Supplemental Methods for

Working with the Slipper Snail, *Crepidula*

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Supplemental Methods A1: *Collection and Transportation of Live Animals*

Adult *Crepidula fornicata* and many other species are easily collected in the intertidal and shallow subtidal. The most common species that can be readily collected in the USA are listed in supplemental **Table ST1**. *Crepidula fornicata,* as well as *C. plana,* can be obtained from the Marine Resources Department of the Marine Biological Laboratory in Woods Hole, MA, USA. Gulf Specimen Marine Lab can also provide some species (Panacea, Florida). Animals can best be shipped wrapped in moist paper towels or wood fibers, but can also be shipped in sealed plastic bags containing seawater if these are topped-off with pure oxygen. The use of moist seaweed or other live packing materials should be avoided since these respire in the dark and compete with the animals for oxygen. Such live material may also begin to decompose rapidly during transit. Animals should not be removed from their natural substrate prior to shipping as this

stresses them, and larger animals have trouble re-attaching. Insulated styrofoam containers with small icepacks are recommended for shipping. Animals should not be exposed to temperatures much above 20 °C during shipping, but care must be taken to insulate the animals from close contact with ice packs. Once received, the animals should be slowly acclimated to their new environment by floating the bags in marine aquaria and by slowly adding or exchanging the seawater.

Supplemental Table ST1: Species of Calyptraeidae that may be readily collected in North America.

Species	**Range**	**Mode of Development**
Crepidula atrasolea	Florida to North Carolina	Direct
Crepidula fornicata	New England, Europe	Planktotrophic
Crepidula depressa	Florida and Gulf of Mexico	Planktotrophic
Crepidula convexa	New England	Direct
Crepidula adunca	California -Washington	Direct
Crepidula plana	New England	Planktotrophic
Crepidula ustulatulina	Florida	Lecithotrophic larvae
Crepidula norrisiarum	California	Direct
Crepidula naticarum	California	Planktotrophic
Crepidula onyx	California and Asia	Planktotrophic
Bostrycapulus aculeatus	Florida	Direct
Crepipatella lingulata	West coast of USA	Planktotrophic

Supplemental Methods B1: *Maintaining Live adult* Crepidula

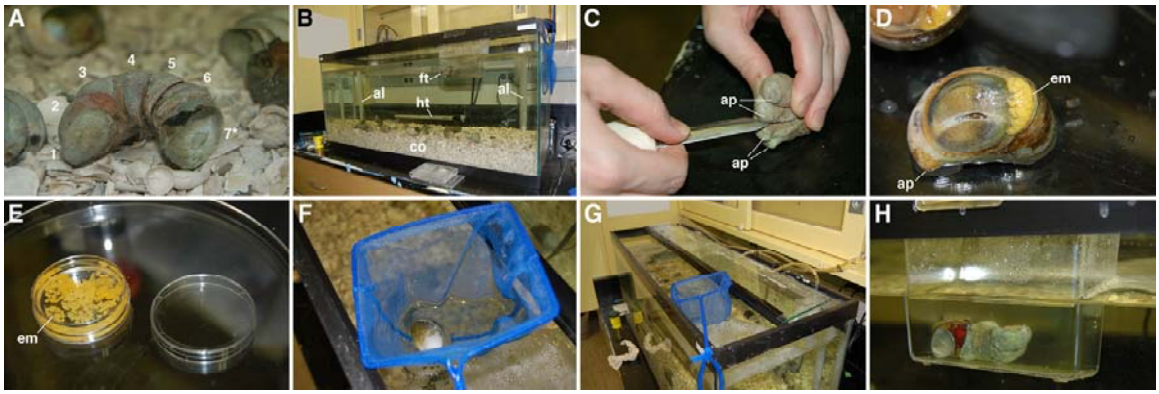
Adult *Crepidula* can be maintained in any laboratory with flowing seawater. They grow well in sea tables with running seawater and may not need any additional food, depending on the filters employed with the system. Individual *Crepidula* or stacks

of animals can also be maintained in small cups or beakers if the water is changed 2 or 3 times a week and food is added to the cultures. We (R.C.) routinely keep 1 or 2 small animals in 300 ml plastic cups, but larger containers are necessary for stacks of *C. fornicata* or large females. Animals can be raised for the entire life cycle on *Isochrysis galbana*, but the optimal amount depends on the species and size of the container. We find that more frequent feedings of less concentrated algal suspensions work the best. Outside the natural range of *C. fornicata* or any other calyptraeids, extreme caution must be used to avoid accidental introduction of these invasive species.

Crepidula fornicata can also be readily maintained in re-circulating inland glass aquaria containing artificial sea water (Instant Ocean, Spectrum Products, Canada) and fed using various types of phytoplankton. In our inland lab (J.H.), we routinely maintain *C. fornicata* animals in 30-gallon marine aquaria (**Supplemental Figure 1A-B**) equipped with undergravel biological filters, as described by Spotte (1970). A high quality, powerful piston air pump ensures good aeration and effective circulation of water through the crushed seashell and dolomite gravel substrate. Instant Ocean sea salts (Spectrum Products, Canada) are prepared with deionized water, and the salinity is adjusted to a specific gravity of 1.023 gm/cm^3. A submersible heater maintains the tank temperature at 25 °C. A hinged glass lid helps prevent the loss of water via spray or evaporation. We inoculate the gravel using commercial marine nitrogen fixing bacteria (Instant Ocean BioSpira, Spectrum Products) following the manufacturer's instructions. Small groups of adult snails have been kept in this way for up to two months without feeding, and over six months with weekly feedings of live marine phytoplankton. We supply a diet of commercial live marine phytoplankton (DTs Live Marine Phytoplankton, Sycamore, IL, see Espinosa and Allam, 2006). We typically keep no more than 40-60 animals per 30-gallon tank and dead animals are removed immediately.

Effective filter feeding requires that the animals be attached to a solid substrate (shells, glass, or plastic). Animals that do not have solid substrates for firm attachment will not thrive. When undisturbed the animals raise their shells slightly to circulate water and food through their mantle cavity and gills. We feed the animals 1-3 times a week by moving each stack with a small aquarium net into a plastic fish-breeding tank hung within the main tank (**Supplemental Figure 1B, H**). 1.5 to 2ml of thoroughly re-

suspended DTs is mixed into the the small tank and the snails are allowed to filter feed for 15-20 minutes, which is typically enough time for them to completely clear the water. If the density of food is too high, the animals will clear the water, but will not consume the majority of the food. When this happens, rejected food-laden mucus will accumulate at the anterior margin of the shell.



Supplemental Figure 1. Laboratory culture of *Crepidula fornicata*. A. Stack of 6 live adult snails living in a 30 gallon recirculating marine aquarium. The bottom of the stack is attached to the empty dead shell of a seventh (7*) animal. B. 30 gallon marine aquarium equipped with an undergravel biological filter equipped with two airlifts (al). The substrate is covered with crushed coral, oyster shells, and other shell debris (co). A heater (ht) maintains the water temperature at 25 ºC. A feeding tank (ft) is also hanging in the aquarium. C. Separation of the snails using an oyster knife inserted between the posterior apices (ap) of adjacent snails. D. Close-up showing yellow egg mass (em) with over 20 egg capsules attached to the back of one shell. E. Petri dish containing egg masses collected from several females. F-G. Recently separated stacks of snails suspended in a fish net to allow them to firmly reattach before being released in the tank. H. A small fish "breeding tank" tank, which is used to feed the snails live phytoplankton.

The normal breeding period for *C. fornicata* in Woods Hole, MA is reported to be mid-June through mid-August (Costello and Henley, 1973), however; it is likely that animals begin spawning in May, and a few animals continue to lay eggs in August. This seasonal limitation is removed by the fact that one can obtain animals from the wild between November and May that will readily spawn within days to a few weeks of being placed in warm (25 °C) sea water. Animals can be kept in chilled sea water tanks at 12°C for up to a month, without feeding, and subsequently warmed to 25 °C when embryos are needed. It is imperative that these "off-season" animals be collected from the cold and shipped with ice packs to prevent them from spawning during transport.

Supplemental Methods C1: ***Obtaining Eggs and Embryos***

Egg capsules are attached to the solid substrates to which the animals are attached. As individual *C. fornicata* are tightly adhered in their stacks, they must be separated using an oyster knife. The edges of the shells may be quite sharp, so care must be exercised to avoid cutting one's hands. The adults are best pried apart at the point closest to the apex, where the superior shell overhangs the surface of the inferior shell within each stack. This avoids injury to the animals.

As slipper snails lack an operculum, it is important that animals remain firmly attached to a solid substrate so that they are protected and can effectively circulate water. It is best to maintain animals in their correct order and orientation within their stacks. Once an animal is lifted they should be replaced quickly. It is possible to go thorough an entire stack of animals very quickly, holding the loosened animals together in one hand while wielding the oyster knife in the other. After a stack is inspected, it should be cradled in a fine dip-net suspended in the seawater so that the animals can become firmly reattached to one another (**Supplemental Figure 1C-G**). This takes 10-20 minutes, and the animals may then be gently returned to the bottom of the tank. If the stacks fall apart the process is simply repeated. The more quickly the animals are replaced in their stacks the sooner they will reattach. In this fashion, stacks of animals can be re-inspected on a daily basis for egg laying. Egg masses can be removed using fine watchmaker's forceps by grasping the point of attachment and peeling this from the substrate. The egg capsules should be quickly transferred to a dish of filtered seawater. Care must be exercised not to smash the embryos or tear the egg capsules. Eggs, embryos, and larvae can be easily released from the egg capsules using watchmaker's forceps. Once removed, the eggs and embryos are rather sticky and will adhere to the charged surfaces of plastic and even glass dishes, slides or Pasteur pipettes. To prevent this from occurring these surfaces should be coated with gelatin (0.1% Knox unflavored gelatin dissolved in deionized water with 0.04% formaldehyde; see Zalokar and Sardet, 1984). Older ciliated embryos are not as sticky, and once the larvae begin to swim there is no need to use gelatin. However, the velar surfaces may remain somewhat sticky.

Supplemental Methods D1: ***Culture of Embryonic Material***

In the case of *C. fornicata*, fertilized eggs and embryos should be reared in 0.2μm filtered natural seawater (or artificial seawater) containing penicillin G (100units/ml) and streptomycin sulfate (200μg/ml). Some tropical species of *Crepidula* develop well with 83 units /ml penicillin and 89 μg/ml streptomycin, and we have found that higher concentrations result in a high proportion of embryos with abnormal development. The seawater and antibiotics should be replaced every few days, as needed. When rearing larger numbers of embryos in small volumes the sea water should be replaced daily. Capsules cannot be cleaned prior to removal of the embryos, so culture contaminants will be introduced at the time when embryos are transferred to individual culture dishes. Bacteria can also grow on the surface of the gelatin coated dishes and it is best to replace these as well. Occasionally, we have found that cultures develop fungal infections, consisting of small colonies of delicate fine white fungal strands that can also grow on the embryos. Unfortunately, there appears to be no good means to eliminate these fungal infestations without also harming the embryos.

The early embryos rely on internal yolk reserves and may be reared up to the stage of normal hatching (approx. one month depending on temperature) without feeding. The embryos possess a pair of "embryonic kidneys" or absorptive cells (Rivest, 1992), which have been shown to absorb ferretin but not iron dextran in several species of calyptraeids (Collin, 2000b; pers. obs.). It is not clear whether these structures are involved in early larval nutrition. The embryonic kidneys and head vesicle are lost prior to hatching in all calyptraeids. Following hatching the veliger larvae need to be reared on a diet of live marine phytoplankton, using the algae described in **Supplemental Methods B1,** above.

Supplemental Methods E1: ***Microinjection and Cell Labeling***

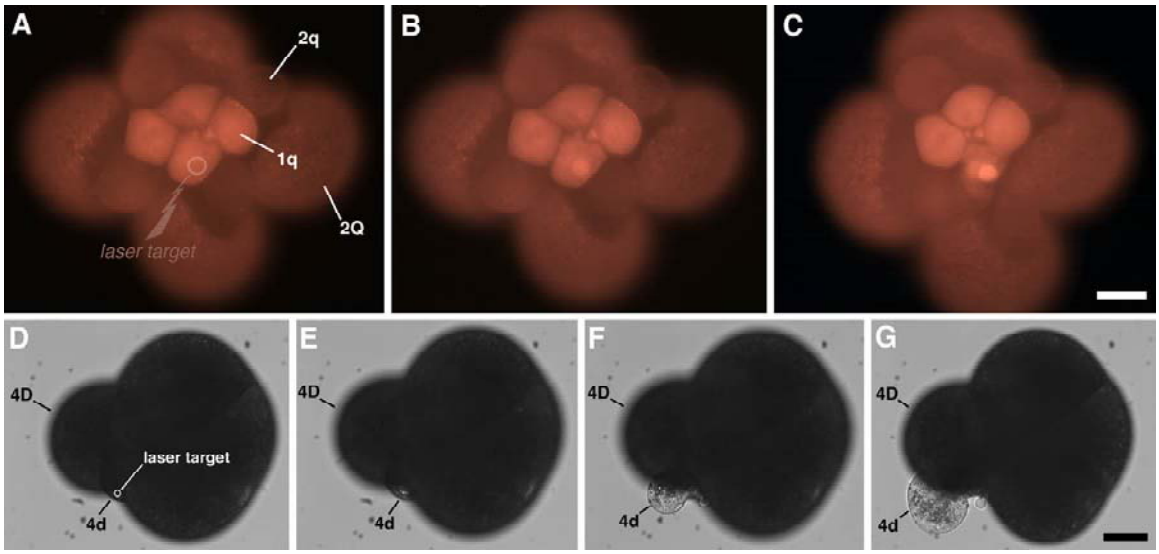
Microinjection of fertilized eggs and early embryonic cells is rather easy. Once the eggs or embryos are removed from the capsules, there are no extracellular investments to interfere with injection and the cells can be penetrated without lysing. Microinjection is accomplished via pressure injection following the protocol of Henry and Martindale (1994, 1998) and Henry *et al*. (2008), using a PLI100 injector (Harvard

Apparatus, Holliston, MA). Nitrogen gas supply pressure is set to 20-30 PSI and pulse durations range from 100 to 600ms depending on the needle and volume required. Glass needles are pulled on a Sutter P97 pipette puller and typically need to be opened by *gently* tapping the glass tips on the bottom of the dish. Borosilicate Glass capillaries with internal filaments, cat. no. TW100F-4 (World Precision Instruments) are used to prepare the needles. Solutions are back-loaded into the pulled micropipettes. The internal glass filament carries the solutions to the tip via capillary action.

Solutions of $DiIC_{18}$ or fluorescent dextrans can be injected for the purpose of lineage tracing, or morpholinos and synthetic RNAs for accomplishing expression assays (see **Supplemental Methods I1**, below). $DiIC_{18}$ is solubilized at a concentration of 5mg/ml in soybean oil (or other vegetable oil) for injection. The $DiIC_{18}$ is first disolved in 100% EtOH at 100mg/ml before being solubilized in the oil using mild heat and sonication. Flourescent dextrans (such as 10,000MW Fluoro Ruby dextran, D-1817, or Rhodamine Green Dextran; Molecular Probes, Eugene, OR) are made up in 0.2M KCl at a concentration of 50mg/ml. Prior to injection the dye solution is passed through a Spin-X microfuge filter (CoStar, Cambridge, MA) to remove any particulates that might clog the needle. A Narishige hydroaulic micromanipulator and a fluorescent dissection microscope may be used to accomplish the injections and the amount of dye injected represents approximately 1-5% of the cell's volume. The embryos are injected under FSW in gelatin coated dishes in which small grooves have been previously etched using the sharp, pointed edge of a broken glass Pasteur pipette.

Due to the transparency of the animal micromeres, which lie on top of the four larger yolky, opaque macromeres, the arrangement of these cells may be difficult to visualize. This can be overcome by labeling these cells using vital, fluorescent dyes, such as rhodamine isothyocyanate, RITC (Sigma, St Louis, MO, cat number, see **Supplemental Figure 2A-C**), following the protocol of Wray and McClay (1988). Initially, 1mg of RITC is dissolved in 20μl of DMSO (some heating or sonication may be required to solubilize the RITC). This is then mixed with 10ml of 0.22μm-filtered natural or artificial seawater (FSW) to make a stock solution. 4μl of this stock solution is diluted in 1ml of sea water to prepare the final working strength solution, which has a light pink color. The embryos are soaked in this solution for 10-30 minutes and then

washed in FSW. The pattern of micromeres may then be observed using fluorescence microscopy (see **Supplemental Figure 2A-C**). This procedure does not effect subsequent development. Alternatively, embryos can be labelled with the bright green dye, CFDA-SE (Invitrogen, see Wu and McClay, 2007); however, this dye has a tendency to be extremely bright, intensifying over time and thus obscuring details of individual cells. Treatments with this dye must be limited to very dilute solutions for very short durations.



Supplemental Figure 2. Techniques to fluorescently label blastomeres using RITC and laser ablation of specific blastomeres. A-C. Fluorescence micrographs showing the ablation of a single first quartet micromeres (1q) at the 12-cell stage. The embryo was pretreated with RITC to label the cells. The target zone is shown in A, prior to firing the IR laser. Notice red fluorescence of the RITC label intensifies immediately after the laser is fired (B) and the micromere subsequently undergoes lysis (C). 2q, second quartet micromere. 2Q, second quartet macromere. D-G. Light micrographs showing stages of lysis of the 4d mesentoblast at the 25-cell stage following IR laser ablation. Scale bar in C equals 40μm and applies to A-C. Scale bar in G equals 30μm and applies to D-G.

Supplemental Methods F1: ***Blastomere Isolation and Ablation***

While feasible, it is somewhat difficult to separate the blastomeres using glass needles alone in *C. fornicata* (Henry *et al*., 2006). However, it is simple to lyse early blastomeres by stabbing or flicking them with sharp needles. Blastomere isolation may be facilitated by treating the embryos in $Ca^{++}Mg^{++}$ free seawater (Cavanaugh, 1956); however, prolonged exposure to $Ca^{++}Mg^{++}$ free seawater causes abnormal development (Henry *et al*., 2006). The early cells are large enough to be removed freehand, using glass

or tungsten microneedles or via a micromanipulator and specific combinations of blastomeres can be isolated using these methods. This approach becomes more difficult at later stages when the cells have undergone multiple divisions. Another approach utilizes microinjection of excess quantities of fluorescent dextran or $DiIC_{18}$ (see **Supplemental Methods I1,** below). Smaller cells or the polar lobe may be removed by suction using a fine bore capillary attached to a mouth pipette or using a laser (**Supplemental Figure 2D-G;** lasers from MicroPoint system, Photonic Instruments, Inc. or the XYClone laser produced by Hamilton-Thorne, Inc., Beverly, MA).

Supplemental Methods G1: ***Anesthesia and Fixation of Embryonic Material.***

The veliger larvae are quite active and will contract their heads and velar lobes into the shells when disturbed. To prevent this, the larvae can be anesthetized using a solution of chloral hydrate (5.94 mg/ml of seawater for 5-10 mins), $MgCl_2$ isotonic with seawater, or by adding Na azide (0.1%) to seawater. These treatments are reversible for up to one hour.

Embryos and larvae can be fixed in 3.7% formaldehyde in filtered seawater for one hour at room temperature, washed three times in phosphate buffered saline (PBS) and stored in either PBS with 0.01% Na Azide at 4°C or in 100% Methanol at –80°C. For scanning electron microscopy (SEM) larvae can be fixed directly in OsO_4 in buffer, or seawater, or post-fixed after initial formalin-fixation (e.g., Collin, 2000a,b). For transmission electron microscopy standard protocols call for fixation in phosphate-buffered (pH 7.6) 2.5% glutaraldehyde and post-fixation in 2% osmium tetroxide buffered in 1.25% sodium bicarbonate (pH 7.2). This protocol also works well for SEM.

Supplemental Methods H1: ***In situ Hybridization***

Following fixation (see **Supplemental Methods G1**), in situ hybridization reactions may be carried out in 24-well cell culture dishes in volumes of 500ul following the protocol of Finnerty *et al*., (2003). Template DNA for various riboprobes can be isolated using RACE (Rapid Amplification of cDNA Ends) and the products ligated into pGEM-T easy plasmid vector (Promega). Digoxygenin-labeled sense and anti-sense probes may be generated using T7 and SP6 RNA polymerase (Invitrogen), where

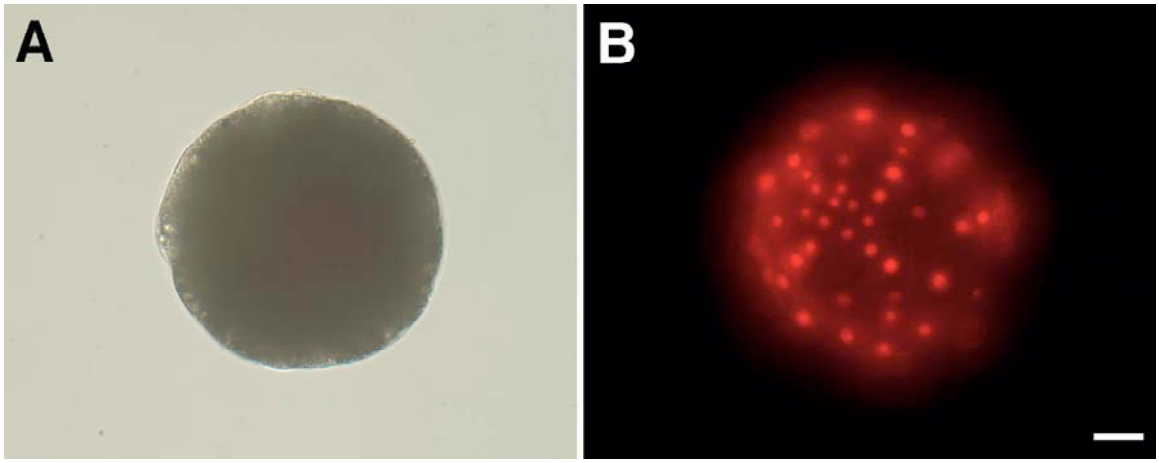
appropriate, and Dig labeling mix (Roche). Probe lengths ranging from 500-1200bp are used at a concentration of 1-2ng/ul. Prehybridization can be carried out for 2-4 hours at 65ºC and hybridizations carried out overnight at 65ºC. To visualize the probe, BM purple (Roche) is recommended and incubated on a rocker at room temperature for 4-6 hours or overnight at 4ºC. Embryos may be mounted in 80% glycerol diluted with 1X PBS.

Supplemental Methods I1: ***Functional Assays***

Morpholinos, chemisynthetic oligonucleotides designed to target either the start site or internal splice sites within the messenger RNAs to prevent translation or disrupt proper splicing of the message, respectively, can be injected into fertilized eggs or specific lineages by targeting individual blastomeres. The effectiveness of the morpholino can be determined with real time PCR following injections. Morpholinos can be tagged with fluorophores to verify injection, and serve as a lineage tracer. To knockdown the translation of specific messages, morpholinos are injected at a concentration of 1mM or less, as recommended by the manufacturer, and 1-5% of the cell's volume (approximately 20-100 picoliters) can be injected. We have found that fluorescent lissamine tagged morpholinos tend to accumulate within the nuclei of embryonic cells in *C. fornicata*, allowing visualization of the pattern of cells at subsequent stages (**Supplemental Figure 3A-B**). Morpholinos directed against *β-catenin* mRNA have been shown to knockdown translation of the message (J. Henry, J. Perry and M. Q. Martindale, in preparation). The presence of control, non-sense tagged morpholinos does not have an adverse effect on development in *C. fornicata*. (J. Henry, J. Perry and M. Q. Martindale, in preparation).

Synthetic RNAs can be injected for gain of function assays, and to localize the distribution of certain tagged proteins. For gain of function assays and *in vivo* expression analyses, RNAs encoding full-length proteins can be injected following the approach of Henry *et al*. (2008). To prepare synthetic RNAs, linearized template DNA from full length clones inserted into pCS2+ vector is made via PCR reactions generate, using SP6/T3 primer pairs. PCR product is verified on an agarose gel, and the products are used in an mMessage mMachine RNA transcription reaction (Ambion, Austin, TX) using either SP6 RNA polymerase or T3 RNA polymerase to generate capped mRNA. The

capped RNA is purified using the Mega Clear kit (Ambion) and RNA pellets eluted to a concentration of approximately 2.0 μg/μl for microinjections. A final concentration of 40% Glycerol and 0.2μg/μl Rhodamine Dextran are added to the RNAs to facilitate microinjection and to trace the injected cells.



Supplemental Figure 3. Corresponding light (A) and fluorescence (B) micrographs showing distribution of red fluorescent, lissamine-tagged, beta-catenin morpholinos, which were injected into a zygote of *Crepidula fornicata*. The animal pole shows the characteristic arrangement of the micromeres, the so-called "molluscan cross." The embryo is shown at a late cleavage stage when gastrulation is being initiated. Note that some of the injected morpholinos tend to accumulate within the nuclei permitting one to discern the arrangement of these cells. Scale bar equals 30μm.

Online, Supplemental Methods J1: ***Protein localization***

Protein localization can be determined readily in whole-mounts using various antibodies (e.g., anti-dpERK1/2, MAPK, anti-seratonin, anti-FMRFamide, etc.; see Hejnol *et al*., 2007; Henry and Perry, 2008). Embryos can be fixed, as described in **Supplemental Methods G1**. The duration of fixation and storage conditions may vary depending on the antigen being examined. To examine the activation of ERK1/2, MAPK, specimens should be stored in 100% MeOH at –80 °C and examined using anti-dpERK1/2, MAPK (diluted 1:200 in 1X phosphate buffered saline (PBS), Sigma, St Louis; Henry and Perry, 2008). Detection of other antigens may require storage only in PBS with 0.1% NaAzide at 4 °C. Primary antibodies are used at different concentrations as determined empirically and incubated overnight at 4ºC. Likewise, secondary antibodies may be used at different concentrations and incubated for 2 hours at room temperature or overnight at 4°C. Finally, embryos should be washed thoroughly in 1X

PBS and mounted in 80% glycerol diluted in PBS. Hoechst 33342, diluted 1:10,000 in the mounting media, can be used to counter-stain the nuclei (Henry *et al*., 2006). Likewise, muscle cells containing filamentous actin may be labeled overnight at 4°C using Alexa 488 labeled phalloidin (2.5 μg/100μl PBS with 0.2% Tween 20, Hejnol *et al*., 2007). Proteins can also be detected using Western Blot following the approaches detailed in Henry *et al*. (2008).

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