Hui et al., 2005). VWFA domains typically contain ~200 amino acids, while the VWFA domain found in Ci-multidom appears to lack 20–25% of the residues generally found in the C-terminus. A structural feature shared by several VWFA domains is the presence of motifs called “metal ion-dependent adhesion sites” (MIDAS), characterized by the non-contiguous amino acid residues DxxxS...T...D. Ci-multidom contains a putative MIDAS motif in its VWFA (boxed in Supplemental Fig. 1A), suggesting that it might bind divalent cations, such as calcium and magnesium. Since both CCP domain and VWFA domains are found in proteins belonging to a variety of different families, it was difficult to infer the function of Ci-multidom from its domain composition. Therefore, we analyzed the subcellular localization of Ci-multidom to gain clues on the nature of this novel protein.

We showed that EGFP-multidom is predominantly localized to the ER, which suggests that Ci-multidom might be an ER resident protein. However, neither a canonical KDEL nor a KXXX endoplasmic retention signal were found in Ci-multidom. Thus, Ci-multidom might be held directly in, or retrieved to, the ER via KDEL- or KXXX-independent retrieval mechanisms (Murshed and Presley, 2004). The observation that EGFP-multidom and KDEL-mRFP are not always colocalized would be consistent with this hypothesis.

Finally, it is noteworthy that a Ci-multidom fragment containing only the first 129 amino acid residues of the N-terminal region (multidom1-129; Fig. 1B) resembles the subcellular localization of the full-length protein (Fig. 2L–N); this region does not contain any known domain, but is predicted to comprise 1 transmembrane region (http://bioinf.cs.ucl.ac.uk/psipred/; Bryson et al., 2005), which is also revealed by the hydropophobicity plot (red arrow in Supplemental Fig. 1B). This region might be responsible for the retention of Ci-multidom in the ER. Despite our efforts, the function of Ci-multidom in development remains elusive. Even though the Ci-Bra> EGFPP-multidom construct is likely to cause an overexpression, as well as an earlier accumulation of Ci-multidom in notochord cells, no evident phenotype was observed in larvae electroporated with this plasmid (Fig. 2).

Similarly, no effect on gross cell morphology and morphogenetic movements
was observed when a Ci-fkh>Ci-multidom construct, which caused misexpression of Ci-multidom in CNS, endoderm, and notochord, was employed (data not shown). Also a morpholino oligonucleotide designed against the sequence encompassing the first ATG of Ci-multidom did not produce any detectable effect when injected in Ciona zygotes in parallel with appropriate controls (data not shown). Considering that the great variability observed by in situ hybridization in the levels of Ci-multidom transcripts among notochord cells does not detectably affect their development, these results seem to suggest that there might be a higher tolerance to variations in the levels of Ci-multidom than there is in the case of other proteins.

Transcriptional Regulation of Ci-multidom Expression

The identification of Ci-multidom as a putative target of Ci-Bra (Takahashi et al., 1999) is per se a strong argument in support of the hypothesis that Ci-multidom might be part of the large gene battery controlled by this transcription factor. We verified this by means of misexpression assays (Fig. 4), aimed to assess whether Ci-Bra is sufficient to ectopically activate Ci-multidom transcription, i.e., whether or not Ci-Bra needs a notochord-specific co-factor or intermediary to control Ci-multidom expression. Our results show that misexpression of Ci-Bra is sufficient to activate transcription of Ci-multidom in CNS, muscle, and endodermal precursors, suggesting that Ci-Bra controls this gene either alone or, alternatively, via a nearly ubiquitous co-factor or transcriptional intermediary. The search for the cis-regulatory elements controlling expression of Ci-multidom has been complicated by the scarce sequence conservation observed when the Ci-multidom sequence was compared to the multidom sequence from Ciona savignyi.

Nonetheless, our analysis of the 5’-flanking region of Ci-multidom (Fig. 8) has identified a distinct cis-regulatory element that is sufficient to direct reporter gene expression in the ANB. The identification of the factor(s) controlling the ANB enhancer of Ci-multidom will be the subject of our future studies; it is noteworthy that the expression of the a/b isof orm of the transcription factor gene Ci-pitx precedes that of Ci-multidom in the ANB (Christiaen et al., 2005). Pitx proteins belong to the bicoid subclass of homeodomain proteins, and we have found four consensus bicoid-related binding sites (TAATCC; Quentien et al., 2002) in the 1.1-kb ANB enhancer (data not shown); these observations make Ci-pitx a candidate regulatory factor for this cis-element.

Our analysis did not allow the isolation of a discernible cis-regulatory module that might mediate the response to Ci-Bra, differently from what was found in the case of another transcriptional target of Ci-Bra, Citropomyosin (Di Gregorio and Levine, 1999). In the case of Citropomyosin, a minimal 114-bp cis-regulatory element, located 5’ of the coding region and containing a small cluster of T-box half-sites, is sufficient to direct robust notochord expression when placed upstream of the Ci-fkh basal promoter. In the case of Ci-multidom, a sporadic notochord staining is only detected in a minor fraction of embryos electroporated with construct 3 (Fig. 8B), which employs the basal Ci-Bra promoter, but such staining is completely absent when either the endogenous Ci-multidom or the Ci-fkh basal promoter are used. The notochord staining seen with construct 3 might have resulted from the combination of the numerous T-box half-sites in the 1.1-kb sequence with the Ci-Bra basal promoter, which is intrinsically prone to function sporadically in notochord cells. We conclude that rather than on a discernible cluster of Ci-Bra binding sites or on a phylogenetically conserved sequence, the expression of Ci-multidom in the notochord relies upon cis-regulatory element(s) with a yet uncharacterized architecture, which are likely dispersed along the transcription unit rather than lying upstream of it.

A Possible Molecular Mechanism Underlying Ci-multidom Expression

Pulsatile regulation of gene expression is a sophisticated mechanism employed by several organisms to respond to subtle variations in certain environmental or internal stimuli by finely modulating the amounts of various molecules, including light-responsive enzymes and hormones (e.g., Tamai et al., 2005; Ferris and Shupnik, 2006). In addition to that, numerous proteins are able to negatively feed back on their own promoters, either directly or indirectly, thus avoiding the cellular damages due to their surplus.

In Drosophila, variations in gene expression levels among cells of the same tissue have been described as position-effect variegation (PEV). PEV occurs when a gene which is normally located in a euchromatic region is juxtaposed to an heterochromatic region by a chromosomal rearrangement, resulting in the continuous transcription of the gene only in a clonal subpopulation of cells (Wakimoto, 1998). The molecular effectors of PEV fall into two classes: enhancers of variegation, encoded by E(var) genes, and suppressors of variegation, encoded by Su(var) genes. Proteins of both families usually contain a chromodomain, which mediates the interaction with the chromatin. The Ciona genome contains several chromodomain proteins, including orthologs of the Su(var) genes HP1 and Su(var)3-9, which play key roles in heterochromatic gene silencing (e.g., Schotta et al., 2003), and preliminary in situ hybridization results indicate that Ci-Su(var)3-9 is weakly expressed in notochord precursors (Jamie E. Kugler and A.D.G., unpublished results).

Although PEV has never been reported in ascidians, this represents an exciting possibility to consider in order to explore how the dynamic, mosaic expression pattern of Ci-multidom is established.

EXPERIMENTAL PROCEDURES

Embryo Rearing and Treatment With Cytochalasin-B

Adult Ciona intestinalis were purchased from Marine Research and Educational Products (M-REP, CA). The animals were kept at ~18°C in recirculating artificial sea water. In vitro fertilization, dechorionation, and cul-
ture of embryos were carried out as described previously (Corbo et al., 1997). For cleavage-arrest experiments, Cytochalasin-B (Sigma C6762), dissolved in Dimethyl Sulfoxide, was added to the developing embryos when they reached the 110-cell stage, at a final concentration of -2 μg/ml. Embryos were subsequently allowed to develop for additional 9 hr at 21°C, then fixed and stored at -20°C.

Cloning of Ci-multidom

Isolation of 204d, an incomplete cDNA clone of Ci-multidom, has been reported previously (Hotta et al., 1999). Rapid amplification of cDNA 5’-ends (5’RACE) was performed using the GeneRacer™ kit (Invitrogen, CA). For 5’RACE, total RNAs from early tailbud stage embryos were reverse-transcribed using oligo(dT)12–18 primers in combination with the following specific primers:

Ci-multili496 5’-CACTGCCTTCG-3’
Ci-multil239 5’-TTGCACTGCCCTGTTACAAGGATTCC-3’.

The relative positions of these primers along the gene model are indicated in Figure 1A.

Whole-Mount In Situ Hybridization

Digoxigenin-labeled antisense RNA probes for Ci-multidom and Ci-Bra were synthesized, respectively, from the 204d-L cDNA clone and from a plasmid containing the full-length Ci-Bra cDNA (NCBI accession number AB210329; Corbo et al., 1997). Whole-mount in situ hybridizations were performed essentially according to the protocol published by Ikuta et al. (2004); embryos were hybridized at 50°C with variable amounts of each probe, ranging from 0.1 to 0.5 μg/ml, for 15–18 hr.

Electroporation

Electroporations were performed according to previously published protocols (Corbo et al., 1997; Bertrand et al., 2003), with some slight modifications. One hundred microliters of fertilized eggs were mixed with 250 μl of 0.96 M mannitol containing 50 μg of plasmid DNA, and electroporated in 4 mm cuvettes with a Gene Pulser Xcell System (BIO-RAD), using a square pulse protocol (50V and 16 ms per pulse). After electroporation, embryos were allowed to develop until the tailbud stage and fixed in filtered seawater containing 0.2% glutaraldehyde for 30 min at room temperature, then washed in PBST (phosphate-buffered saline containing 0.1% Tween20) twice and washed once in 1 mM MgCl₂, 3 mM potassium ferrocyanide and 3 mM potassium ferricyanide in PBST for 5 min. The staining reactions were carried out in PBST containing 400 ng/ml 5-bromo-4-chloro-3-indolyl—D-galactopyranoside (X-gal), 1 mM MgCl₂, 3 mM potassium ferrocyanide, and 3 mM potassium ferricyanide at 25°C for ~12 hr, then stopped by washing in PBST.

Plasmid Construction

To construct the Ci-Bra>EGFP plasmid, the EGFP coding sequence was PCR-amplified from the EGFP-C1 plasmid (Clontech) using the following primers:

5’-GAAGATCCCGATGTTGACCAGGCGAGG-3’ (EGFP forward) and
5’-TTAGCGGCCGCTTACTTGACAGTGCTGCTCATGCCGC-3’ (EGFP reverse) primers.

The relative positions of these primers along the gene model are indicated in Figure 1A.

To construct the Ci-Bra>EGFP-multidom plasmid, the EGFP coding sequence was amplified using the EGFP forward primer and the following reverse primer:

5’-TTAGATCCCTTGATGAGCCTGCCCTTGACTGCTGCTCATGCCGC-3’ and then cloned into the BamHI site of the Ci-Bra>EGFP (NotI) vector to make the Ci-Bra>Ci-multidom plasmid.

To generate the Ci-Bra>EGFP-multidom plasmid, the EGFP coding sequence was amplified using the EGFP forward primer and the following reverse primer:

5’-TTAGATCCCTTGATGAGCCTGCCCTTGACTGCTGCTCATGCCGC-3’ and then cloned into the BamHI site of the Ci-Bra>Ci-multidom plasmid.

To create the Ci-Bra>Nup50-mRFP plasmid, the Ci-Nup50 coding sequence was PCR-amplified from EST clone CiGC29123, obtained from the Ciona intestinalis Gene Collection Release 1 (Satou et al., 2002; courtesy of Dr. Nori Satoh), using the following primers:

5’-CTAGGATCCATGCGAGAGGAGGTAGCTGA-3’ and
5’-AGTGGCCGCCGCTAAAGTTCGACCCAGTTGGCTT-3’.

The resulting PCR product was then inserted into the BamHI and NotI sites of the Ci-Bra>mRFP plasmid (Rhee et al., 2005).

To create the Ci-Bra>KDELr-mRFP, the mRFP coding sequence and Ci-KDELr coding sequence were inserted into the BamHI/NotI sites of Ciana-MCS, respectively. To make the Ci-Bra-MCS plasmid, in which a multicloning site is inserted downstream of the 3.5-kb Ci-Bra regulatory region, a PsI/BglII-BamHI/NotI linker was inserted into the PsI and NotI sites of the Ci-Bra>GFP (NotI) vector. The mRFP coding sequence was PCR-amplified from plC-myrmRFP1 plasmid using the following primers:

5’-TTGATCCATGCGCTCTCTGCGAGGAGGACG-3’ and
5’-ATGGGCCGCGCCATGGACCCGCTGGAGATTG-3’.

To construct the Ci-Bra>ST>mRFP plasmid, the membrane anchor sequence from rat sialyltransferase (Bovin et al., 1998) was amplified from the ST-aGFPM5 plasmid (Zeller et al., 2006) using the primers:

5’-ATCGGATCCATGATCACACACACCCTGAAGA1-3’ and
5’-TGGAGGCCGCCAAGCTCTTGTGTGT-GCTAAC-CA-3’ and then inserted into the BamHI and NotI sites of the Ci-Bra>mRFP plasmid (Rhee et al., 2005).

To create the Ci-sna1-Ci-Bra plasmid was prepared by cloning the Ci-Bra coding region into the PsI and BglII sites located at the 3’-end of a 1-kb fragment from the Ci-sna1 (c1001515261) 5’-flanking region (in pSPT2-1.27; Corbo et al., 1997). The Ci-sna1 1-kb fragment encompasses the 504-bp muscle enhancer described by Erives et al. (1998).

To create the enhancer construct 1, which includes the putative endogenous Ci-multidom promoter, a 1.1-kb
fragment upstream of Ci-multidom was PCR-amplified from Ciona genomic DNA using the following primers:

5'-ATGGGATCCATTACGGATCCTGTTTCTTAA-3' (1.1 forward) and 5'-TGTGCCCCGCGCTTGTGTCGTTATTTTCTC-3' then cloned into the BamHI and NotI sites of the pFBasp6 plasmid, which carries the LacZ reporter gene. The pFBasp6 plasmid contains 155 bp of the Ci-fkh/HNF-3β/Ci-fkh; JGI gene model ci0100153163; Di Gregorio et al., 2001) basal promoter and part of the first exon of Ci-fkh fused in frame to the lacZ reporter gene and inserted into the pSP72-1.27 vector (Harafuji et al., 2002). Since the SP6 site originally present in pSP72-1.27 was found to contain a T-box binding site, the Ci-fkh basal promoter was removed and replaced by the alternative promoter regions. To create the enhancer construct 1, which contains the −1.1-kb intergenic fragment upstream of the Ci-fkh basal promoter, the 1.1-kb fragment was PCR-amplified from Ciona genomic DNA using the −1.1 forward primer and the following reverse primer:

5'-GGTCCCATGTTACCCGCTAACATTCTTGCA-3' and then cloned into the BamHI and NotI sites of the pFBasp6 plasmid.

Finally, as a first step to create enhancer construct 3, the Ci-Bra promoter region was PCR-amplified from Ciona genomic DNA using the primer:

5'-TTCCAGGTTGTATTAGTATAAACCTGACCCCCAG-3' and then cloned into the BamHI and NcoI sites of the pBrapro plasmid.

The resulting product was cloned into the BamHI and NcoI sites of the pBrapro plasmid.

**Semi-Quantitative RT-PCR**

Total RNAs were isolated from 10 µl of mid-tailbud embryos using the RNeasy Protect Mini Kit (Qiagen), according to the manufacturer’s instructions. RNA samples were resuspended in RNase-free water and quantified spectrophotometrically at 260 nm. Five hundred ng of total RNAs were reverse transcribed in a 20-µl total volume, using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) and the supplied oligo(dT)12-18 primer; a negative control, which was incubated in parallel but did not contain reverse transcriptase, yielded no products.

All PCR amplifications were performed in a 25-µl total volume using Taq DNA polymerase (New England Biolabs), 5× Green GoTaq Reaction Buffer (Promega) and 0.5 µl of the synthesized cDNA sample as a template. The number of cycles was optimized for each primer pair to remain within the linear range of amplification.

Control PCR reactions, performed in parallel without reverse transcriptase, did not amplify any product. Gel electrophoresis of 1 or 2 µl of each PCR reaction was carried out on 2.0% agarose gels. After electrophoresis, digital images of the gels were acquired using a Kodak EDAS 290 imaging system. The relative levels of intensity of the bands obtained were quantified using the Multi-Analyst version 1.0.2 software (Bio-Rad). The primers used for RT-PCR were as follows:

- For Elongation factor 2 (EF2): 5'-CAAAAGGGGTTGTCTTTTG-3' and 5'-GGATCTCCAGGTTATGTCG-3'; for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5'-GGCAGGCTGGCAATGCAC-3' and 5'-ACAGTAAAGTTGCAAGCAG-3'; for Ci-multidom: 5'-TGCTTCGGACCGGGCATAC-3' and 5'-GGGGTGCCTCCTTATCC-3'.

**Histochemistry and Confocal Microscopy**

Embryos were fixed with 4% paraformaldehyde in 0.5 M NaCl and 0.1 M MOPS pH 7.0 at room temperature for 30 min. To visualize the cell membranes by staining the actin cytoskeleton, embryos were rinsed with PBST (phosphate-buffered saline containing 0.1% Tween20) 3 times and incubated in 1 U (200 µl) of Rhodamine-phalloidin (Invitrogen) in PBS/0.2% Triton X100 for 3 hr at room temperature. After incubation, embryos were washed 3 times with PBST and mounted on microscope slides with Vectashield Mounting Medium (Vector Laboratories, CA). To visualize the nuclei, embryos were rinsed 3 times with PBST and incubated in 5 µM TO-PRO-3 iodide (Invitrogen) in PBS/0.2% Triton X100, overnight at 4°C. After incubation, embryos were washed and mounted as described above. Embryos electroporated with Ci-Bra>ST-mRFP were fixed with 100% acetone for 30 min at −20°C.

Laser scanning confocal images were acquired with a Zeiss LSM 510 on a Zeiss Axiowert 200 widefield microscope, using either a 63× or a 25× oil immersion lens. Confocal microscopy was carried out at the Weill Cornell Medical College Optical Microscopy Core Facility.

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