Embryonic regeneration by relocalization of the Spemann organizer during twinning in *Xenopus*

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Contributed by Edward M. De Robertis, April 3, 2018 (sent for review February 16, 2018; reviewed by Makoto Asashima, Atsushi Suzuki, and Naoto Ueno)

The formation of identical twins from a single egg has fascinated developmental biologists for a very long time. Previous work had shown that *Xenopus* blastulae bisected along the dorsal–ventral (D-V) midline (i.e., the sagittal plane) could generate twins but at very low frequencies. Here, we have improved this method by using an eyelash knife and changing saline solutions, reaching frequencies of twinning of 50% or more. This allowed mechanistic analysis of the twinning process. We unexpectedly observed that the epidermis of the resulting twins was asymmetrically pigmented at the tailbud stage of regenerating tadpoles. This pigment was entirely of maternal (oocyte) origin. Bisecting the embryo generated a large wound, which closed from all directions within 60 minutes, bringing cells normally fated to become Spemann organizer in direct contact with predicted ventral-midline cells. Lineage-tracing analyses at the four-cell stage showed that in regenerating embryos midline tissues originated from the dorsal half, while the epidermis was entirely of ventral origin. Labeling of D-V segments at the 16-cell stage showed that the more pigmented epidermis originated from the ventral-most cells, while the less-pigmented epidermis arose from the adjoining ventral segment. This suggested a displacement of the organizer by 90°. Studies with the marker Chordin and phospho-Smad1/5/8 showed that in half embryos a new D-V gradient is intercalated at the site of the missing half. The displacement of self-organizing morphogen gradients uncovered here may help us understand not only twin formation in amphibians, but also rare cases of polyembryony.

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nimal embryos have a remarkable capacity of self-organization that has fascinated biologists since the beginning of experimental embryology. In 1891, Hans Driesch separated the first two blastomeres of a sea urchin embryo and found that each one was able to self-organize and give rise to complete, although smaller, embryos (1). Later, it was found that each of the first four blastomeres of a sea urchin could form normal larvae (2). In 1895, Thomas Hunt Morgan removed one of the two blastomeres of a frog embryo and generated a complete embryo from half an egg (3). In amphibians it was possible, by constricting with a hair loop at the two-cell stage, to obtain two embryos of half size but normal proportions from the same egg (4, 5). Duplicated embryos were also obtained by partially sectioning insect eggs (6), indicating that the intriguing self-organizing ability of developmental systems is widespread. These experiments showed that when an embryo is damaged, it tends to self-regulate toward formation of the whole.

Spemann showed that at the start of gastrulation the newt embryo could be subdivided by constriction into a dorsal and ventral fragment, generating a dorsal half embryo scaled to normal proportions and a ventral half embryo consisting of ventral tissues devoid of an axis, while sagittal constriction did not form twins (7). *Xenopus* is the model organism for these studies in modern times. It has many advantages, such as a large number of eggs produced throughout the year. A key experimental advantage is a cortical rotation in the fertilized egg (8) that results in the displacement of the maternal oocyte pigment, forming a dorsal crescent, which reliably marks the dorsal (back) side of the embryo in regularly cleaving embryos (9). The opposite, darker side of the embryo gives rise to the ventral (belly) side. The displacement of egg cytoplasmic determinants along microtubules toward the dorsal side triggers an early Wnt signal (10), which is responsible for localizing the subsequent formation of the Spemann organizer signaling center in the marginal zone at the gastrula stage. The Spemann organizer is a tissue that secretes a mixture of growth factor antagonists, such as Chordin, Noggin, Follistatin, Cerberus, Frzb1, and Dickkopf, which are able to induce embryonic cell differentiation (11). Diffusion of these antagonists establishes a dorsal–ventral (D-V) gradient of bone morphogenetic protein (BMP) activity, and a ventral center secreting other molecules, such as BMP4/7, Sizzled, and Tolloid is formed at the side of maximal BMP signaling (12, 13).

In *Xenopus*, dorsal- and ventral-half embryos can be generated by simply cutting the embryo with forceps or a surgical blade (14). The ventral half lacks an organizer and develops as a mass of ventral tissues with very high BMP signaling (12). Embryos bisected sagittally at the gastrula stage fail to form twins. In 2006, we reported that identical twins could self-organize after sagittal bisection of *Xenopus* embryos (using forceps or a metal knife) at the 4,000-cell blastula stage, before Spemann’s organizer is formed (15). Although remarkable, this regeneration occurred only at very low frequency (about 1%), precluding meaningful mechanistic analyses. The mystery of how the embryo achieves this ultimate regeneration of an entire missing body half has remained an enduring puzzle.

Significance

Many animals, including humans, can generate identical twins from a single egg. We perfected a method by which a frog (*Xenopus*) egg cut in half along the dorsal–ventral (back to belly) axis at the 4,000-cell stage produced twins at high frequency. The large wound generated by bissection healed within an hour, juxtaposing cells that would normally form the most dorsal and ventral tissues in the intact embryo. Tracing the fate of microinjected cells showed that the dorsal Spemann organizer was formed 90° away from its original location in bisected embryos. A new gradient of dorsal–ventral signaling was generated by this displacement, explaining the regeneration of the missing half. The experiments help explain twinning in a classic model system.

Author contributions: Y.M. and E.M.D.R. designed research, performed research, analyzed data, and wrote the paper.

Reviews: M.A., National Institute of Advanced Industrial Science and Technology; A.S., Hiroshima University; and N.U., National Institute for Basic Biology.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1802749115/-/DCSupplemental.
In the present study, we improved the bisection technique of blastula embryos, resulting in increased frequency of twinning after sagittal bisection, obtaining 50% or more twinning. This made the regeneration process amenable to analysis. We unexpectedly noted that in properly regenerating twins the maternal pigment of the egg was concentrated on one side of the tailpole epidermis in preference to the other. Normally, the epidermis on the left and right sides of the embryo have equal pigmentation, which is entirely of maternal (ege) origin in *Xenopus*. Investigation of this asymmetry led to the finding that, following healing of the bisected blastula, the organizer is formed at a distance from the place where it would normally develop. This displacement in the position of the organizer explains how the missing half of the embryo is regenerated.

**Results**

We noted a higher frequency of identical twins by bisecting *Xenopus laevis* stage 8 blastulae (16) along the sagittal plane (dividing the dorsal crescent) when an eyelash (from Y.M.) was used to cut the embryo instead of using metal instruments (Fig. 1A and SI Appendix, Movie S1). The large vegetal cells could still be separated using forceps, but the initial use of the eyelash knife greatly improved regeneration. Other improvements were provided by performing the surgery in 0.3× modified Barth solution (MBS) (17), allowing the half embryos to heal for 60 min, and transferring to 0.1× MBS for further culture. Using this improved technique up to 50% of twins, complete with developmen of two eyes (and more rarely of one eye), was observed.

After bisection, cells rapidly migrate from all directions to close the large gap left by the bisection wound, which becomes smaller and smaller until 60 min later only a small healing point remains (Fig. 1B–E and SI Appendix, Movie S2). This healing process results in half embryos in which the most dorsal region becomes directly juxtaposed to the cells that would become the most-ventral region during the course of normal development.

**Pigment Asymmetry in the Epidermis of *Xenopus* Twins.** We were surprised to find that a striking asymmetry in epidermal pigmentation was observed in most twins that healed properly (Fig. 2A). The pigmentation during the first 2 d of development in *Xenopus* embryos is entirely derived from the cortical pigment granules provided by the oocyte, and the pigment is uniformly distributed on both sides of the wild-type tailbud tadpole (Fig. 2B). When twins are formed, a darker pigmentation was found at either the left or right side of the half embryos (Fig. 2C and D; note that both half embryos derived from same egg developed complete axes with two eyes). This unexpected observation provided the starting point of the following investigations on the developmental mechanics by which the missing half of the embryo is regenerated.

The Entire Epidermis Is Derived from Ventral Lineages. We investigated the regeneration process by lineage tracing of dorsal or ventral halves of the embryos. This was achieved by microinjection of a mixture of GFP or RFP mRNAs consisting of soluble, membrane-targeted, and histone H2B fluorescent protein fusions into the two ventral or dorsal blastomeres of regularly cleaving embryos (9). Embryos were cultured until early tailbud (stage 22), embedded in low-melting agarose, and serially sectioned with a Vibratome (Fig. 3A). We naïvely presumed that the less-pigmented epidermis would be dorsal in origin, but found otherwise.

In the whole embryo, the dorsal blastomeres give rise to the notochord, dorsal endoderm, and most of the CNS in the trunk region (Fig. 3A–F). Part of the somite lying next to the notochord was also found to be of dorsal origin, and we designated this region the medial somite. The rest of somite was of ventral origin. We were surprised by this but should not have been, because in the chicken embryo it has long been known that the medial somite derives from regions close to Hensen’s node and the lateral somite from more posterior (i.e., ventral) regions in the primitive streak (18, 19). Lineage studies at neurula stages (Fig. 4) revealed that in *Xenopus* the medial somite is formed as a result of convergence and extension movements in the embryo by which cells of lateral origin move toward the midline above and below the medial somite. In fact, the entire somite was found to have an exquisite fate map according to the D-V origin of its cells during cleavage. At the 16-cell stage individual D-V segments can be
In conclusion, these experiments in which four-cell embryos were lineage-traced in the dorsal and ventral halves suggested a displacement of the Spemann organizer in sagittally bisected embryos.

Origin of the Pigmented and Less-Pigmented Epidermis. We next traced the origin of the intriguing epidermal difference in pigmentation in regenerating twins. At the 16-cell stage the embryo can be subdivided into four segments via four injections of lineage tracer (Fig. 5A and B). We labeled each of the four 16-cell segments in this way, which were then sagittally bisected at stage 8 (Fig. 5C and D). We observed that the more pigmented epidermis invariably derived from segment 4, which is the most ventral one (n = 36) (Fig. 5E and G). The less-pigmented epidermis of the twins, which we had speculated might arise from the dorsal side, instead originated from descendants of segment 3 (Fig. 5F and H). Segment 3 is the second-most ventral one and labeled by four injections (SI Appendix, Fig. S1A). These segments are designated 1–4 (for example, segment 1 is the one that gives rise to dorsal blastomeres A1, B1, C1, and D1 at the 32-cell stage) (20). Labeling each segment in different colors at the 16-cell stage showed that the Xenopus somites (and presumably the presomitic mesoderm from which they derive) had highly organized cell origins (SI Appendix, Fig. S1 B–I). Perhaps more importantly for the present analysis, the epidermis was found to be mostly of ventral origin (Figs. 3D and 4 C and D).

In bisected embryos, the dorsal region gave rise to the trunk midline, while the epidermis derived from the ventral lineages, which was also the case in the whole embryo (Fig. 3 G–L). Given that the dorsal-most and ventral-most tissues of the blastula become juxtaposed during healing, this suggested that profound rearrangements in cell fate must take place during twinning. As shown in SI Appendix, Fig. S2 and the corresponding SI Appendix, Movie S3, while the healing point is located precisely at the intersection of dorsal and ventral tissues, the dorsal blastopore lip forms at a distance of about 90° within the dorsal region (SI Appendix, Fig. S2D′, arrowhead). Gastrulation directs involution starting from the dorsal lip, and by the end of the process both the left and right epidermis were derived from the ventral side in half embryos (SI Appendix, Fig. S2F and Movie S3). This indicated that although the original dorsal-most tissue was located at the healing point, the site of formation of the Spemann organizer was displaced. A similar indication was provided by the fact that the neural plate was mostly of dorsal origin and that its midline, under which the axial mesoderm involutes, is flanked by dorsal tissues (SI Appendix, Fig. S3 and Movie S4).
The Dorsal and Ventral Signaling Centers Are Displaced in Regenerating Twins. To investigate further whether the Spemann organizer formation is displaced after healing of half embryos, segment 1 was lineage labeled with nlacZ mRNA at the 16-cell stage, cultured until gastrula, and embryos analyzed by in situ hybridization for the organizer marker chordin (chd). The domain of expression of chd was included within the segment 1 lineage in the whole embryo, but in sagittally bisected half embryos chd expression was found in segment 1 and in adjoining un.injected regions corresponding to segment 2 (Fig. 7 A–B'). Similarly, labeling of the dorsal C1 blastomere at 32-cell stage showed lineage tracing overlapping with chd in the whole embryo but displaced from the C1 lineage (and the healing point scar) in regenerating embryos (Fig. 7 C–D').

We also examined the formation of the BMP signaling gradient, which is maximal in the ventral side and can be followed by

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nuclear phospho-Smad1/5/8 (12, 25). A phospho-Smad1/5/8 gradient was formed both in whole embryos and half embryos on the ventral side opposite the dorsal lip (Fig. 7 E–H). Because in the wild-type embryo the ventral center is formed in segment 4 and the dorsal center in segment 1 (Fig. 5D), these two regions would have been expected to lie next to each other in the regenerating twin. Instead, what was found was that the new dorsal and ventral centers were formed 180° from each other.

These results imply that the position of both the dorsal and ventral centers was respecified in the regenerating embryo (Fig. 7I). In the half embryo, organizer formation was displaced by 90°, which corresponds to 45° in the whole embryo. At early stages after healing, cells fated to develop into the highest BMP signaling regions of prospective high and low BMP signaling. The key observation that prompted our subsequent analysis was that in regenerating half embryos the maternal pigmentation was stronger on one side, instead of being uniform as in the intact embryo (Fig. 2). Lineage-tracing studies showed that the entire epidermis derived from the ventral side. The lighter part of the twinned embryo derived from segment 3, while the darker half derived from segment 4 (Fig. 5). These and other experiments indicated that in twin embryos the Spemann organizer was formed about 90° from its original position. It still forms within the dorsal side, which is primed by βCat for dorsal development (10, 22). The ventral center, which is marked by maximal BMP activity and nuclear phospho-Smad1/5/8, was also displaced 90° in the opposite direction. As summarized in Fig. 7I, healing of the embryo juxtaposes early on—before the Spemann organizer is formed—regions of prospective high and low BMP signaling. The D-V gradient is respecified, so that a new gradient is intercalated before the start of gastrulation, leading to the separation of 180° between the new dorsal organizer and the ventral centers.

**Possible Molecular Mechanisms.** The findings reported here are embryological in nature, and do not address the molecular pathways by which the D-V gradient is respecified. The Spemann organizer requires low BMP signaling for its formation, while the opposite is true for the ventral center (11, 26). It is conceivable that as BMP levels start rising in the ventral side, they displace the formation of the Spemann organizer. The role of BMP signaling in this reprogramming could be tested, for example, by injecting BMP-MOs (14) or dominant-negative BMP receptors (27) into segment 4 (the predicted result being that the organizer would not be displaced). In the reciprocal reaction, once the organizer is formed, it will secrete BMP and Wnt inhibitors that will dampen BMP signals. The Chd–BMP pathway is regulated by the metalloproteinase Tolloid and its inhibitor Sizzled (12, 26), and it can be expected that these molecules might play a role as well.
While BMP signaling is likely to be an important player in twinning, other pathways are likely to be involved. The Nodal-related molecules Xnr5 and -6 are some of the earliest growth factor genes activated zygotically in the dorsal endoderm of the *Xenopus* embryo (28). *Xenopus* nodals are required for the induction of the organizer genes by the Nieuwkoop center, in combination with the dorsal $\beta$Cat signal (29). In the chick embryo, removal of the hypoblast causes multiple embryonic axes to form in the overlying epiblast. The chick hypoblast secretes Cerberus, a multifunctional antagonist of Nodal, Wnt, and BMP signaling, and this twinning can be prevented by inhibiting Nodal signaling (30).

Another important player is xWnt8, which has a major role in promoting ventral development in *Xenopus* (31) and is turned on transcriptionally by BMP signaling (32). Wnt signaling reinforces BMP and Nodal/TGF-$\beta$ signaling by decreasing inhibitory phosphorylations in Smads mediated by glycogen synthase kinase 3 (33, 34). Unraveling the relative roles of this network of intertwined D-V patterning signals will require much work. The present results concern only the cellular mechanisms by which the patterning gradient, by displacing the positioning of the Spemann organizer, results in the induction of the missing half of the embryo.

The recent completion of the *X. laevis* genome has greatly increased the value of this model organism for gene discovery (35). We have performed preliminary RNA-seq analyses with pools of regenerating sagittally bisected embryos, but these did not reveal reproducible changes in up-regulated or down-regulated transcripts. Analyses of individual regenerating half embryos are under way, but it may well be that the D-V system is respecified by repositioning signaling centers rather than by changes in the transcription of any particular regeneration gene.

**Twinning in Other Animals: Polyembryony.** Animal stem cells have the property of self-organizing into complex organoids (36, 37). The ultimate example of regulatory development is twinning, in which the transcriptional and signaling mechanisms involved in this highly unusual process deserve further attention.
which an entire body is formed. In humans monozygotic twins occur in 3 of 1,000 live births, the majority of which are thought to result from spontaneous splitting of the inner cell mass of the blastocyst (38). In the chick, the early blastoderm, which consists of 20,000–60,000 cells, can be sliced into four fragments, with each one giving rise to an embryo (39). The chick embryo has active signaling mechanisms that prevent formation of multiple embryos (30, 40). Interestingly, the chick organizer, called Hensen’s node, is not defined by a fixed population of cells; blastoderm cells are continuously entering and leaving the Chd-expressing node and have inductive properties only while residing within the node (30). Thus, organizer formation is not predetermined.

The most extreme examples of self-organization are provided by species with obligate polyembryony, in which a single zygote gives rise to multiple individuals every generation. In mammals, the nine-handed armadillo gives rise to identical quadruplets sharing a common amniotic cavity (41). The parasitic wasp Copidosoma floridanum lays a single egg into the fertilized egg of a moth, and develops into up to 2,000 monozygotic embryos in the nutritious environment of the caterpillar (42). Unlike other insects, this wasp embryo hatches from its chorion, proliferates, and self-organizes into hundreds of morulae consisting of round, undifferentiated cells that subsequently gastrulate as the caterpillar molts. The most extreme example of making many from one is provided by the tapeworm Echinococcus granulosus. It produces eggs in the intestine of dogs. A single egg can colonize several hosts such as sheep or humans, forming a hydatid cyst in liver and other organs. Each cyst can develop up to a million secondary hosts such as sheep or humans, forming a hydatid cyst. The formation of twins has interested experimental embryologists since the beginning of the discipline (7). Studying the amphibian embryo, we found an unexpected asymmetry in the maternal pigmentation of the epidermis in twin tadpoles. Cell lineage studies indicated that the dorsal organizer and the ventral center are displaced in opposite directions from their expected site of appearance in the embryo. A new D-V gradient is generated, leading to the regeneration of the missing half of the body through the remarkable inductive activity of Spemann’s organizer.

Materials and Methods

Embryo Manipulations and mRNA Synthesis. X. laevis were purchased from Nasco and kept on a diet of minced calf liver. All animal experiments were approved by the University of California, Los Angeles animal research review board. Embryos were generated through in vitro fertilization, those with clear D-V polarity (9) selected at the two- and four-cell stages, cultured in 0.1× Marc’s modified Ringer’s (MMR) (44), and staged according to Nieuwkoop and Faber (16). For in vitro mRNA synthesis, pCS2-nLacZ, pCS2-EGFP-Flag, pCS2-RFP-HA, pCS2-mGFP (containing a Gap43 membrane signal), pCS2-mRFP, pCS2-H2B-GFP, and pCS2-H2B-RFP were linearized with NotI and transcribed with SP6 RNA polymerase using the Ambion mMessage mMachine kit; pRNS-Siamois was linearized with SfiI and transcribed with T3 RNA polymerase (24). To obtain ventralized embryos, a total of 24 ng of antisense MO against Xanops pCat1 (21) was injected four times marginally at the two-cell stage.

Lineage-Tracer Injections and Whole-Mount in Situ Hybridization. Synthetic mRNAs used for lineage tracing were injected into each blastomere (four injections were required to label an entire segment at 16-cell) in 4 nl containing the following amounts: 400 pg of nLacZ; 333 pg of soluble GFP or RFP; 133 pg of membrane-targeted GFP or RFP; or 6.7 pg of histone H2B-GFP or H2B-RFP. Fluorescent dyes conjugated to dextran amine (10 kb, all from Fisher Scientific) were injected into each blastomere as follows: 1.3 ng of Alexa Fluor 568, 2.5 ng of Rhodamine, Cascade blue or Alexa Fluor 647; or 5 ng of Fluorescein (F-DA). All embryos injected with fluorescent lineage tracers were cultured in the dark until the stages indicated. To relocalize the Spemann’s organizer in ventralized embryos, 50 pg of Siamois mRNA were microinjected into two blastomeres of the lateral marginal zone at the 16-cell stage. To prepare an in situ hybridization probe, a pBlueScript-Xcdh (pBS-59) was linearized with EcoRI and transcribed with T7 RNA polymerase. In situ hybridizations were performed as described (24). After hybridization, whole-mount z-stack images were acquired with an Axio Zoom.V16 Stereo Zoom Zeiss microscope and in-focus images stacked using the Zen 2.3 pro Zeiss software.

Generation of Sagittally Bisected Twins. Midblastula embryos (stage 8) were dechorionated manually and bisected in 0.3x MBS saline (17). The animal and marginal regions of embryos were cut along the midline of the dorsal crest with an eyelash knife, and the vegetal cells then fully separated with fine forceps (SI Appendix, Movie S1). Half embryos were washed free of dead cells with a pulled glass pipette, transferred within 3 min into fresh 2% agar-coated plates, placed cut face up in depressions previously made with a heated Pasteur pipette (their position being marked on the outside of the plastic Petri dish with a felt-tip pen), and allowed to heal for 60 min in 0.3x MBS solution (17). The regenerating half embryos were then transferred into fresh agar-coated plates (with depressions) and cultured in 0.1x MBS until they reached gastrula or tailbud stage. Embryos that did not heal completely within 60 min were discarded. The frequency of twins can be increased by selecting those embryos in which dorsal is scored 90° away from the healing point at gastrula, and those that show pigment asymmetry at neurula stage 13.

Vibratome Sections. Lineage-traced whole or half embryos were fixed in 0.5x MEMFA (44) overnight at 4 °C. After washing in PBS, single embryos or halves were transferred into 5 ML of 6% (wt/vol) NuSieve GTG (Lonza) low-melting agarose in PBS in a glass vial (Fisher #303-339-258) at 60 °C, and immediately embedded in an plastic mold (Electron Microscopy Sciences #62352-07). Samples were oriented head down with forceps under the dissection microscope until the agarose solidified. After incubating for 10 min floating on ice water, the excess agarose was trimmed with a Vibratome blade (Electron Microscope Sciences #71990), the embedded sample was removed from the mold, attached with super glue to a specimen block (Leica #3905374), and serially sectioned with a Vibratome (Lancer Vibratome Series 1000) at 100 μm. Sections were picked up from PBS with a cut pipette tip, placed on clean microscope slides, excess PBS removed, and mounted in antifade reagent (Molecular Probes #P36934). Images were acquired with an LSM880 Zeiss confocal microscope equipped with four lasers.

LacZ Staining. Whole or half embryos were fixed with 0.5 × MEMFA for only 20 min and washed in PBS for 10 min at room temperature four times. Embryos were stained in Red-Gal solution [8 μL of 0.5M K4Fe(CN)6, 8 μL of 0.5M K3Fe(CN)6, 4 μL of 0.5M MgCl2, 25 μL of 0.12M Red-Gal, in 1 mL of PBS final volume] for 6 h at room temperature. After staining, samples were washed for 5 min at room temperature twice and fixed again overnight in MEMFA.

X. laevis Immunostaining. The embryos were collected and fixed in MEMFA at stage 12 and bisected with a surgical blade. The Wallingford immunostaining process was followed (45), and only those embryos resulting in a clean horizontal or sagittal cut were imaged. A rabbit polyclonal antiphospho-Smad1/5/8 (Cell Signaling #511L; 1:100) was used to identify the BMP gradient. An anti-Chd rabbit polyclonal antisemur raised against the amino terminus of Xenopus Chd was affinity-purified, as described previously (12), and used at 1:100. Cy3-conjugated goat anti-rabbit antibody (from Jackson ImmunoResearch) was used at 1:1,000 as the secondary antibody. Stacked images of whole and half embryos were acquired with an Axio Zoom.V16 Stereo Zoom Zeiss microscope with Apotome function and stacked images reconstructed using the Zen 2.3 pro Zeiss software.

Movies. SI Appendix, Movie S1 was taken with a DCR-PC350 Sony Handicam and edited using iMovie software. Time-lapse images for SI Appendix, Movie S2 were taken in a Leica stereo microscope using an AxioCam HRc Zeiss camera controlled by Zeiss Axiovision 4.6 and assembled with Fiji software. SI Appendix, Movies S3 and S4 were taken with an inverted Zeiss Observer Z.1 microscope with fluorescence filters controlled by Axiovision 4.6 software.

ACKNOWLEDGMENTS. We thank Sir John Gurdon for the Siamois plasmid; John Wallingford for histone H2B-GFP and H2B-RFP; the staff of the Advanced Xenopus Imaging Workshop at the Woods Hole Marine Biology Laboratory.


Promotion of Science. This work was supported by Norman Sprague Endowment and the Howard Hughes Medical Institute, of which E.M.D.R. is an Investigator.