PATTERNS & PHENOTYPES

A Homologue of *Snail* Is Expressed Transiently in Subsets of Mesenchyme Cells in the Sea Urchin Embryo and Is Down-Regulated in Axis-Deficient Embryos

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Vertebrate members of the zinc finger transcription factor family related to *Drosophila snail* are expressed in neural crest and paraxial mesoderm along the left–right axis of the embryo. As simple deuterostomes, echinoderms are an important sister phylum for the chordates. We have identified populations of patterned, nonskeletogenic mesenchyme in the sea urchin *Lytechinus variegatus* by their expression of a sea urchin member of the *snail* family (*Lv-snail*). *Lv-snail* mRNA and protein are detectable at the midgastrula stage within the archenteron. At the late gastrula stage, a contiguous cluster of cells on the left side of the tip of the archenteron is *Lv-snail*–positive. At the early prism stage, two small clusters of mesenchyme cells near the presumptive arm buds are also *Lv-snail*–positive. At the pluteus stage, staining is detectable in isolated mesenchyme cells and the ciliated band. Based on fate mapping of secondary mesenchyme cells (SMCs) and double-label immunostaining, these patterns are consistent with expression of SNAIL by novel subsets of SMCs that are largely distinct from skeletogenic mesenchyme. In radialized embryos lacking normal bilateral symmetry, mesenchymal expression of Lv-SNAIL is abolished. These results suggest that transient expression of *Lv-snail* may be important for the differentiation of a subset of axially patterned nonskeletogenic mesenchyme cells and suggest conserved functions for *snail* family members in deuterostome development. *Developmental Dynamics 235:3121–3131, 2006.* © 2006 Wiley-Liss, Inc.

Key words: *snail*; secondary mesenchyme; sea urchin; gastrulation

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INTRODUCTION

It has become clear that, despite their widely divergent body plans, metazoans use regulatory molecules in surprisingly similar ways to produce differentiated tissues in appropriate places and at appropriate times (Mann and Carroll, 2002; Pearson et al., 2005; Davidson and Erwin, 2006). One fundamental feature of metazoan development is the conversion of epithelial cells to mesenchymal cells, followed by the migration of mesenchymal cells to distant sites during and after gastrulation. Transcription factors related to the *Drosophila* zinc finger transcription factor *snail* have been implicated in the specification of mesenchyme in both protostomes and deuterostomes (Manzanares et al., 2001; Nieto, 2002; Morales and Nieto, 2004). In addition to their initial characterization in *Drosophila* (Boulay et al., 1987; Alberga et al., 1991; Leptin, 1991; Ip et al., 1992; Whiteley et al., 1992), members of this family of proteins have been characterized in other arthropods (Sommer et al., 1992; Sommer and Tautz, 1994), ascidians (Corbo et al., 1997; Wada and Saiga,

1999), the cephalochordate *Amphioxus* (Langeland et al., 1998), *Xenous* (Sargent and Bennett, 1990; Mayor et al., 1995), zebrafish (Hammerschmidt and Nusslein-Volhard, 1993; Thisse et al., 1993, 1995), chick (Nieto et al., 1994; Isaac et al., 1997; Sefton et al., 1998), and mouse (Nieto et al., 1992; Smith et al., 1992).

Vertebrate homologues of *snail* are expressed in multiple tissues and at various times during development. In *Xenopus*, *snail* is expressed in the dorsal marginal zone (the region containing the Spemann organizer) before gastrulation (Sargent and Bennett, 1990). Two different *snail*-related genes have been identified in zebrafish; the *snail1* gene is expressed in paraxial mesoderm and later in cephalic neural crest (Hammerschmidt and Nusslein-Volhard, 1993; Thisse et al., 1993), while the *snail2* gene is expressed in mesendoderm, and subsequently in regions similar to that of its amphibian counterpart, including the neural crest (Thisse et al., 1995). A *snail* homologue in the mouse is expressed in presumptive mesodermal tissue, neural crest, and later in limb bud mesenchyme (Nieto et al., 1992; Smith et al., 1992). Another *snail*-related gene, designated *slug*, has been identified in several vertebrates; *slug* transcripts localize to neural crest cells at the time of their emigration from the dorsal surface of the neural tube in chick and *Xenopus* embryos (Nieto et al., 1994; Mayor et al., 1995).

One way in which *snail* appears to function is by controlling the epithelial–mesenchymal transition (EMT; reviewed in Morales and Nieto, 2004). In *Drosophila*, loss of *snail* function results in failure to down-regulate DE-cadherin expression in presumptive mesoderm (Oda et al., 1998). Mouse knockouts show similar EMT failure (Carver et al., 2001) and enhance cranial defects in other mouse mutants (Oram and Gridley, 2005). When expressed in mammalian epithelial cells, snail is capable of inducing EMT (Batlle et al., 2000; Cano et al., 2000). In addition, antisense disruption of chicken *snail* function results in randomization of heart coiling and associated left–right axis defects (Isaac et al., 1997). Disruption of *slug* gene function in chick embryos by means of antisense oligonucleotides perturbs

ingression of cells through the primitive streak and neural crest differentiation and migration (Nieto et al., 1994). Overall, these studies support a role for *snail*-related proteins in controlling the development of populations of mesenchymal cells during early development in vertebrates.

The deuterostomes include not only vertebrates, but nonvertebrate chordates such as ascidians and cephalochordates, which lack neural crest, as well as echinoderms. A *snail* orthologue in the ascidian *Ciona intestinalis* is expressed in bilateral strips of tissue at the border between presumptive neural and epidermal ectoderm, a position consistent with these cells being similar to the ectodermal cells that ultimately generate the neural crest in vertebrates (Corbo et al., 1997). In addition, *Ciona sna* is expressed in muscle cells and other mesenchyme. *Ciona sna* is thought to repress the expression of the T-box gene *Brachyury* in tail muscle, an event that is important for establishing the muscle/notochord boundary (Fujiwara et al., 1998). The neural plate expression of an *Amphioxus snail* homologue is similar to that in *Ciona* (Langeland et al., 1998).

Thus far, there have been no reports of the cloning or characterization of snail-related genes in any non-chordate deuterostome sister-group. By studying the role of snail-related proteins in such organisms, it may be possible to assess basic functions of snail-related proteins in deuterostomes. The sea urchin embryo is a convenient system for studying pattern formation by mesenchyme cells in a simple deuterostome (Ettensohn et al., 2004). Much is known regarding the cellular basis of morphogenesis and differentiation of mesenchyme in this system (reviewed in Hardin, 1996), and the simple organization of the sea urchin embryo makes it a convenient system for phylogenetic studies (Wray and Lowe, 2000). Here we describe the cloning and expression pattern of a *snail* homologue in the sea urchin, *Lytechinus variegatus*. The transient expression of *Lv-snail* in populations of mesenchyme engaging in pattern formation in this simple deuterostome suggests that the functions of *snail* have been conserved across considerable phylogenetic distances.

RESULTS

Lv-snail Transcripts Are Expressed by Small Populations of Mesenchyme Cells in the Gastrula

We used a DNA fragment corresponding to the zinc finger domain of *Drosophila snail* to clone a *Lytechinus variegatus snail* homologue (*Lv-snail*; see Supplementary Materials, which are available at www.interscience. wiley.com/jpages/1058-8388/suppmat). Using an *Lv-snail* cDNA as a probe, only one 4.2-kb band was seen on Northern blots of *L. variegatus* polyA+ RNA (Fig. 1A). Given the size of the deduced *Lv-snail* open reading frame, this result suggests that the Lv-snail mRNA possesses an unusually long 3′ untranslated region (UTR). There is precedent for extremely long 3′ UTRs in the sea urchin, including the sea urchin myogenic factor SUM-1, which possesses a 3′ UTR of comparable length (Venuti et al., 1993). Unlike the 5′ end of the *Lv-snail* cDNA, all attempts at 3′ rapid amplification of cDNA ends (RACE) were unsuccessful (data not shown), which is consistent with the presence of a long 3′ UTR. *Lv-snail* mRNA levels increase massively at the late gastrula stage (Fig. 1A). A similar temporal profile was seen in RNAse protection assay experiments (data not shown).

We next assessed the spatial expression of *Lv-snail* during archenteron invagination and in larvae using whole-mount in situ hybridization (Fig. 1B–J). At the early gastrula stage, *Lv-snail* may be expressed above background by cells in the invaginating archenteron (Fig. 1B). At the midgastrula stage, individual cells in the wall of the archenteron can clearly be identified that transcribe detectable levels of *Lv-snail* (Fig. 1C). However, as the archenteron nears the animal pole, staining becomes very intense in a small group of cells at the tip of the archenteron (Fig. 1D). When the tip of the archenteron is just beginning to attach to the animal pole, there are occasionally cells visible near the vegetal pole that also have signal above background in (Fig. 1E). The cells expressing *Lv-snail* at the tip of

the archenteron are embedded within the tip, and asymmetrically situated there. Based on the location of the ventrolateral clusters of primary mesenchyme cells, which serve as convenient bilaterally symmetric landmarks on the ventral side of the embryo, the patch of *Lv-snail*–expressing cells is on the left side of the tip of the archenteron (Fig. 1F).

Shortly after this stage, that is, at the completion of gastrulation, two additional bilateral clusters of cells express *Lv-snail*. That these new clusters of cells express *Lv-snail* simultaneously with the cells at the tip of the archenteron is clear when embryos are compressed to view both populations in the same focal plane (Fig. 1G). These clusters of approximately six to eight cells each form at the sites where the arm buds of the pluteus will form. The period during which staining persists simultaneously in both locations is not likely to be more than 2 hr at 22°C. Eventually the staining at the tip of the archenteron is completely lost; the staining in the two ventrolateral clusters becomes more intense when embryos reach the definitive prism stage (Fig. 1H). The clusters that begin to accumulate *Lv-snail* transcripts slightly later are clearly mesenchymal, based on through-focus observations of whole-mounts. Some of these cells appear to lie within the ventrolateral chains of PMCs (Fig. 1F,H). PMCs that are part of the ventral ring appear to lie above (i.e., animal to) other *Lv-snail*–expressing cells, suggesting that most of the *Lv-snail*–positive cells are not primary mesenchyme cells (Fig. 1I). At the pluteus stage, cells in the ciliated band, which forms at the border between dorsal and ventral ectoderm, appear to express *Lv-snail*. In addition, cells embedded in the ectoderm appear to express detectable levels of *Lv-snail* mRNA (Fig. 1J). These cells are distributed in the aboral ectoderm, including near its boundary with the ciliated band/oral ectoderm, and their distribution resembles that of chromogenic mesenchyme (i.e., pigment cells). Sense probes give no detectable signal (data not shown).

Lv-SNAIL Protein Is Transiently Expressed by Small Groups of Mesenchyme

We next assessed the distribution of *Lv-snail* protein in postmesenchyme blastula embryos, using affinity purified anti–Lv-SNAIL antibodies, which recognize a single faint band of approximately 37 kDa on Western blots that corresponds closely to the size of the *Lv-snail* conceptual translation product (data not shown). Little or no signal could be detected before the late gastrula stage (Fig. 2A), although this may simply reflect low sensitivity of our antibodies (S. Wu and D. McClay, personal communication). At the late gastrula stage, a cluster of mesenchyme cells in the left side of the tip of the archenteron expresses high levels of Lv-SNAIL protein (Fig. 2B). Slightly later, cells in the anal region begin expressing Lv-SNAIL protein (Fig. 2C); the number and location of these cells is similar to that obtained using riboprobes. In addition, some migratory cells within the blastocoel express Lv-SNAIL protein (Fig. 2C,D). The cluster of cells eventually disperses from the tip of the archenteron, and cells expressing Lv-SNAIL appear within the ectoderm. These cells appear highly constricted initially, suggesting that they are invading the ectoderm (large arrow, Fig. 2D). There does not appear to be a dorsoventral bias to the arrangement of cells near the base of the archenteron that express Lv-SNAIL (data not shown). By the prism stage, clusters of cells in the blastocoel in the vicinity of the growing arm buds are also visible (Fig. 2E). In later prism stage embryos, more cells scattered within the ectoderm appear that produce Lv-SNAIL protein, including higher concentrations of Lv-SNAIL–expressing cells in the arm bud ectoderm (Fig. 2F). At the pluteus stage, cells expressing high levels of Lv-SNAIL protein can be identified in the ciliated band; cells scattered within the ectoderm can also be identified (Fig. 2G). Mesenchyme associated with the midgut and the ciliated band of the oral hood also express Lv-SNAIL (Fig. 2H). Two different *Drosophila* antibodies give essentially the same pattern of staining as that obtained with anti–Lv-SNAIL

antisera (Fig. 2H). Preimmune sera gave no staining (data not shown).

Lv-SNAIL–Expressing Cells Are Largely Distinct From Skeletogenic Mesenchyme

Double labeling of embryos with anti–Lv-SNAIL antibodies and antibodies directed against a cell surface component of primary mesenchyme cells (1d5) was performed to determine whether cells expressing Lv-SNAIL are skeletogenic mesenchyme (Fig. 3I–M). Most Lv-SNAIL–expressing cells localized to the ectoderm near the blastopore, to isolated cells within the blastocoel that do not express the 1d5 antigen, and to cells near sites of ventral arm bud outgrowth that do not express the 1d5 antigen (Fig. 2I–K). In plutei, Lv-SNAIL–expressing cells localize to the ciliated band in the postoral arms, as well as to isolated mesenchymal cells within the arms. The isolated cells do not appear to be part of the chains of PMCs that generate the postoral or oral skeletal rods (Fig. 2L,M). A small number of PMCs at the very tips of growing rods may simultaneously express both Lv-SNAIL and the 1d5 antigen (Fig. 2M), but there is little overlap between patterns of 1d5 and anti–Lv-SNAIL expression patterns in the pluteus.

Pigment cell progenitors are released from the archenteron early in gastrulation (Gibson and Burke, 1985). Other nonskeletogenic mesenchyme are likely released from the tip of the archenteron in *L. variegatus* late in gastrulation (Ettensohn and Ruffins, 1993). Although fate mapping indicates that nonskeletogenic mesoderm cells are derived from the vegetal plate (Ruffins and Ettensohn, 1993), it is not clear what subpopulations of cells are generated from secondary mesenchyme cells (SMCs) that leave the archenteron late in gastrulation. To determine whether the expression pattern of Lv-SNAIL is consistent with the fates of SMCs that emanate from the tip of the archenteron late in gastrulation, we performed SMC transplantations by removing cells directly from the tip of the archenteron of rhodamine-labeled *L. variegatus* late gastrulae and transplanting these into unlabeled host gastrulae. We then examined the pattern of labeled cells as they migrated within the host (Fig. 2N,O). A small cluster of cells localizes
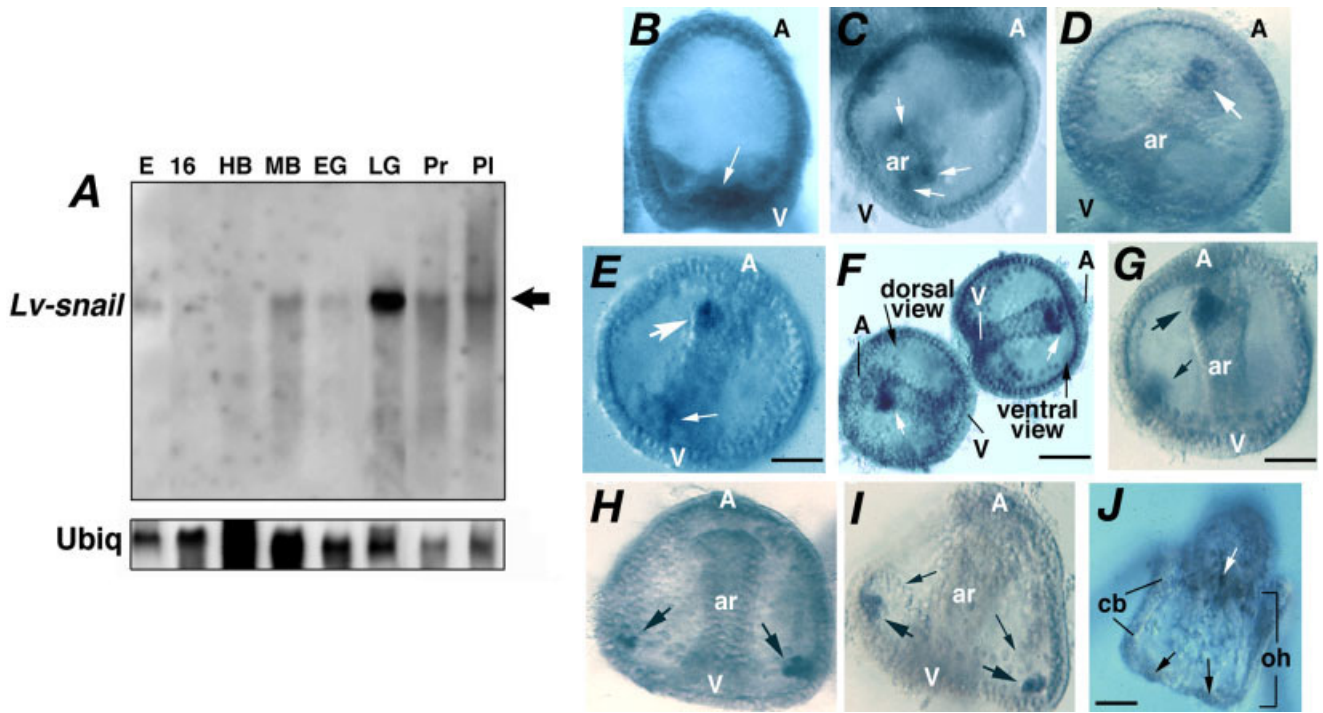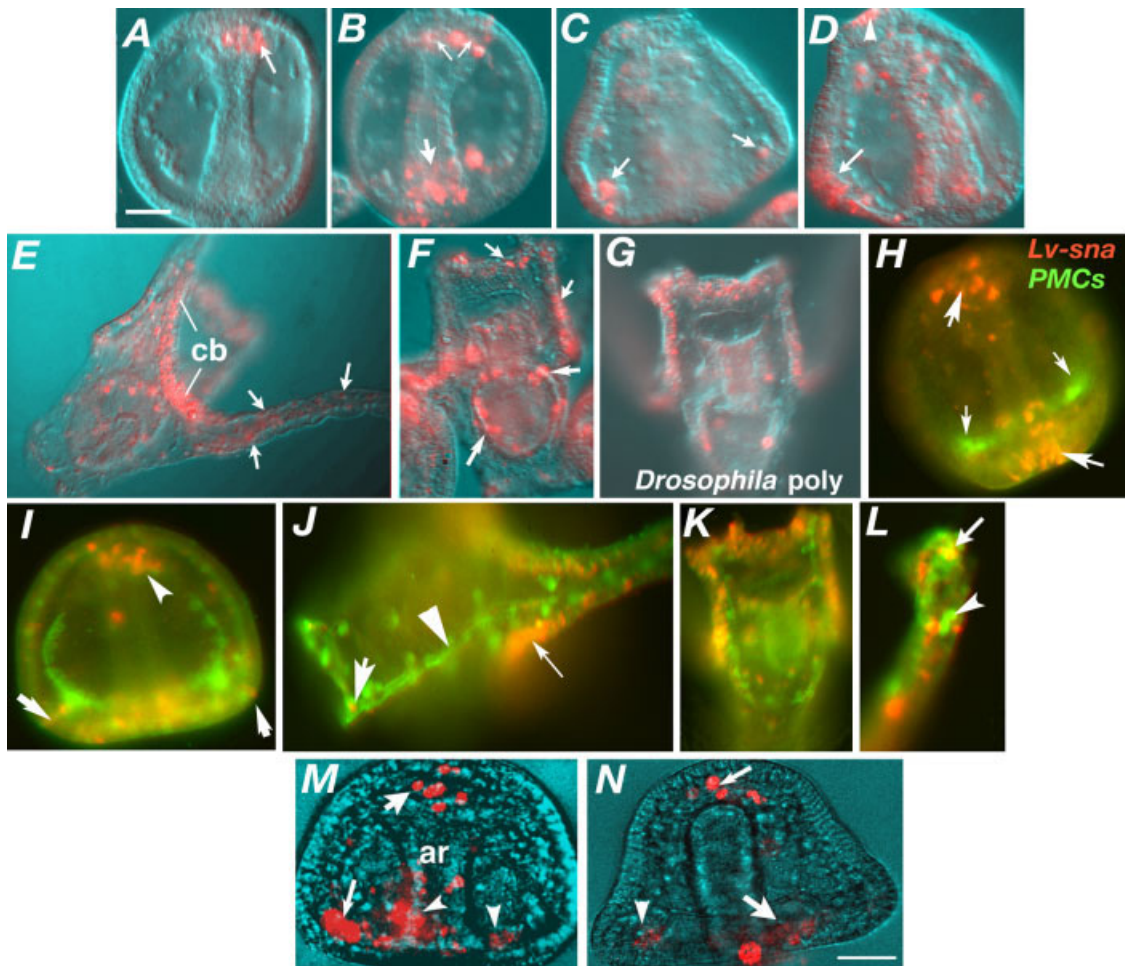
Fig. 1.

Fig. 2.

near the tip of the archenteron, near the site where the archenteron attaches to the animal pole ectoderm (Fig. 2N). In addition, other cells appear in the vicinity of the forming arm buds, and others localize near the base of the archenteron in the vicinity of the anal ectoderm (Fig. 2O). The similarity of the Lv-SNAIL immunostaining pattern to the pattern of fates obtained in these experiments suggests that *Lv-snail* is expressed in subsets of SMCs derived from the tip of the archenteron late in gastrulation.

Alterations in *Lv-snail* Expression Follow Perturbations of Gastrula Stage Differentiation

To examine regulation of expression of *Lv-snail* in experimentally manipulated embryos, we treated embryos with various chemicals known to have profound effects on the differentiation and patterning of mesenchyme cells in the sea urchin embryo. These treatments included (1) β-aminoproprionitrile (BAPN), an inhibitor of collagen crosslinking known to inhibit differentiation of mesendoderm in sea urchin embryos (Butler et al., 1987; Wessel and McClay, 1987); (2) zinc, an "animalizing" agent known to suppress development of "vegetal" structures (i.e., gut and mesenchyme; Nemer, 1986); (3) lithium, a "vegetalizing" agent known to result in overproduction of mesendoderm (Nocente-McGrath et al., 1991) and known to shift the position of skeletogenic mesenchyme cells in tandem with shifts in the position of the endoderm–ectoderm boundary (Wolpert and Gustafson, 1961); and (4) nickel (II), which radializes embryos, apparently as a consequence of conversion of much of the ectoderm to a ventral fate (Hardin et al., 1990), similar to phenotypes obtained by disrupting Nodal signaling in the early embryo (Flowers et al., 2004; Duboc et al., 2005). Figure 3A shows a Northern blot of polyA+-selected RNA derived from embryos treated with these four chemicals as well as from normal gastrulae, probed with *Lv-snail* cDNA. Both BAPN and zinc treatment partially suppressed expression of *Lv-snail* transcripts. Nickel treatment reduced *Lv-snail* expression even further. In contrast, polyubiquitin transcripts are expressed at levels comparable to controls. Of the four treatments used in this study, only lithium chloride had no effect on the overall levels of *Lv-snail* expression (Fig. 3B).

Because LiCl and $NiCl_2$ are known to alter the normal distribution of mesenchyme cells in sea urchin embryos, the position of Lv-SNAIL–expressing cells was assessed by means of immunostaining in embryos treated with these chemicals. In embryos vegetalized with 30 mM LiCl, large numbers of cells accumulating Lv-SNAIL protein are evident in the gut rudiment by the late gastrula stage and persist through the pluteus stage (Fig. 3C,D). Fewer isolated cells in the ectoderm express significant levels of Lv-SNAIL. Occasionally, there is detectable Lv-

Fig. 1. *Lv-snail* transcript accumulation during *Lytechinus variegatus* development. **A:** Northern blot analysis of *Lv-snail* transcripts using a *Lv-snail* probe. The blot was then stripped and reprobed with a *L. pictus* polyubiquitin cDNA probe (Ubiq). The position of the RNA standards are indicated on the right. A single band corresponding to the *Lv-snail* mRNA migrates at 4.2 kb (arrow). Note the massive accumulation of *Lv-snail* transcripts at the late gastrula stage. B–J: Spatial localization of *Lv-snail* transcripts using whole-mount in situ hybridization. The animal pole (A) and vegetal pole (V) are indicated in B–I. **B:** Early gastrula. Some staining may be present in the invaginating archenteron (arrow). **C:** Midgastrula. Cells in the archenteron (ar) express *Lv-snail* transcripts (arrows). **D:** Late gastrula. Cells strongly positive for *Lv-snail* are visible at the tip of the archenteron (ar) in a contiguous cluster (large arrow). **E:** Embryo after completion of gastrulation; simultaneous expression of *Lv-snail* transcripts in cells at the tip of the archenteron (large arrow) and in mesenchyme cells near the base of the archenteron (small arrow). **F:** Low-magnification views of complete gastrulae. Dorsal and ventral views can be assigned by the position of the large ventrolateral aggregations of primary mesenchyme in these embryos. The cluster of *Lv-snail*–expressing cells is visible in each (white arrows), embedded in the left-hand tip of the archenteron. **G:** Gastrula slightly later than F, left view. Staining is still evident at the tip of the archenteron (large arrow), and it is simultaneously visible in two ventrolateral clusters, one of which is in focus in this view (small arrow). **H:** Early prism stage embryo. Staining is evident in two bilateral clusters (arrows; ar, archenteron). Staining at the tip of the archenteron is lost by this stage. **I:** Late prism stage embryo. Staining persists in bilateral clusters (large arrows), which lie below (i.e., vegetal to) the main chains of primary mesenchyme cells (small arrows). **J:** Pluteus larva. Staining is visible in the ciliated band (cb) and in the aboral ectoderm (white arrow), but is absent from most of the oral hood (oh). Sense probes yielded no detectable signal. Scale bar = 25 μm.

Fig. 2. A–H: Lv-SNAIL protein expression pattern in gastrula, prism, and pluteus embryos. SNAIL protein expression is shown in red, brightfield in blue. Embryos in A–K were stained with an anti–Lv-SNAIL polyclonal antiserum. **A:** Late gastrula. Note the cluster of cells in the tip of the archenteron (arrow). **B:** Late gastrula, somewhat later than B. The cluster has dispersed somewhat (small arrows), and SNAIL-expressing cells are visible near the base of the archenteron (large arrow). **C:** Prism stage embryo. Bilateral clusters of SNAIL-expressing cells are apparent (arrows). **D:** Later prism stage embryo. Cells embedded in the presumptive arm buds (small arrow), the animal pole ectoderm (arrowhead), and the dorsal (aboral) ectoderm express SNAIL. **E:** Pluteus larva. Intense staining is visible in the ciliated band (cb). Staining of mesenchyme is also visible in the arms (arrows). **F:** Oral hood of a pluteus larva. Staining is evident in the ciliated band (small arrows) and in cells associated with the midgut (arrows). **G:** Oral hood of a pluteus larva stained with a polyclonal antibody that recognizes *Drosophila* SNAIL. H–L: Double-label immunostaining for Lv-SNAIL protein (red) and skeletogenic (primary) mesenchyme (green). Colocalization is indicated by yellow. **H:** Note the prominent ventrolateral clusters of skeletogenic mesenchyme (small arrows), which are clearly distinct from SNAIL-expressing cells in the anal region and the animal pole (large arrows). **I:** Late gastrula, ventral view. SNAIL-positive cells have invaded the ventrolateral ectoderm (arrows), but SNAIL-positive cells are not found in the ventrolateral clusters or ventral chain of skeletogenic mesenchyme. Some SNAIL-positive cells remain in the left side of the tip of the archenteron (arrowhead). **J:** Pluteus, "Scheitel" region. Most SNAIL-positive cells are in the ciliated band of the arms (small arrow), whereas skeletogenic mesenchyme are associated with skeletal rods (arrowhead). One cell at the vertex between skeletal rods may stain with both markers (medium arrow). **K:** Pluteus, oral hood region. Most SNAIL-positive cells are in the ciliated band. **L:** Pluteus, tip of a postoral rod. One or two cells may stain for both markers (arrow). M,N: Fate mapping of secondary mesenchyme cells derived from the tip of the archenteron of the *L. variegatus* late gastrula. Cells were removed from the tip of rhodamine-labeled donor late gastrulae and inserted into the blastocoel of unlabeled host late gastrulae as described in the Experimental Procedures section. **M:** Late gastrula/early prism. A cluster of cells (large arrow) localizes near the tip of the archenteron (ar). Other cells are visible near the base of the archenteron (arrowheads) and in the ventrolateral region (small arrow). **N:** Prism stage embryo. A group of somewhat dispersed cells remain near the ectoderm that was near the animal pole of the embryo (small arrow). Several cells are visible near the right presumptive arm bud (arrowhead). Other cells remain near the base of the gut rudiment; some appear embedded in the ectoderm (large arrow). Scale bars = 25 μm.

SNAIL in mesenchymal cells in the blastocoel, and in the region corresponding to the anal sphincter, that is, at the connection between the ectoderm and the base of the enlarged, everted archenteron (Fig. 3C). Reduced staining is observed in the remnants of the ciliated band compared with normal embryos (arrowhead, Fig. 3D). In all tissues where staining was detected, there appeared to be little nuclear Lv-SNAIL protein in lithium-treated embryos. The increased number of cells expressing Lv-SNAIL in the gut rudiment suggests ingression of SNAIL-expressing cells may be retarded and/or a greater number of cells fated to express SNAIL may be present in the archenteron as a consequence of lithium treatment.

Embryos treated with 0.5 mM NiCl$_2$ show no Lv-SNAIL staining when processed together with control embryos at stages when the normal embryos express Lv-SNAIL (data not shown). At the pluteus stage, Lv-SNAIL protein is detectable, but its distribution is altered. Figure 3E,F shows that Lv-SNAIL is found in the radialized tissue that is known to correspond to the ciliated band in NiCl$_2$-treated embryos (Fig. 3E, arrow; Fig. 3F, arrowhead). This includes the small knob of material at the extreme animal pole of the embryo, which is lost after fusion of the archenteron with the ectoderm to make the mouth (Fig. 3E, arrow), and which is known to express a ciliated band marker (Hardin et al., 1990). In contrast to normal embryos, there is little if any detectable staining in the rest of the ectoderm, and no *Lv-snail* expression could be detected in the tip of the archenteron (data not shown).

DISCUSSION

Developmental Expression of Sea Urchin *Snail* Suggests a Conserved Role Among the Deuterostomes

Members of the *snail* family of proteins have been identified within the two major groupings of triploblastic eucoelomates, and the protostomes and deuterostomes (reviewed in Manzanares et al., 2001; Nieto, 2002; Morales and Nieto, 2004). Among the vertebrates, there appear to be multiple *snail* family members that have distinct roles in early development. Two
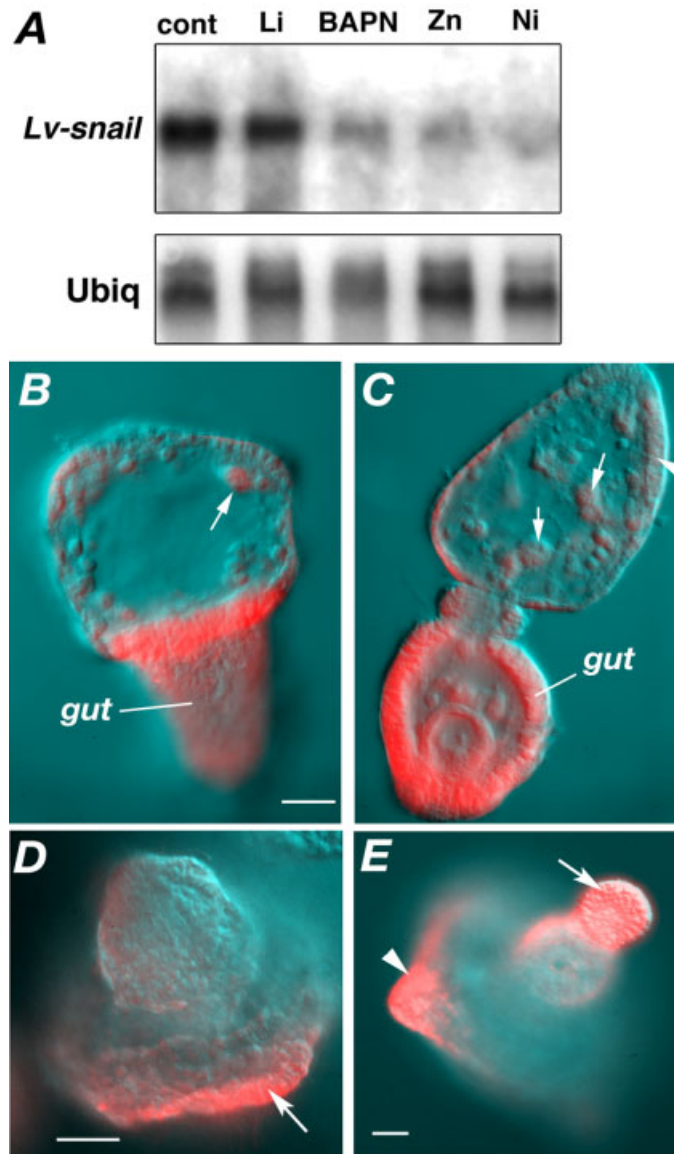
Fig. 3. Perturbation of *Lv-snail* expression in axially disrupted embryos. **A:** Northern blot of poly A+ RNA isolated from control and experimentally treated embryos at the late gastrula stage probed with radiolabeled *Lv-snail* cDNA (*Lv-snail*). The blot was subsequently reprobed using a polyubiquitin cDNA probe (Ubiq) as a loading control. For details of the chemical treatments, see the Experimental Procedures section. cont, control late gastrula; Li, embryos vegetalized by treating embryos with 30 mM Li$^+$; BAPN = embryos treated with 0.5 mM β-aminoproprionitrile; Zn = embryos treated with 10 mM Zn^{2+}; Ni = embryos radialized with 0.5 mM Ni^{2+}. B–E: *Lv-snail* protein distribution in LiCl- and NiCl$_2$-treated embryos. Whole-mount immunostaining for Lv-SNAIL was performed on *Lytechinus variegatus* embryos treated with 30 mM LiCl from the two-cell to mesenchyme blastula stages (B,C) or 0.5 mM NiCl$_2$ from the late one-cell to early gastrula stages (D,E). Lv-SNAIL staining is shown in red; brightfield is in blue. **B:** LiCl-treated late gastrula/prism. The gut stains brightly, as do isolated mesenchyme cells in the blastocoel (arrow). **C:** LiCl-treated pluteus equivalent. The gut shows intense fluorescence; some mesenchymal cells also exhibit staining (small arrows). What appears to be oral ectoderm (based on its thickened appearance) is indicated with an arrowhead. **D:** NiCl$_2$-treated prism stage embryo. Intense staining is visible in the ciliated band (arrow). No staining was detected in mesenchymal cells. **E:** NiCl$_2$-treated pluteus. Intense staining is visible in the ciliated band (arrowhead) and has appeared in the knob of oral ectodermal tissue at the animal pole (arrow). Scale bar = 25 μm.

snail family members have been identified in both teleosts (*snail1* and *snail2*; Hammerschmidt and Nusslein-Volhard, 1993; Thisse et al., 1993, 1995) and amphibians (*Xsna* and *slug*; Sargent and Bennett, 1990; Mayor et al., 1995), suggesting that multiple gene duplication events may

underlie the multiplicity of *snail* superfamily members among the vertebrates. In nonvertebrate chordates, a single *snail* gene appears to function in differentiation of the lateral neural plate and various types of mesoderm (Corbo et al., 1997; Langeland et al., 1998). Echinoderms are among the simplest extant deuterostomes (Wray et al., 1996), so by examining the functions of *snail* homologues in well studied representative echinoderms, it may be possible to infer basic functions for *snail* homologues that have been elaborated in the chordates. The expression of *snail* in mesenchymal cells in sea urchin embryos indicates that expression in such tissues is a basic feature of the *snail* family of proteins in all deuterostomes thus far examined.

Functions of *snail* During Secondary Mesenchyme Cell Differentiation

Lv-snail transcripts are expressed in a subpopulation of presumptive SMCs within the archenteron wall. SMC derivatives include pigment cells, which ingress at various times during gastrulation in different species (Gibson and Burke, 1985; Takata and Kominami, 2004), blastocoelar cells (Tamboline and Burke, 1992), esophageal muscle precursors (Ishimoda-Takagi et al., 1984; Wessel et al., 1990; Venuti et al., 1993), and cells expressing the 5-hydroxytryptamine receptor (Katow et al., 2004) At least some of the SMC derivatives that express *Lv-snail* mRNA and protein are likely to be pigment cells, because some *Lv-snail*–expressing cells are embedded in the ectoderm, and because in nickel-treated embryos, which largely lack pigment (Hardin et al., 1990; Takata and Kominami, 2004), *Lv-snail* is down-regulated. The small number of SMCs that express *Lv-snail* compared with other more general markers for SMCs, such as *Frizzled5*/8 (Croce et al., 2006) and most SMC markers identified using macroarray approaches (Calestani et al., 2003) suggests that *Lv-snail* is only expressed in a subset of SMCs. Based on our situ hybridization results, *Lv-snail* is detectable mainly in SMCs that ingress late in gastrulation, a population that has been suggested to be a reserve population of cells capable of "converting" to the skeletogenic fate (Ettensohn and Ruffins, 1993). *Lv-snail* is also expressed in two small, bilateral clusters of mesenchymal cells at the prism stage. These may be "plug" skeletogenic mesenchyme cells, which eventually reside at the tips of the growing arms (Ettensohn and Malinda, 1993), or they may be SMC derivatives. Of interest, small bilateral clusters of cells express the myogenic factor SUM-1 (Beach et al., 1999). The embryological function of these clusters of cells will require further studies.

Position of *snail* in the Endomesodermal and Left–Right Gene Regulatory Networks

Endodermal and mesodermal differentiation in the sea urchin embryo has been described in terms of an endomesodermal gene regulatory network (GRN; Davidson et al., 2002). How *snail* fits into the SMC-specific subset of the endomesodermal GRN is unclear. Expression of *snail*, like other SMC-specific genes, presumably requires a Notch-dependent signal produced by micromeres, the skeletogenic founder cells in the 16-cell embryo, which is transmitted to neighboring macromeres (Sherwood and McClay, 1999; Sweet et al., 1999, 2002; McClay et al., 2000; Peterson and McClay, 2005). In addition, *snail* expression is perturbed by nickel treatment, a known disruptor of oral–aboral axis specification (Hardin et al., 1990), including Nodal signaling (Duboc et al., 2004; Flowers et al., 2004).

In addition to its role in oral–aboral axis specification, Nodal signaling is part of a conserved "left–right cassette" in the sea urchin embryo, along with *Lefty* and *pitx2*, which, in distinction to vertebrates, are expressed on the right side of the larva (Duboc et al., 2005). Although the position of the axes relative to the expression of these conserved signaling molecules appears reversed relative to other deuterostomes, these results suggest a fundamental conservation of left–right specification machinery in echinoderms. One intriguing aspect of the spatial pattern of expression of *snail* is its transient, asymmetric distribu-tion in the left side of the tip of the archenteron, a feature it shares in common with *sox9* (Duboc et al., 2005). Such an asymmetric expression pattern is consistent with the expression of *snail* family members in amniotes, which are downstream components in the process of left–right axis specification (Isaac et al., 1997). In the sea urchin, right-sided expression of Nodal appears to regulate left-sided expression of *sox9*; one simple prediction is that left-sided expression of *snail* is similarly regulated. However, the functional role of *snail* as part of such a left–right cassette in sea urchin larvae remains to be determined.

Snail also likely plays a role in the EMT in mesenchyme cells in the sea urchin embryo. In both PMCs and SMCs, loss of epithelial markers, including the cadherin complex (Miller and McClay, 1997) and the surface marker Epith-1 (Kanoh et al., 2001), occurs immediately before EMT. Although these observations suggest that SNAIL may act as a repressor of cadherin expression, tissue-specific or temporal loss of function experiments would be required to definitively show that SNAIL performs a similar function in sea urchin mesenchyme as well.

Similarities Between Sea Urchin and Vertebrate Mesenchyme

The utilization of *snail* family members reveals surprising similarities between their expression in echinoderms and chordates. In the chordates that have been examined, *snail* family members appear to be expressed initially in presumptive axial mesoderm or mesendoderm (Nieto et al., 1992; Smith et al., 1992; Hammerschmidt and Nusslein-Volhard, 1993; Thisse et al., 1993, 1995), and later in mesenchymal tissue that will generate the neural crest (Nieto et al., 1994; Mayor et al., 1995; Isaac et al., 1997). In sea urchins, *snail* is expressed in a subpopulation of secondary mesenchyme cells. Based on several criteria, sea urchin SMCs function in a manner analogous to the neural crest of vertebrates: (1) they are a pluripotent or heterogeneous population of cells that form many different structures, including mesodermal and pigment de-

rivatives; (2) they arise at the edge of an epithelial invagination; and (3) they constitute a population of cells that migrates a large distance within the embryo before producing differentiated cell types. It is intriguing that at least some of the cells used in similar ways in the two phyla both express *snail* and further emphasizes the importance of *snail* family members in mesenchymal differentiation among the deuterostomes.

EXPERIMENTAL PROCEDURES

Cloning, Library Screening, and Sequence Analysis

The 835-bp *Bgl*1–*Eco*R1 fragment of the *Drosophila snail* cDNA was used to probe a *L. variegatus* cDNA library (courtesy of Dr. D. McClay, Duke University) essentially as described (Sambrook et al., 1989). A single positive clone was recovered and subcloned into pBluescriptIIKS$^+$/SK$^+$. The nucleotide sequence of both strands of CI107 and CI640 was determined using the dideoxy chain-termination method (Sanger et al., 1977). Sequence analysis was performed using the GeneWorks software package (IntelliGenetics, Inc., Mountain View, CA). The nucleotide sequence data originally appeared in GenBank under the accession number AF013243; a subsequent, corrected coding region sequence appears under accession number DQ665364 (S. Wu and D. McClay, personal communication).

3′ RACE

Polymerase chain reaction (PCR) primers SnaPVU2 (CAA GAA GTA CAG CTG C) and T$_{60}$ (CAT GTC GTC CAG GCC GCT CTG GGA CAA AAT ATG AAT TCT T$_{18}$) were used to PCR amplify the 3′ end of the *Lv-snail* cDNA using the *L. variegatus* late gastrula cDNA library as a template. SnaPVU2 is 195 bp from the 3′ end of CI107. A total of 3 ml of cDNA library, 50 pmol of primer SnaPVU2, and primer T$_{60}$ were mixed and denatured for 5 min at 90°C. PCR reaction conditions were (20 mM Tris-HCl pH 8.2, 10 mM KCl, 6 mM (NH$_4$)$_2$SO$_4$, 1.5 mM MgCl$_2$, 0.1% Triton X-100, 1 mM dNTP and 2.5 units of PFU, Strat-

agene) with a 100-ml reaction volume. The PCR reaction profile was 35× (95°C 1 min, 45°C 2 min, 72°C 2 min) with the last cycle having an extra 8 min 72°C. DNA fragments generated by PCR were phenol extracted, restricted with *Pvu*II and *Eco*RI, gel purified, and ligated into pBluescriptI-IKS$^+$. The positive clone CI640 overlapped the 3′-most 195 bp of CI107 and extended 264 bp further 3′.

Northern Blotting

L. variegatus embryos were grown to various developmental stages or under different chemical growth conditions and polyA+ mRNA was recovered using the Pharmacia Quickprep mRNA Purification Kit. Northern blotting was performed according to Sambrook et al. (1989). For each RNA sample, 7 μg were fractionated through an agarose/formaldehyde gel and blotted onto a nitrocellulose membrane (Schleicher and Schuell BA85). The filter was prehybridized at 42°C in 0.2 ml/cm^2 of 25 mM KPO$_4$ pH 7.4, 5× standard saline citrate (SSC), 5× Denhardt's solution, 5 mg/ml single-stranded salmon sperm DNA, and 50% formamide. Hybridization was carried out overnight at 42°C, and was performed as above with the addition of 10% dextran sulfate and denatured CI107 probe labeled with [^{32}P]CTP whose specific activity was typically 0.5 to 2 × 10^9 cpm/mg (Promega Random Priming Kit), to a final probe concentration of 1 × 10^6 cpm/ml. The blot was then washed twice at room temperature (2× SSC and 0.1% sodium dodecyl sulfate [SDS], 15 min each), twice at 65°C (2× SSC and 0.1% SDS), and twice at 65°C (0.2× SSC and 0.1% SDS). A *Pst1* fragment of the *Lytechinus pictus* polyubiquitin cDNA (Gong et al., 1991) was used as a control probe as described in Hardin et al. (1990).

Sea Urchin Growth and Chemical Treatments

L. variegatus embryos were reared using stirred cultures as described in Hardin and Cheng (1986). Treatment with 0.1 mM BAPN (β-aminoproprionitrile) was performed as described in Butler et al. (1987), treatment with 30 mM LiCl was performed as de-

scribed in Hardin and Cheng (1986), treatment with 0.5 mM NiCl$_2$ was performed as in Hardin et al. (1990), and treatment with 10 mM ZnSO$_4$ was performed as described in Nemer (1986).

RNase Protection

Total RNA isolation from *L. variegatus* embryos was performed essentially as described by Sambrook et al. (1989) with guanidinium isothiocyanate buffer and pelleting through cesium chloride. A *Lv-snail* specific probe that spans 317 nucleotides (the 877- to 1,195-bp *Pst*I fragment from CI107 cloned into pBluescriptIIKS$^+$) was synthesized with T3 RNA polymerase after linearization with *Bam*HI. RNase protection experiments were performed according to the RPA kit protocol (Ambion, Inc., Austin, TX).

In Situ Hybridization

In situ hybridization to whole-mount embryos was carried out as described by Harkey et al. (1992) with modifications for RNA probes as described by Ransick et al. (1993). Digoxigenin-labeled RNA was synthesized according to the Boehringer–Mannheim protocol with T7 RNA polymerase from clones of CI107 in opposite orientations in pBluescriptIIKS+, or from SUM-1 cDNA in pBluescriptSK linearized with either Asp718 or *Not*I to give sense or antisense orientations. Hybridized embryos were mounted for viewing in aqueous medium and photographed using Kodak Ektachrome Plus film using a Nikon Diaphot microscope and a Nikon 35 mm SLR camera.

Generation of Antisera

Glutathione-*S*-transferase fusion protein was produced using the last two thirds of the *Lv-snail* coding region by digestion with *Cla*I and *Eco*RI, filling in the resulting 5′ overhangs with T4 DNA polymerase, followed by blunt-end ligation into the pGEX -2T expression vector (Pharmacia) previously digested with *Sma1*. The reading frame of the resulting clones was verified by sequencing through the junction between vector and insert. Recombinant

protein production was induced in *Escherichia coli* transformed with the resulting clone, using standard protocols (Sambrook et al., 1989). Expressed protein was affinity purified from sonicated bacteria by means of glutathione–agarose beads; the resulting 22-kDa fusion protein was purified by means of polyacrylamide gel electrophoresis and electroelution. Rabbit polyclonal antisera were raised by immunizing rabbits with recombinant *Lv-snail* protein fragments and affinity purified against the 22-kDa fusion protein according to standard protocols (Harlow and Lane, 1988). Western blotting was performed using nitrocellulose membranes (Schleicher & Schuell, Keene, NH) according to standard protocols (Coffman and Mc-Clay, 1990) using a Genie blotting apparatus (Idea Scientific, Minneapolis, MN) and a Bio-Rad electrophoresis power supply (Bio-Rad, Hercules, CA). Western blots indicated that affinity purified antibody recognized a single major band of approximately 37 kDa. In addition to polyclonal antisera generated against *Lv-snail*, two different antibodies against *Drosophila snail* were also used: (1) a monoclonal antibody produced by Dr. A. Alberga, and (2) a polyclonal antibody, both kindly provided by Dr. Sean Carroll, Univ. of Wisconsin–Madison.

Immunostaining

Embryos were stained as wholemounts according to described procedures (Hardin et al., 1990). Embryos were fixed in artificial sea water (ASW) with 3.7% formaldehyde for 2 hr on ice, rinsed 3–4 times in ASW, and then acetone extracted for 6 min at −20°C. After acetone extraction, an equal volume of ice-cold ASW was added, followed by several rinses in ASW until all trace of organic odor was gone. Embryos were incubated in primary antibody diluted 1:20 in phosphate buffered saline (PBS) for a minimum of 2 hr at room temperature, rinsed 3 times in PBS/Tween-20, incubated in secondary antibody diluted 1:20 in PBS (either lissamine rhodamine-conjugated goat anti-rabbit or fluorescein-conjugated goat anti-mouse, Sigma) for 1 hr at room temperature, and finally washed 2 times in PBS–

Tween-20. In some cases, primary antibody was applied overnight at 4°C.

Mesenchyme Cell Transplants

Rhodamine labeled embryos were prepared as described (Armstrong et al., 1993), and cells were aspirated from the tip of the archenteron of labeled midgastrulae using mesenchymal transplant techniques adapted from Ettensohn and McClay (1986), as described in Armstrong et al. (1993). Recipient embryos were allowed to develop to the prism stage, and the pattern of fluorescence was analyzed using wide-field epifluorescence microscopy.

Microscopy

Stained or chimeric embryos were mounted on footed coverslips and viewed using epifluorescence optics on a Nikon Optiphot II equipped with a silicon-intensified target camera (Hamamatsu); images were acquired using a Scion LG-3 frame grabber and a Macintosh computer, using the public domain software NIH Image, available at http://rsb.info.nih.gov/nih-image. Overlays of fluorescent images were performed using Adobe Photoshop software.

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