Xbves is a regulator of epithelial movement during early *Xenopus laevis* development

Anna N. Ripley*, Megan E. Osler*, Christopher V. E. Wright, and David Bader†

Program in Developmental Biology, Vanderbilt University, 2220 Pierce Avenue, Nashville, TN 37232-6300

Edited by Eric N. Olson, University of Texas Southwestern Medical Center, Dallas, TX, and approved November 18, 2005 (received for review July 19, 2005)

Bves/pop1a is a unique, highly conserved integral membrane protein expressed in embryonic epithelia and striated muscle. Although studies have proposed a role in epithelial morphogenesis, the function of Bves/pop1a in development is completely unknown. Here we show that *Xenopus laevis* Bves (Xbves) RNA and protein are expressed in epithelia of the early embryo. Transfection of Xbves into nonadherent mouse L cells confers cell/cell adhesion. Global inhibition of Xbves function by morpholino injection into two-cell embryos arrests development at gastrulation by deregulating the epithelial movements of epiboly and involution. Clonal inhibition of Xbves activity within the A1 blastomere and its derivatives completely randomizes movement of its progeny within otherwise normally differentiating embryos. These data demonstrate that Bves/pop1a proteins play a critical role in epithelial morphogenesis and, specifically, in the cell movements essential for epithelial rearrangements that occur during *X. laevis* development.

cell adhesion | gastrulation | embryo | convergent extension morpholino

Movement and reshaping of epithelia are essential during embryogenesis. In vertebrates, some of the first and most fundamental examples of epithelial morphogenesis are those that drive epiboly and gastrulation (1–3). In *Xenopus laevis*, these movements are governed, in part, by rearrangements of epithelial cells (4, 5). Epiboly in *X. laevis* is regulated by the intercalation of deep cells and thinning of epithelial cells (6). These movements governed by convergence and extension, which ultimately contribute to the closure of the yolk plug and neurulation, depend on many different signaling and adhesive systems that permit repositioning of epithelial sheets (4, 6). Although many players involved in these processes have been identified, there is still an incomplete understanding of the cell biological and biochemical processes that balance plasticity with structural integrity in epithelial sheets during morphogenesis (2, 7–14). Identification of new classes of molecules that regulate movement is critical for an understanding of early development and epithelial morphogenesis.

Bves is an integral membrane protein identified in avians and mammals (15–17). The Brand group subsequently cloned three genes and named them the *popeye* family (17). Bves, identified by T. Brand as Pop1a, is a product of the *bves* (*pop1*) gene, and Bves is the accepted name for the product of this gene (Mouse Genome Informatics, The Jackson Laboratory, HUGO Gene Nomenclature Committee, Bar Harbor, ME). The gene family has been renamed as the *popdc* family. Bves has a short extracellular N terminus with two conserved N-linked glycosylation sites, three transmembrane domains with two small intervening loops, and a long intracellular C terminus (17, 18). Although the sequence and structure of Bves are highly conserved between species, computer modeling detects no structural homology with other proteins. No functional motifs, outside the transmembrane domains, have been identified, and, thus, predictions of Bves function from proposed protein structure are speculative.

Bves traffics to points of cell/cell contact during early epithelial sheet formation *in vitro* (19–21). Transfection of chicken *bves* into L cells confers adhesive activity (19), whereas morpholino oligonucleotide knockdown disrupts epithelial sheet formation *in vitro*

(21). *In vivo* expression studies detect the Bves in epithelia undergoing extensive remodeling during morphogenesis (16, 19, 21–23). Although Andree *et al.* (17, 24) have demonstrated that *pop1-3* are highly expressed in cardiac myocytes, knockout of the *pop1* gene in mice did not produce an embryonic phenotype, perhaps because of functional compensation by the other *pop* genes. Thus, no studies have demonstrated a function for any *popdc* family gene *in vivo*, particularly during early development.

Here we demonstrate the expression of *X. laevis* Bves (Xbves) in epithelia during the earliest phases of frog development and the conservation of its adhesive function. Global disruption of Xbves function arrests development at gastrulation with disrupted epithelial movement. Clonal analysis of Xbves knockdown coupled with lineage tracing demonstrates that Xbves-depleted cells lose normal patterns of movement during and after gastrulation and disperse randomly within normally developing embryos. Taken together, these studies demonstrate a fundamental role for Bves proteins in the regulation of amphibian gastrulation and in overall epithelial morphogenesis.

Results

Conservation of Protein Sequence, Adhesive Function, and Subcellular Localization of Xbves. Sequence analysis predicted a 338-aa protein termed Xbves that contained the canonical Bves/Pop structure, including a short N terminus with two invariant N-linked glycosylation sites, three hydrophobic domains separated two intervening loops, and a long C terminus (18). Xbves protein shared >90% amino acid similarity with mouse and chick Bves. Computational analyses did not identify known protein motifs or significant structural similarity to other proteins. To determine whether Xbves retained the adhesive function described for the chicken protein (19), Xbves cDNA was transfected into mouse L cells for cell adhesion analyses. As seen in Fig. 8A, which is published as supporting information on the PNAS web site, Xbves-transfected L cells readily adhered to each other, forming a statistically significant number of aggregates when compared to control cells (Fig. 8C, 66.83 ± 3.45 vs. 24.00 ± 4.18 ; $P < 0.001$ by Student's *t* test).

To determine whether Xbves traffics to points of cell/cell contact during epithelial sheet formation, *X. laevis* A6 kidney cells were plated a low density and allowed to form epithelial sheets. α -Xbves reactivity was observed at cell/cell boundaries and not at the free surface of these cells (Fig. 8D). In confluent sheets, Xbves was seen at the cell surface with a distribution overlapping that of E-cadherin at points of cell/cell contact (Fig. 9, which is published as supporting information on the PNAS web site).

Maternal and Early Stage Embryonic Expression of Xbves. The distribution of Xbves mRNA and protein during early embryogenesis was determined. Xbves transcripts were detected throughout the

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviation: MO, morpholino.

*A.N.R. and M.E.O. contributed equally to this work.

†To whom correspondence should be addressed. E-mail: david.bader@vanderbilt.edu.

© 2006 by The National Academy of Sciences of the USA

Fig. 3. Injection of *Xbv* morpholino into a two-cell *X. laevis* embryos inhibits gastrulation movements. (A, C, E, and G) Uninjected embryos serve as a control. Arrows indicate the blastopore (D) and the forming neural plate (E, G, and H). At stage 6, *Xbv* MO-injected embryos develop in an apparently normal fashion (B). By stage 11, experimental embryos are delayed or inhibited in yolk plug closure (D, arrow denotes the edge of the yolk plug), and by stage 14 (F), most embryos lack closed yolk plugs and have become necrotic. Control sense MO-injected embryos (H) complete gastrulation and undergo neurulation (neural plate, white arrows) at the same rate compared with uninjected embryos (G).

and became necrotic thereafter (Fig. 3F). Control MO had no effect on gastrulation and neural plate formation (arrows, Fig. 3H).

Mesoderm formation is critical for gastrulation but is independent of sustained epithelial movements necessary for development (26, 27). Thus, *Xbves* function was disrupted and expression of two mesodermal markers, *goosecoid* and *Xbra* (28, 29), was examined. At the onset of gastrulation, *goosecoid* and *Xbra* expression were similar in experimental and control embryos (Fig. 12B, which is published as supporting information on the PNAS web site). In controls, *goosecoid*-expressing cells were observed at stage 9/9.5 as a patch of cells near the dorsal lip, and bisection of stage 10.5 embryos showed that this expression shifted to the deep cell layers after involution (Fig. 4 A and C). Normal *Xbra* expression was characterized by transcription around the blastopore and in chordamesodermal cells moving anteriorly (arrows, Fig. 4B; refs. 30–32). Bisection of stage 12 control embryos demonstrated movements of involuting mesendoderm, formation of the archenteron, and clear definition of the yolk plug as indicated by *Xbra* expression (Fig. 4D).

Beginning at stage 10/10.5, *goosecoid* hybridization of *Xbves* MO-injected embryos was more abundant in an area juxtaposed to the dorsal lip (Figs. 4E and 12). Bisection revealed that the intense

Fig. 5. Xbves depletion enhances extension of animal cap explants. Animal cap assays were performed as described in *Materials and Methods*. In the absence of Activin-A, no extension was observed in control or Xbves MO-injected samples. In the presence of Activin-A, control caps extended as predicted, whereas experimental caps exhibited even greater extension ($P < 0.005$; Student's *t* test) during the course of study.

staining was concentrated at the embryo surface (Fig. 4G). *Xbra* expression was observed in *Xbv*es MO-injected embryos in the mesoderm around the blastopore at stage 9.5 (Fig. 12). However, at stage 10/10.5, *Xbra* staining in *Xbv*es MO-injected embryos was broader and more irregular, and staining of the axial chordamesodermal rod was completely absent in the vast majority of the embryos (Figs. 4F and 12). *Xbra*-expressing cells in *Xbv*es MO-injected animals remained at the surface, suggesting altered involution (Fig. 4H).

Animal cap assays are used to determine whether specific molecules influence gastrulation. Isolated embryo caps from control and *Xbv*es MO-treated embryos were treated with Activin-A, a factor that induces convergence/extension causing an elongation of the dissected tissue. Control and *Xbv*es MO embryos cultured in Activin-A exhibited epithelial movements and animal cap elongation, whereas control embryos did not elongate (Fig. 5). Interestingly, we observed a 178% greater elongation of *Xbv*es MO-injected caps, suggesting that *Xbv*es impacts gastrulation movements. The increase in extension is compatible with previous work showing that inhibition of *Xbv*es function potentiates cell movement (discussed below).

RNA Rescue and Protein Knockdown. To verify that the phenotype observed with MO injection was specific to the knockdown of *Xbves* protein, we coinjected MO with *Xbves* rescue RNA and monitored embryos for restoration of development. Rescue RNA-injected embryos developed normally, proceeding through gastrulation to

Fig. 4. *In situ* hybridization analysis of mesodermal markers *goosecoid* and *xbra*. Expression of *goosecoid* and *xbra* markers is unaffected by injection of embryos with control MO at stage 10.5 (A and B). (Six examples are given for each group.) Bisection of whole mount-stained embryos shows the diffuse subsurface distribution of *goosecoid* reactivity in gastrulated cells (C, arrow), whereas *xbra*-stained samples reveal the movement of mesendoderm away from the blastopore and the formation of the archenteron (D). In contrast, injection of *Xbves* MO leads to an abnormal accumulation of *goosecoid* staining immediately anterior to the dorsal lip of the blastopore (E) and the lack of *xbra* in midline mesendoderm (F, arrows). Note that *goosecoid* and *xbra* staining are restricted to the surface of the embryo position of the dorsal lip of the blastopore in all bis-

Fig. 6. Injection of *Xbv es* MO into the A1 blastomere alters movements of progeny during gastrulation. Three embryos were imaged to show the blastopore (A–C) and reimaged after a 90° rotation (D–F). Injection of control MO with lacZ tracer into the A1 blastomere demonstrates the convergence of A1 progeny toward the blastopore at stage 12 (A and D). Progeny of *Xbv es* MO-injected A1 blastomeres do not follow normal paths of movement (B, C, E, and F). Cross-sections of these embryos demonstrate that progeny of the control MO-injected A1 blastomeres remain closely associated with the embryo surface (G), whereas derivatives of *Xbv es* MO-injected cells are often detected below the surface (H, arrows). Fig. 14 shows additional examples.

tailbud and tadpole stages with only minor tail malformations and a slightly arched dorsum (Fig. 13 G–L, which is published as supporting information on the PNAS web site). Next, we analyzed control MO- and *Xbv es* MO-injected embryos for a reduction in *Xbv es* protein expression by using immunofluorescence. Fig. 13 illustrates that control MO-injected embryos expressed *Xbv es* protein at high levels and trafficked the protein to the cell surface (Fig. 13 M and N). In contrast, *Xbv es* MO-injected embryos had significantly reduced levels of *Xbv es* with little, if any, membrane staining (Fig. 13 O and P). Solid-state ELISA was also used to quantify the amount of *Xbv es* protein depletion after MO treatment. As seen in Fig. 13Q, the relative activity of α -*Xbv es* was significantly reduced, >10-fold, in embryo extracts ($P < 0.0073$, ANOVA), whereas α -actin reactivity was unaffected ($P > 0.05$, ANOVA). It is interesting to note that *Xbv es* knockdown also results in diminished levels of E-cadherin and occludin, both components of cell junctions (data not shown).

Progeny of *Xbv es*-Depleted Blastomeres Display Rogue Cell Movements Within Developing Embryos. We next determined whether depletion of *Xbv es* protein within a marked clone of cells would alter their movement relative to normally developing cells. The A1 blastomere of the 32-cell embryo was injected with control and experimental MO along with a nuclear-targeted lineage tracer.

Gross morphology of control and experimental groups was indistinguishable, but striking differences in the distribution of A1 progeny between control and experimentally injected cells were observed. In the controls (uninjected or control MO-injected), labeled A1 descendants formed a tightly compacted continuous strip of cells at stage 12. This strip was narrowest at the blastopore, where cells had begun involution and broadest near its anterior margin (Fig. 6 A and D; see also Fig. 14A, which is published as supporting information on the PNAS web site). This tightly packed array of cells was observed in 73% of control embryos (117 of 161; Fig. 6 A and D; see also Fig. 15, which is published as supporting information on the PNAS web site). In contrast, progeny of *Xbv es*

MO-injected A1 blastomeres had a completely different distribution. Most clones had a highly irregular shape and did not extend to the blastopore. Some clones were located on the pole opposite the blastopore and did not possess a discernable narrowed leading edge (Figs. 6 B and E and 14). Other *Xbv es* MO-injected clones had a diffuse appearance with many interspersed nonlabeled cells (Fig. 6 C and F). Morphometric analysis of these clones determined that 73% (55 of 72 embryos with 5 ng of injected MO) and 82% (29 of 35 embryos with 10 ng of injected MO) had an irregular shape that did not extend to the blastopore (Figs. 6 and 15). The average widest point of 34 randomly selected *Xbv es* MO-injected clones was 855 μ m, compared to 585 μ m in control embryos ($P < 0.0001$; Student's *t* test).

Histological analysis of stage 12 control and experimental groups revealed that lacZ-labeled cells had differing distributions relative to the embryo surface. As seen in Fig. 6G, control-labeled cells were closely compacted near the outer surface of the blastocoel roof. In contrast, a substantial number of *Xbv es* MO-injected cells were located in deeper layers of the animal cap (Fig. 6H, arrows). The location of labeled nuclei relative to the surface was measured in 20- μ m increments. Also, as seen in Fig. 16, which is published as supporting information on the PNAS web site, the distribution of cells was such that *Xbv es* MO-injected blastomere progeny was displaced from the surface.

A1 progeny contribute to anteriorly located tissues in the head region, including the skin, connective tissue, and brain, as well as to anterior somites (33). To determine whether cell fate and location was altered by *Xbv es* depletion, control and experimental embryos were allowed to develop to stage 37. Derivatives of lacZ/control MO-injected cells faithfully differentiated into predicted structures with little “scatter” of labeled cells (Fig. 7). Note that β -gal staining in the head totally obscured visualization of the eye and that somites were labeled (Fig. 7A). Sectioning of embryos demonstrated the extensive incorporation of lacZ-labeled cells in the eye, skin, brain, and connective tissue of the head, even in the deepest regions of the embryo. In contrast, the progeny of *Xbv es* MO-injected blastomeres showed a wide and inconsistent range of distributions throughout the embryo. In most cases, β -gal-positive cells were dispersed without concentration in any particular structure and, notably, with no enrichment in the head or somites (Fig. 7 B and C). Additionally, a subset of embryos had labeled cells located exclusively in the caudal region, a position normally devoid of A1 progeny (compare to Fig. 7A). In sections, the β -gal-stained nuclei were scattered primarily in surface structures, including the epidermis and adjacent connective tissue space. Cells were almost always absent from deep structures such as the brain, spinal cord, and digestive system (Fig. 7 E–G). Importantly, viable cells were always observed in experimental animals, demonstrating that *Xbv es* inactivation was not generally toxic to cells. These data suggest that inhibition of *Xbv es* function resulted in loss of regulated cell movement and an apparently random distribution of progeny throughout the embryo.

Discussion

Expression and Adhesive Properties of *Xbv es*. Previous data demonstrated *Bves* expression in epithelial structures of the postgastrulated embryo and in epithelial cell lines (15, 19–23). *pop* gene products are also highly expressed in developing and adult striated muscles (17, 22, 24, 34). Still, a comprehensive analysis of RNA and protein expression has not been reported. The current cDNA cloning, PCR, *in situ*, and immunochemical analyses detect *Xbv es* mRNA and protein in the cleaving embryo. *Xbv es* protein is detected in all, or nearly all, epithelia in the gastrulating embryo and is predominantly localized to the interface between epithelial cells. The broad pattern of *Xbv es* expression in the early frog embryo is consistent with our previous studies that find this protein in epithelia undergoing significant shape changes during a myriad of morphogenetic processes in the embryo (19, 21, 22). Here, we also demonstrate conservation of *Bves* adhesive properties in *Xbv es*.

Fig. 7. *Xbv es* MO injection randomized movement of A1 progeny. At stage 37, (A) A1 derivatives are observed in head structures and in somites of control-MO injected animals. (B and C) A1 progeny are randomly distributed throughout the embryo after *Xbv es* MO injection. Note the absence of labeled cells from the head region with *Xbv es* MO injection. (D) A cross section through the head region of control embryos reveals blue cells in various cranial structures and tissue types. (E) In sections through *Xbv es* MO-injected embryos, few labeled, if any, cells are detected in the head region. (F and G) Trunk cross-sections show that most blue cells reside in the outer epidermis of the embryo.

These data predict a broad role for *Xbv es* in epithelial movements during gastrulation.

Inactivation of *Xbv es* Function Inhibits Gastrulation Movements. To date, there are no functional studies of Bves proteins during development. Global MO knockdown of *Xbv es* during early development leads to a general arrest of gastrulation characterized by the failure of epiboly, yolk plug closure, involution, and mesodermal patterning. Furthermore, by using a classic amphibian signaling system as a model, we demonstrate the convergence/extension movements of animal cap explants are altered and, in fact, are more robust after *Xbv es* MO treatment. Although this finding is seemingly contradictory to the aforementioned data, this outcome was predicted by recently published work showing that *Xbv es* depletion results in loss of tight junction integrity and an initially accelerated rate of epithelial movement in a wound-healing assay (21, 35). Thus, the potentiation of cap extension seen here due to Activin-A signaling may reflect the deregulation of cell adhesion at the junctional level. This finding, in turn, led us to postulate that cells with impaired *Xbv es* function respond to Activin-A and/or extension signals with accelerated movement. Overall, our data suggest that the loss of proper cell–cell interactions within embryonic epithelia lead to misdirected morphogenetic movements and arrest in

development with either clonal or global *Xbv es* knockdown. All of these anomalies have been previously attributed to defects in epithelial movement influenced by the essential roles of cell/cell and cell/matrix adhesion molecules in *X. laevis* gastrulation (2, 7, 13, 25, 36). Although these phenotypes vary, global inhibition of any of these molecules results in a cessation of epithelial movement and a failure to gastrulate. Thus, it is reasonable that inhibition of a regulator of epithelial adhesion, such as *Xbv es*, may result in a similar phenotype.

Epithelial movement in *X. laevis* gastrulation is driven by convergence and extension (4, 6, 37, 38). Here, the convergence of epithelial cells along one axis results in the concomitant extension of cells along the perpendicular axis, resulting in the net movement or expansion of the surface cells over the yolk plug and the ingression of cells to form mesendoderm (4, 6, 13). This rearrangement of cells within the epithelial sheet is typified by the pyramidal shape of superficial epithelial cells labeled in control lacZ injections of the A1 blastomere (Fig. 6). When *Xbv es* function is inhibited within the same population of cells, this precise rearrangement or reorganization of cells is severely disrupted, suggesting that the process of convergent/extension has been inhibited. Further, the displacement of *Xbv es* MO-treated cells from the embryo surface indicates that these cells do not properly intercalate into the advancing epithelium. Taken together, these data suggest that *Xbv es* is essential for the appropriate interaction and movement of neighboring epithelial cells during *X. laevis* gastrulation.

***Xbv es* Plays a Role in Proper Cell Fate Decisions.** Clonal disruption of *Xbv es* function leads to the randomization of cell movement, that, in turn, may lead to alterations in cell differentiation. The production of mesendoderm in the frog depends on the involution of epithelial cells from the embryo surface (6, 32). In the absence of *Xbv es* function in our clonal analysis studies, cells are apparently incapable of producing mesendoderm in significant amounts. We postulate that this result is due to the disruption of coordinated cell movement within epithelial sheets leaving the cells unable to reach deeper positions in the embryo. It is possible that *Xbv es*-depleted morphant cells normally fated to mesendodermal lineages may regulate and assume an ectodermal phenotype or alternatively undergo apoptosis in the absence of proper differentiative signals. Although global inactivation of *Xbv es* function is not incompatible with mesoderm production (Fig. 4), we cannot exclude the regulative capacity of morphant cells within a normally differentiating embryo. In either case, surviving cells appear to favor epidermal over neural fates within derivatives of the ectoderm. Furthermore, preliminary studies that examine the ultrastructural detail of *Xbv es*-depleted embryos suggest that changes in cell shape/survival may be important factors in the outcome we observed (35). Thus, although its influence may be direct or indirect, proper *Xbv es* function is critical for the morphogenetic movement, differentiation, and/or survival of cells fated to mesendodermal lineages.

Materials and Methods

Xenopus Methods. *X. laevis* eggs were harvested and fertilized by standard methods (39). Animal caps were dissected from devitellinized control or MO-injected embryos at stage 8.5–9 in Steinberg's solution with 0.01% BSA. Half of the embryos were placed in 10 ng/ml Activin A. Caps were cultured to stage 18–20 and scored for elongation.

cDNA Cloning and Antibodies. EST database searches revealed an expressed tag in a two-cell *X. laevis* library. This 500-bp cDNA was used as a probe to screen tailbud, dorsal lip, and oocyte libraries (gifts from Bruce Blumberg, University of California, Los Angeles). Thirteen independent cDNAs were cloned, aligned, and deposited in the National Center for Biotechnology Information (NCBI) database (AF 527799). These cDNAs were the only *Xbv es* sequences isolated after screening 10^8 independent clones and were

identical to all those reported in the NCBI database (September 2004) and by Hitz *et al.* (40). Only one *Xbves* transcript was identified, and no related family members were isolated. Still, our analyses are directed specifically toward the *Xbves* transcript and do not address possible function of related family members, should they exist. Two *Xenopus* peptide antisera designed to specifically recognize the *Xbves* protein (Ab1, CENWREIHHLVFHLANT; Ab2, KLYSLNDPTLGKKRKLDT) were generated in rabbits (Biosynthesis) and affinity purified (22). The antiserum used in this study does not react on a Western blot. α -E-cadherin was also used in these studies.

Cell Lines and Assays. A6 *X. laevis* kidney epithelial, CHO, and L6 cells were obtained from American Type Culture Collection (ATCC) and grown according to ATCC protocols. Mouse fibroblast L cells were stably transfected with control and full-length FLAG-tagged *Xbves* cloned into pCIneo vector (Promega), and adhesion assays were conducted according to Thoreson *et al.* (41). The ratio of cells in aggregates vs. the total cell number was tabulated and summarized by GraphPad (San Diego) PRISM 3.0. All values are reported as mean $\pm$ SEM.

RT-PCR. RNA preparation and RT-PCR analysis was performed according to Osler *et al.* (22) by using primer set 5'-ATGAAGGTGTCCTACCGAGGCCAT and 3'-CAGTGTGGGATCATTGAGCGAGTA (266-bp product) with an annealing temperature of 58°C and extension time of 1 minute. Reverse transcriptase was excluded from the negative control reactions.

MO Treatment and Rescue. A series of antisense MO were synthesized (Gene Tools, Carvalis, OR). The most efficacious MO in terms of eliminating protein synthesis in developing embryos was ATCTTTCTTATACCTGGATGTGCAG, which contains the reverse complement to the AT of the start codon and sequences immediately 5'. Control and experimental MO was dissolved in water and injected at 5 nl into blastomeres at varying concentrations of oligonucleotide. The sense complement had no influence on development up to 80 ng per blastomere. In one set of experiments, control or *Xbves* MO was injected into both cells of the two-cell embryo (40 ng per cell). An *Xbves* cDNA that lacks 5' UTR (the first 17 bp of the MO) and begins with the start ATG codon was used to rescue MO treatment. mRNA was synthesized by mMESSAGE mMACHINE (Ambion, Austin, TX) and coinjected into both cells of the two-cell embryo (50 pg of rescue mRNA with 40 ng of MO). In a second set of

experiments, 5 or 10 ng of control and *Xbves* MO plus 1 ng of lacZ tracer RNA were injected into the A1 blastomere of the 32-cell embryo. Animals were maintained for selected periods of time up to stage 35, and β -gal-expressing cells were identified (42). Whole-mount, lacZ-stained embryos were photographed and captured by Magnafire (National Institutes of Health).

Solid-State ELISA Protocol. Control and *Xbves* MO-injected embryos were collected at stage 12 and snap frozen. Thawed samples were then vortexed in Steinberg's solution, and proteins were collected by centrifugation. After one additional round of washing and centrifugation, proteins were extracted in 0.5 M NaCl with 0.5% Triton X-100 for 30 min on ice. After centrifugation, the supernatant was collected and the protein concentration determined. Fivefold serial dilutions of extracts beginning at 50 μ g per well were applied to 96-well plates according to Gonzalez-Sanchez and Bader (43). Solid-state ELISA was conducted by using fixed amounts of primary [α -Bves (1:200) and α -actin (1:100)] and alkaline phosphatase-labeled goat anti-rabbit antibodies (1:5,000) by using standard methods (43). Four reactions were analyzed for each data point.

In Situ, Immunochemical, and Histological Analyses. Three separate *Xbves* probes (bp 70–670, 870–1210, and full-length 1–1738), *Xbra* (29), and *goosecoid* (28) were used for hybridization (44). To characterize α -*Xbves* antisera, A6 cells and CHO cells were transiently transfected with pCIneo/FLAG/*Xbves* (Fig. 9). Immunofluorescence analysis was standard (11, 22). Nuclear lacZ staining was conducted according to Sive *et al.* (45). For clonal analysis of A1 progeny, images of cross-sectioned stage 12 embryos were analyzed to determine the location of labeled nuclei relative to the embryo surface. Two hundred fifty or more randomly selected blue nuclei were counted for each group and were scored as 0–20, 20–40, 40–60, and >60 μ m from the surface, and significance was determined by χ^2 analysis.

We thank Kristin Price for her expert technical assistance; Shuji Takahashi, Yuki Ohi, and J. J. Westmoreland for helpful advice with *Xenopus* protocols and methodology; Angela Matthe, Hillary Hager, and Farideh Bowles for technical aid; and members of our laboratory for critical assessment of these studies. This work was supported by National Institutes of Health Grants P01 HL-76105, R01 HL-37675, R01GM-56238, and R03 EY-15211 and by American Heart Association Grant 0415184B.

- Keller, R. E. (1980) *J. Embryol. Exp. Morphol.* **60**, 201–234.
- Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C., Noro, C. Y., & Wylie, C. (1994) *Cell* **79**, 791–803.
- Nieuwkoop, P. D. (1973) *Adv. Morphog.* **10**, 1–39.
- Wallingford, J. B., Fraser, S. E., & Harland, R. M. (2002) *Dev. Cell* **2**, 695–706.
- Keller, R., Davidson, L. A., & Shook, D. R. (2003) *Differentiation* **71**, 171–205.
- Keller, R. (1991) *Methods Cell Biol.* **36**, 61–113.
- Brieher, W. M., & Gumbiner, B. M. (1994) *J. Cell Biol.* **126**, 519–527.
- Broders, F., & Thiery, J. P. (1995) *Cell Adhes. Commun.* **3**, 419–440.
- Bradley, R. S., Espeseth, A., & Kintner, C. (1998) *Curr. Biol.* **8**, 325–334.
- Cordenonsi, M., Mazzon, E., De Rigo, L., Baraldo, S., Meggio, F., & Citi, S. (1997) *J. Cell Sci.* **110**, 3131–3139.
- Fagotto, F., & Gumbiner, B. M. (1994) *Development (Cambridge, U.K.)* **120**, 3667–3679.
- Heasman, J., Kofron, M., & Wylie, C. (2000) *Dev. Biol.* **222**, 124–134.
- Marsden, M., & DeSimone, D. W. (2001) *Development (Cambridge, U.K.)* **128**, 3635–3647.
- Wallingford, J. B., Vogeli, K. M., & Harland, R. M. (2001) *Int. J. Dev. Biol.* **45**, 225–227.
- Reese, D. E., Zavaljevski, M., Streiff, N. L., & Bader, D. (1999) *Dev. Biol.* **209**, 159–171.
- Reese, D. E., & Bader, D. M. (1999) *Mamm. Genome* **10**, 913–915.
- Andree, B., Hillemann, T., Kessler-Ickson, G., Schmitt-John, T., Jockusch, H., Arnold, H. H., & Brand, T. (2000) *Dev. Biol.* **223**, 371–382.
- Knight, R. F., Bader, D. M., & Backstrom, J. R. (2003) *J. Biol. Chem.* **278**, 32872–32879.
- Wada, A. M., Reese, D. E., & Bader, D. M. (2001) *Development (Cambridge, U.K.)* **128**, 2085–2093.
- Wada, A. M., Smith, T. K., Osler, M. E., Reese, D. E., & Bader, D. M. (2003) *Circ. Res.* **92**, 525–531.
- Ripley, A. N., Chang, M. S., & Bader, D. M. (2004) *Invest. Ophthalmol. Visual Sci.* **45**, 2475–2483.
- Osler, M. E., & Bader, D. M. (2004) *Dev. Dyn.* **229**, 658–667.
- Vasavada, T. K., DiAngelo, J. R., & Duncan, M. K. (2004) *J. Histochem. Cytochem.* **52**, 371–378.
- Andree, B., Fleige, A., Arnold, H. H., & Brand, T. (2002) *Mol. Cell. Biol.* **22**, 1504–1512.
- Hitz, M. P., Pandur, P., Brand, T., & Kuhl, M. (2002) *Mech. Dev.* **115**, 123–126.
- Niehrs, C., Keller, R., Cho, K. W., & De Robertis, E. M. (1993) *Cell* **72**, 491–503.
- Vodicka, M. A., & Gerhart, J. C. (1995) *Development (Cambridge, U.K.)* **121**, 3505–3518.
- Cho, K. W., Blumberg, B., Steinbeisser, H., & De Robertis, E. M. (1991) *Cell* **67**, 1111–1120.
- Smith, J. C., Price, B. M., Green, J. B., Weigel, D., & Herrmann, B. G. (1991) *Cell* **67**, 79–87.
- Cunliffe, V., & Smith, J. C. (1992) *Nature* **358**, 427–430.
- Green, J. B., New, H. V., & Smith, J. C. (1992) *Cell* **71**, 731–739.
- Kwan, K. M., & Kirschner, M. W. (2003) *Development (Cambridge, U.K.)* **130**, 1961–1972.
- Moody, S. A. (1987) *Dev. Biol.* **122**, 300–319.
- DiAngelo, J. R., Vasavada, T. K., Cain, W., & Duncan, M. K. (2001) *Hybrid. Hybridomics* **20**, 377–381.
- Osler, M. E., Chang, M. S., & Bader, D. M. (2005) *J. Cell Sci.* **118**, 4667–4678.
- Levine, E., Lee, C. H., Kintner, C., & Gumbiner, B. M. (1994) *Development (Cambridge, U.K.)* **120**, 901–909.
- Heasman, J., Ginsberg, D., Geiger, B., Goldstone, K., Pratt, T., Yoshida-Noro, C., & Wylie, C. (1994) *Development (Cambridge, U.K.)* **120**, 49–57.
- Keller, R., & Danilchik, M. (1988) *Development (Cambridge, U.K.)* **103**, 193–209.
- Wallingford, J. B., Ewald, A. J., Harland, R. M., & Fraser, S. E. (2001) *Curr. Biol.* **11**, 652–661.
- Danilchick, M., Peng, H. B., & Kay, B. K. (1991) *Methods Cell Biol.* **36**, 679–681.
- Thoreson, M. A., Anastasiadis, P. Z., Daniel, J. M., Ireton, R. C., Wheelock, M. J., Johnson, K. R., Hummingbird, D. K., & Reynolds, A. B. (2000) *J. Cell Biol.* **148**, 189–202.
- Morasso, M. I., Sargent, T. D., & Jamrich, M. (1994) *Mech. Dev.* **46**, 63–70.
- Gonzalez-Sanchez, A., & Bader, D. (1984) *Dev. Biol.* **103**, 151–158.
- Harland, R. M. (1991) *Methods Cell Biol.* **36**, 685–695.
- Sive, H. L., Grainger, R. M., & Harland, R. M. (2000) *Early Development of Xenopus laevis: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).