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BRIEF COMMUNICATION

Expression of Brachyury During Development of the Dendrobatid Frog *Colostethus machalilla*

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ABSTRACT **The expression of Brachyury (Bra) during development of *Colostethus machalilla* was analyzed with a polyclonal antibody. The observed molecular mass of Bra was of 48 kDa, as in *Xenopus laevis*. During cleavage, low levels of Bra were expressed. In contrast, in the blastula Bra became up-regulated, and Bra protein was present in a wide ring of surface cells. The surface expression of Bra disappeared in the gastrula, and a new ring of Bra-positive nuclei was detected in deep cells around the closing blastopore. The *C. machalilla* external and internal rings of Bra-positive nuclei apparently mark the prospective mesoderm in the blastula and gastrula, respectively. The two Bra expression rings were dissociated in time in the fairly slow developing embryos of this frog. Brachyury expression in the notochord became visible only after the blastopore closed, in contrast with *X. laevis*. In addition, Bra expression in the notochord indicated that dorsal convergence and extension occurred after blastopore closure. The *C. machalilla* Bra-positive notochord was originally exposed on the gastrocoel roof, in agreement with a superficial component of the prospective mesoderm.**

Key words: archenteron; notochord; prospective mesoderm; *Eleutherodactylus coqui*; *Gastrotheca riobambae*

INTRODUCTION

The current understanding of development in the frog *Xenopus laevis* and other model organisms, such as the zebrafish and the mouse, provide a firm basis for the comparative analysis of development in other vertebrates. Comparative studies of frog development are of particular interest, because there is a wide range of reproductive adaptations among frogs (reviewed in Duellman and Trueb, 1986), which may be related to developmental changes. For example, gastrulation in the marsupial frog *Gastrotheca riobambae* is the most derived mode among frogs and produces an embryonic disk (del Pino and Elinson, 1983). The dendrobatid frog *Colostethus machalilla* provides another example of reproductive and developmental modification (Coloma, 1995; Ávila et al., unpublished observations).

The small frog *C. machalilla* (approximately 17 mm in length) produces terrestrial egg clutches of 15 eggs on average. The eggs have a moderate size of 1.6 mm in diameter, and their development is slower than in *X. laevis*, requiring almost 3 days for the process of gastrulation. A striking feature of *C. machalilla* development is the formation of prominent filamentous external gills. In contrast with *X. laevis* eggs, the eggs of *C. machalilla* and other dendrobatids are deposited on the ground (Duellman and Trueb, 1986; Coloma, 1995). In *C. machalilla*, the father looks after the egg clutch for approximately 20 days until hatching, which occurs at a tadpole stage. Then the father carries the tadpoles on his back to the water (Quiguango-Ubillús, 2000; Ávila et al., unpublished observations). The normal table of *C. machalilla* development and the methods for the culture of its embryos and embryo explants (Ávila et al., unpublished observations) provided the groundwork for this comparative study of embryogenesis.

Well studied and conserved genes, such as the gene *Brachyury* (*Bra*), provide excellent tools for comparative analyses of development among vertebrates. Moreover, *Bra* homologs were found in ascidians, tunicates, and echinoderms (Holland et al., 1995; Gross and McClay, 2001). *Brachyury* was first described in the mouse, where mutant heterozygous individuals had a short tail, and mutant homozygotes lacked posterior mesoderm and were not viable (reviewed in Tada and Smith, 2001). *Brachyury* was cloned by Herrmann et al. (1990); it is the founder of the T-box gene family and encodes a transcription activation factor that binds to a palindromic DNA sequence (reviewed in Smith, 2001; Tada and Smith, 2001). In *X. laevis*, *Brachyury* (*Xbra*) is expressed in the prospective mesoderm of the mar-

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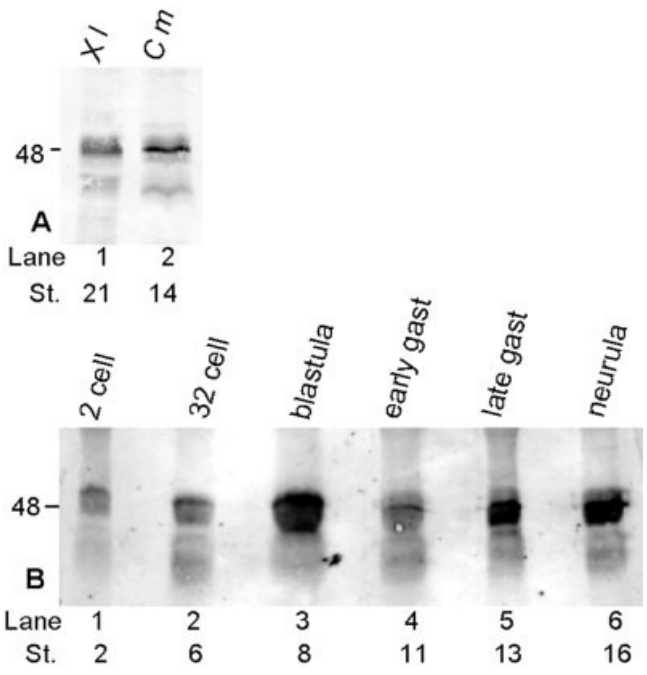

Fig. 1. The Bra expression in embryos of *Colostethus machalilla*. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis with 10% acrylamide. Each lane was loaded with the proteins from two embryos of *C. machalilla* or three embryos of *Xenopus laevis*. **A:** Comparison of Bra expression in the neurula of *X. laevis* (Xl, lane 1) and *C. machalilla* (Cm, lane 2). Brachyury in *C. machalilla* has the same electrophoretic mobility (48 kDa) as Xbra (Cunliffe and Smith, 1994). **B:** Brachyury expression during *C. machalilla* development. Stages of development are according to Nieuwkoop and Faber (1997) and Ávila et al. (unpublished observations). early gast, early gastrula; late gast, late gastrula; St., stage.

ginal zone and later in the prospective mesoderm around the blastopore, and in the notochord and tail bud (Smith et al., 1991). It was demonstrated that *Xbra* expression in the prospective mesoderm of *X. laevis* is an immediate early response to mesoderm induction (Smith et al., 1991). Besides *X. laevis*, the pattern of Bra expression was analyzed only in two additional frogs, the marsupial frog *G. riobambae* (del Pino, 1996), and the frog without tadpoles *Eleutherodactylus coqui* (Ninomiya et al., 2001). In this study, we found a novel pattern of Bra expression in the early embryos of *C. machalilla*, which differs from *X. laevis*, *G. riobambae*, and *E. coqui*.

RESULTS AND DISCUSSION

Bra- Expression in *C. machalilla* Embryos

The polyclonal antibody against the N-terminal domain of the mouse Brachyury protein (Kispert and Herrmann, 1994) recognized Bra in *C. machalilla*, as demonstrated by immunoblotting and immunostaining (Figs. 1, 2). Brachyury in *C. machalilla* had the same electrophoretic mobility of the 48 kDa demonstrated for Xbra (Fig. 1A; Cunliffe and Smith, 1994). Faint levels of Bra expression were detected in cleavage stage embryos (Fig. 1B, lanes 1, 2). In *X. laevis*, similarly, low levels of maternal *Xbra* were detected during cleavage (Smith et al., 1991). In *C. machalilla*, the initial up-regulation of Bra expression in the blastula (Fig. 1B, lane 3) was followed by a down-regulation in the gastrula (Fig. 1B, lane 4). As gastrulation advanced, a new up-regulation occurred (Fig. 1B, lane 5). In the neurula, similarly, Bra was strongly expressed (Fig. 1B, lane 6). Although the extracts from two embryos were loaded per lane (Fig. 1B), in the absence of loading control, it is unknown whether the observed difference represents the normal regulation of Bra or an artifact resulting from difficulty in extraction or degradation.

The changes in Bra expression observed by immunoblotting (Fig. 1B), were accompanied by different spatial distributions of Bra-positive cells during development (Fig. 2). In the blastula, a wide ring of Bra-positive nuclei was observed in the marginal zone, and only the animal and vegetal poles were Bra-negative (stage 9; Fig. 2A,B). The Bra-positive nuclei were located on the embryonic surface (Fig. 2C). During blastopore formation, the Bra-positive signal of the marginal zone decreased. In contrast, this signal remained strong in the area of the dorsal blastopore lip (stage 10, Fig. 2D). This expression pattern differs from *X. laevis*, as the up-regulation of *Xbra* expression occurs in the late blastula to early gastrula. Moreover, the *Xbra*-positive signal has a deep location (Smith et al., 1991).

In the early gastrula of *C. machalilla*, a down-regulation of Bra expression was observed by immunoblotting (stage 11; Fig. 1B, lane 4) and immunostaining, and the few Bra-positive nuclei were concentrated near the blastopore (Fig. 2E). These nuclei were located in the surface cells of the blastopore lip (Fig. 2F). In the midgastrula, the expression of Bra was again up-regulated (stage 12; Fig. 2G–I). The Bra signal, however, was not observed on surface cells (Fig. 2G); instead, it was located in a deep ring around the blastopore (Fig. 2H). The deep location of the Bra-positive nuclei was revealed in cross-sections of the embryos (Fig. 2I). The same pattern of expression was observed in the small blastopore stage (stage 12.5). On the surface, only few Bra-positive nuclei were detected (Fig. 2J), whereas internal cells formed an intense ring of Bra-positive nuclei around the closing blastopore (Fig. 2K,L).

A similar down-regulation of Bra-expression in surface cells before the appearance of a deep Bra-positive signal was observed during gastrulation in *G. riobambae* (del Pino, 1996). Brachyury in *G. riobambae* and *Ecbra* in *E. coqui* are expressed in the early gastrula in a ring of superficial cells, followed by a deep Bra expression after blastopore closure (del Pino, 1996; Ninomiya et al., 2001). The superficial and internal rings of Bra-positive cells occur during gastrulation in these frogs and between the blastula and gastrula in *C. machalilla*. In contrast, *Xbra* and the prospective mesoderm have deep locations in embryos of *X. laevis* (Smith et al., 1991; Winklbauer and Schürfeld, 1999).

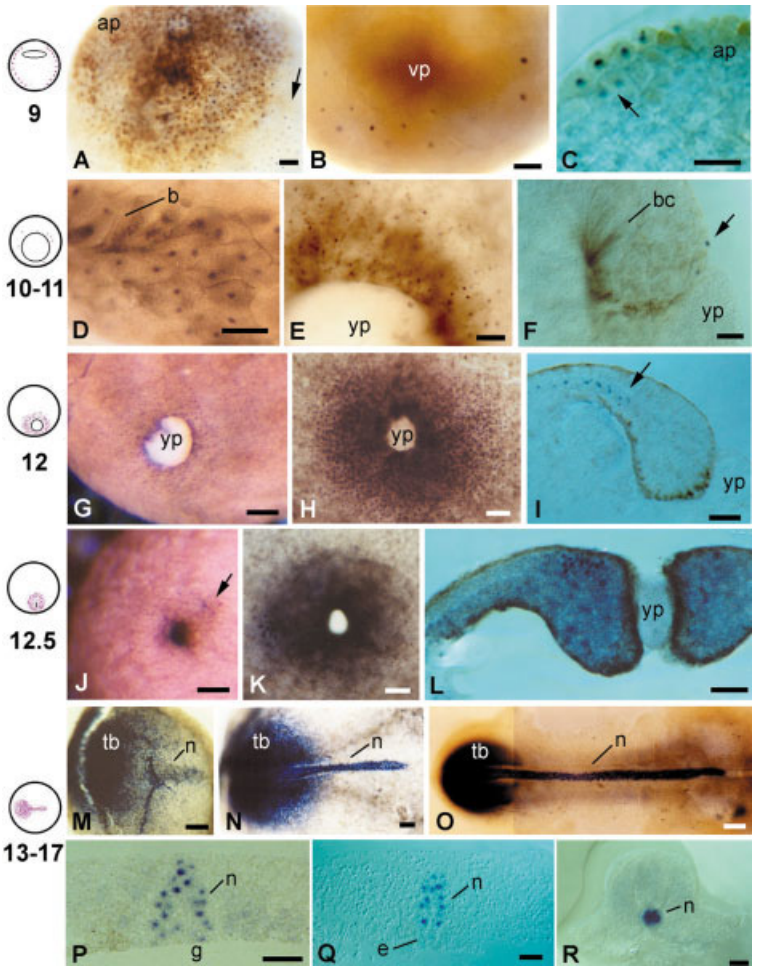

Fig. 2. The immunostaining against Bra of *Colostethus machalilla* embryos. Stages of development on the left, and the age of embryos are according to Ávila et al. (unpublished observations). The external morphology of *C. machalilla* embryos is comparable to *X. laevis* until stage 13 (Nieuwkoop and Faber, 1997). The diagrams on the left show the Bra-positive regions in purple. The embryos were immunostained in whole-mount with anti-Bra. The dark purple spots correspond to Bra-positive nuclei, whereas the brown color corresponds to the natural pigment that remained after bleaching. Embryos could not be bleached totally because elimination of the pigment also removed the Bra alkaline phosphatase signal. All embryos were cleared in bb/ba to eliminate the opacity of the yolk, except for the embryos shown in (G) and (J). Embryos were photographed in brightfield (A,B, D,E,G,H,J,K,M–P). Embryo sections were viewed with differential interference contrast (C,F,I,L,Q,R). The sections in C,F,L,R were 100 μm thick, whereas section I was 50 μm thick, and P,Q were 7 μm thick. The animal pole is oriented toward the upper left in A and toward the top in C. Anterior is toward the top in D,E,G,H,J,K and toward the right in M–O. The external surface is toward the right in F,I and toward the top in L,P–R. A–C: the blastula (stage 9; age, 1.1 days). **A:** Animal view of a cleared blastula that was partially bleached. The animal pole (ap) was Bra-negative. Bra-positive nuclei extend to the marginal zone (arrow). **B:** Vegetal view of the embryo shown in A. The vegetal pole (vp) did not contain Bra-positive nuclei, whereas the surrounding cells were Bra-positive. The cell outlines and the low density of Bra-positive nuclei in this region showed the large size of vegetal blastomeres. **C:** Parasagittal section of a blastula. Bra-positive nuclei were detected in surface cells. The animal pole (ap) was Bra-negative. The two faintly positive nuclei (arrow) were located on the embryonic surface but were positioned in a different focal plane. D–F: The early gastrula (stages 10–11). The drawing represents a stage 11 embryo. **D:** Surface view of the dorsal blastopore lip of a stage 10 embryo (age, 1.4 days). The area of the dorsal blastopore lip (b) contained nuclei that were strongly Bra-positive. The Bra-positive signal of the marginal zone decreased in comparison with the blastula (stage 9). **E:** The blastopore region of a stage 11 embryo (age, 1.9 days). Brachyury-positive nuclei were observedonly near the blastopore lips. The yolk plug and the marginal zone were Bra-negative. **F:** Parasagittal section through the blastopore of a late stage 11 embryo, showing the ventral lip. The Bra-positive nuclei were superficial (arrow). Bottle cells (bc) and those of the yolk plug were Bra-negative. G–I: The midgastrula (stage 12; age, 3 days). **G:** Surface view of the blastopore in an uncleared embryo. Some nuclei of the blastopore lips were Bra-positive. The yolk plug (yp) is Bra-negative. **H:** The same embryo shown in G after clearing. The Bra-positive nuclei formed an internal ring around the blastopore. **I:** Section through the blastopore of an advanced stage 12 embryo. Bra-positive nuclei were found in a deep layer (arrow), in contrast to stage 11 (F). The yolk plug (yp) was Bra-negative. J–L: The small blastopore stage (stage 12.5; age, 3.5 days). **J:** Surface view of the blastopore in an uncleared embryo. The yolk plug has been retracted, but the blastopore was still open. Few Bra-positive nuclei were observed in the surface near the blastopore (arrow), in comparison to stage 12 (G). **K:** The same embryo shown in J after clearing. The Bra-positive nuclei formed an internal ring around the blastopore as in H. **L:** Parasagittal section through the blastopore of a slightly earlier embryo. The small yolk plug was not retracted. The internal nuclei of the blastopore lips were Bra-positive. M–O: The slit blastopore stage and the tail bud (stages 13 and 17). **M:** The slit blastopore stage (early stage 13 embryo; age 4.1 days). After closure of the blastopore, the notochord began to elongate. The nascent notochord (n) and the tail bud (tb) were Bra-positive. **N,O:** A late stage 13 (age, 4.4 days) embryo in N and a tail bud stage embryo (stage 17; age, 8 days) in O. In both embryos, Bra-positive nuclei were found in the notochord (n) and in the tail bud (tb). P–R: Cross-sections through the notochord of a late stage 13 embryo and a tail bud stage embryo (stage 17). The sections in P,Q were from an embryo equivalent to N, and the section in R was from an embryo equivalent to O. The sections represent the posterior (P) and anterior (Q) regions of the notochord of a late stage 13 embryo. **P:** Cross-section through the posterior region of the notochord (n) of a late stage 13 embryo. The notochord lined the roof of the original cavity of the gastrula, the gastrocoel (g). **Q:** Cross-section through the anterior region of the notochord of the same late stage 13 embryo in P. The notochord n was internalized and covered with endodermal cells (e). **R:** Cross-section through the midregion of the notochord of a tail bud embryo (stage 17). The notochord (n) is located under the neural tube and above the endodermal roof of the archenteron; this cavity was totally covered with endoderm. Scale bars = 200 μm in G,J,M, 100 μm in A,B,D,E,H,I,K,L,N–P, 50 μm in C,F,Q,R.

The notochord was not detected by morphologic criteria or by Bra expression in *C. machalilla* embryos of stages 12–12.5 (Fig. 2G,H,J,K). Once the blastopore closed, the onset of notochord elongation was detected by the expression of Bra (early stage 13; Fig. 2M). Somewhat older embryos (late stage 13) had a longer Bra-positive notochord (Fig. 2N). Expression of Bra in the entire notochord was detected also in stage 17 embryos (tail bud stage; Fig. 2O). In addition to the notochord, the tail bud was Bra-positive in the analyzed embryos (stages 13–17; Fig. 2M–O). At later stages, the Bra-positive signal remained in the tail bud and the posterior region of the notochord (not shown). As in *C. machalilla*, the notochord can be detected by

the expression of Bra only after blastopore closure in *G. riobambae* and *E. coqui* (del Pino, 1996; Ninomiya et al., 2001). In contrast, the notochord elongates in the *X. laevis* midgastrula, as shown by *Xbra* expression (Gont et al., 1993). In the neurula of *X. laevis* and *G. riobambae*, the notochord and tail bud are Bra positive, and the signal in the anterior region of the notochord disappears as development advances (Gont et al., 1993; del Pino, 1996). In *E. coqui*, *Ecbra* is similarly expressed in the notochord and tail bud of the neurula; however, the expression was faint in the notochord (Ninomiya et al., 2001).

Prospective Mesoderm and Notochord Location

The location of the prospective mesoderm varies among amphibians, and influences morphogenesis. In *X. laevis* the mesoderm originates primarily from the deep mesenchymal layer of the involuting marginal zone, whereas in other frogs the superficial epithelium contributes to the mesoderm (reviewed in Keller, 1999). In frogs that have superficial prospective mesoderm, such as *Ceratophrys* and *Hymenochirus*, the notochord, which is originally exposed in the roof of the gastrocoel, becomes internalized by morphogenetic mechanisms (Purcell and Keller, 1993; Minsuk and Keller, 1996; use of the term gastrocoel according to Keller, 1999). In contrast, the internal location of the prospective mesoderm in *X. laevis* results in internal location of the notochord (reviewed in Keller, 1999). The expression of Bra in *C. machalilla* was analyzed to determine the location of the notochord in the neurula (Fig. 2P–R). In the late stage 13 embryo, the notochord was exposed in the posterior region of the gastrocoel roof (Fig. 2N,P) and was internalized in the anterior region (Fig. 2N,Q). In a stage 17 embryo, all regions of the notochord had an internal location (Fig. 2O,R). As in most amphibians (reviewed in Keller, 1999), the mesoderm in *C. machalilla* may have a superficial component, which results in the notochord being originally exposed in the gastrocoel roof.

Perspectives

The Bra expression pattern observed in *C. machalilla* differs not only from *X. laevis* but also from *G. riobambae* and *E. coqui*. The striking difference is the presence of two rings of Bra-positive cells in the likely prospective mesoderm of the blastula and gastrula of *C. machalilla*. The superficial and internal Bra-positive rings are separated in time, and the superficial expression in the blastula is apparently down-regulated before the onset of the deep Bra expression in the gastrula. This separation of developmental events may be possible in the relatively slow developing embryos of *C. machalilla*. The superficial and deep rings of Bra expression, which have been observed in *C. machalilla*, may require independent or different regulatory mechanisms. To understand mesoderm formation in *C. machalilla*, the fate of the superficial and internal rings of Bra-positive cells needs to be determined.

In contrast with *X. laevis* and as in *G. riobambae* and *E. coqui*, Bra expression in the notochord of *C. machalilla* became visible only after the blastopore closes. This evidence suggests that the dorsal convergence and extension movements, which elongate the notochord, occur after blastopore closure in all of these frogs. Further analysis is required to determine the movements of gastrulation, the onset of the dorsal convergence and extension movements, and notochord formation in *C. machalilla* embryos.

EXPERIMENTAL PROCEDURES

The dendrobatid frog *Colostethus machalilla* was successfully maintained in glass terraria according to Ávila et al. (unpublished observations). *C. machalilla* and other dendrobatid frogs are suited for developmental work because these tiny frogs are easily maintained in captivity (Zimmermann and Zimmermann, 1987; Ávila et al., unpublished observations). Moreover, several species of dendrobatid frogs are commercially available. *C. machalilla* has the advantage of frequent reproduction throughout the year (Ávila et al., unpublished observations). Its embryos can be cultured in vitro in two ways: in a humid chamber or submerged in a dish filled with 15% Steinberg saline solution (Rugh, 1962). Steinberg's solution contains 58 mM NaCl, 0.65 mM KCl, 0.85 mM $MgSO_4$, 5 mM Tris (pH 8.0), 0.34 mM $Ca(NO_3)_2$.

Proteins were obtained by gently squeezing two embryos of *C. machalilla* or three embryos of *X. laevis* in a saline solution that contains ethylenediaminetetraacetic acid (EDTA) and ethyleneglycoltetraacetic acid (EGTA), to prevent the degradation of proteins (135 mM NaCl, 10 mM Na_2HPO_4, 1 mM EDTA, 1 mM EGTA, 1% Triton, pH 6.7). The homogenate was centrifuged briefly to spin down the yolk. The supernatant was mixed with sample buffer (Laemmli, 1970). Proteins were separated in 5% stacking and 10% resolving sodium dodecyl sulphate-polyacrylamide gel electrophoresis according to Laemmli (1970). The separated polypeptides were electrophoretically transferred from gels to nitrocellulose membranes and processed for immunoblotting as described in Lang et al. (1999). The polyclonal anti-Bra antibody (Kispert and Herrmann, 1994) was diluted 1:1,000 for immunoblotting and whole-mount immunostaining of embryos. The sheep anti-rabbit immunoglobulin (IgG) conjugated to alkaline phosphatase (Boehringer Mannheim GmbH, Mannheim, Germany) was used as the secondary antibody. The presence of the bound secondary antibody was detected with NBT/BCIP (nitro blue tetrazolium, and 5-Bromo-4-chloro-3-indolyl phosphate) color reaction, as described in del Pino et al. (2002). The nitrocellulose membranes were stored wet inside plastic bags and scanned with a Hewlett Packard ScanJet 5300C (Palo Alto, CA) using the Adobe Photoshop version 5.5 (Adobe Systems, Inc., San Jose, CA).

For immunostaining, the embryos were fixed in MEMFA buffer (Harland, 1991) for 2 hr at room tem-

perature and stored in methanol at −20°C until processing. The procedure for immunostaining described by del Pino (1996) was modified according to Kuratani and Horigome (2000). The fixed embryos were equilibrated in dimethyl sulfoxide and methanol (1:1) for at least 1 hr at −20°C, and incubated for 30 min in 10% Triton X-100. Nonfat milk (5%) was added to the blocking reagent. The embryos were immunostained with a rabbit polyclonal antibody against the Bra protein, diluted 1:1,000 (Kispert and Herrmann, 1994). The secondary antibody was sheep anti-rabbit IgG conjugated to alkaline phosphatase (Boehringer Mannheim GmbH, Mannheim, Germany). The presence of the bound secondary antibody was detected with NBT/BCIP color reaction. After immunostaining, the embryos were bleached in 10% H_2O_2 in 70% methanol.

Thick sections of 50–100 μm of immunostained embryos were produced with a Vibratome 1000 (Technical Products International, Inc., St. Louis, MO) according to del Pino (1996). The sections were dehydrated in methanol and transferred to benzyl benzoate/benzyl alcohol (2:1; bb/ba) and mounted in bb/ba. To produce 5- to 7-μm sections, the thick Vibratome sections were embedded in plastic (Technovit 7100, Kulzer Histo-Tec, Wehrheim, Germany) and sectioned with a glass knife in a Supercut 2050 Reichert-Jung microtome (Cambridge Instruments GmbH, Heidelberg, Germany), and mounted in immersion oil. Whole-mount preparations and sections were analyzed and photographed with an Axiophot (Carl Zeiss, Oberkochen, Germany).

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