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

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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.

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ovement 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 (1–5). In X. 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 invariant N-linked glycosylation sites, three hydrophobic domains separated two intervening loops, and a long C terminus (18). Bves 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 Bves retained the adhesive function described for the chicken protein (19), Bves 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, Bves-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

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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.
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unfertilized egg (data not shown), consistent with EST analysis of oocyte libraries. The distribution of Xbves mRNA changed in the two- and four-cell embryos as hybridization was stronger in the animal region (Fig. 1 A–C). As cleavage continued, this region remained strongly positive (Fig. 1 D–F). During gastrulation (stages 10–12), all animal pole cells were positive for Xbves, and with the establishment of the blastopore, a clear distinction between surface cells and nonreactive yolk plug cells was apparent (Fig. 1 G and H). From stage 12.5 to 35, expression persisted in the dorsal regions of the embryo from the neural plate. Staining extended around the blastopore, neural plate, and somites but was reduced on the ventral surface (Fig. 1 I–N). Later, the posterior, ventral regions had reduced expression as Xbves became progressively restricted to the heart, somites, cement gland, and eyes by stage 35 (Fig. 1O).

Because our data greatly varied from the previously published study (25), RT-PCR confirmed the presence of Xbves mRNA in the oocyte and cleavage, gastrulation, and neurulation stage embryos (Fig. 10A, which was published as supporting information on the PNAS web site). Whole-mount analyses were repeated by using three separate Xbves RNA probes, and the same general pattern of hybridization was observed (Fig. 10B).

Immunohistochemistry showed that regions of protein distribution corresponded to those that express Xbves mRNA. For example, at stage 11.5, surface cells were strongly stained by α-Xbves, whereas yolk cells were unreactive (Fig. 24; compare to Fig. 1 G and I). In addition, newly involuted cells were also positive, and higher magnification revealed that this reactivity was confined to regions of cell/cell contact and was absent from the apical surface of cells (Fig. 2 B and B’). At stage 19, protein expression persisted in the dorsal ectoderm/epidermis and the underlying neural tube and optic vesicle (Fig. 2 C and D). Other epithelial structures derived from mesoderm, such as the notochord and somites, were also positive for α-Xbves. Later, epithelial elements, such as the velar plate, pronephros, somatically derived striated muscle, eye, and heart, expressed Xbves (Fig. 11, which is published as supporting information on the PNAS web site).

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Disruption of Xbves Function Inhibits Epithelial Movements During Gastrulation but Not Mesoderm Formation. To determine whether Xbves regulates epithelial morphogenesis, Xbves morpholinos (MOs) were injected into both cells of the two-cell embryo. Xbves MO-injected embryos appeared to undergo normal cleavage (Fig. 3B) and are indistinguishable from control MO-injected and non-injected embryos until stage 9+1. At stage 10, differences in yolk plug size and position of the advancing ectoderm suggested retardation or inhibition of the cell movements of epiboly. Xbves MO-injected embryos began to form a dorsal blastopore lip, but by stage 11, epithelial movement was greatly inhibited. Xbves MO-injected embryos had a large yolk plug, did not form a neural plate,
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, Xbra 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. 4A and C). Normal Xbra expression was characterized by transcription around the blastopore and in chordomesodermal 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 Xbra MO-injected embryos was more abundant in an area juxtaposed to the dorsal lip (Figs. 4E and 12). Bisection revealed that the intense staining was concentrated at the embryo surface (Fig. 4G). Xbra expression was observed in Xbra MO-injected embryos in the mesoderm around the blastopore at stage 9.5 (Fig. 12). However, at stage 10/10.5, Xbra staining in Xbra MO-injected embryos was broader and more irregular, and staining of the axial chordomesodermal rod was completely absent in the vast majority of the embryos (Figs. 4F and 12). Xbra-expressing cells in Xbra 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 Xbra MO-treated embryos were treated with Activin-A, a factor that induces convergence/extension causing an elongation of the dissected tissue. Control and Xbra 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 Xbra MO-injected caps, suggesting that Xbra impacts gastrulation movements. The increase in extension is compatible with previous work showing that inhibition of Xbra 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 Xbra protein, we coinjected MO with Xbra rescue RNA and monitored embryos for restoration of development. Rescue RNA-injected embryos developed normally, proceeding through gastrulation to
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 Xbves 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 Xbves MO-injected clones was 855 μm, compared to 585 μm 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 Xbves 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-μm 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 Xbves 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 Xbves 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 Xbves 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 Xbves inactivation was not generally toxic to cells. These data suggest that inhibition of Xbves function resulted in loss of regulated cell movement and an apparently random distribution of progeny throughout the embryo.

Discussion

Expression and Adhesive Properties of Xbves. 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 immunohistochemical analysis detected Xbves mRNA and protein in the cleaving embryo. Xbves 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 Xbves 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 Xbves.

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 Xbves 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 Xbves MO-injected clones was 855 μm, compared to 585 μm in control embryos (P < 0.0001; Student’s t test).

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Control-MO injected animals. A1 derivatives are observed in head structures and in somites of that the loss of proper cell–cell interactions within embryonic development signals with accelerated movement. Overall, our data suggest with impaired Xbves function respond to Activin-A and signaling may reflect the deregulation of cell adhesion at the potentiation of cap extension seen here due to Activin-A treatment. Although this finding is seemingly contradictory to the aforementioned data, this outcome was predicted by recently published work showing that Xbves 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 Xbves 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 morphogenic movements and arrest in development with either clonal or global Xbves 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 Xbves, 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 Xbves 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 Xbves 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 Xbves is essential for the appropriate interaction and movement of neighboring epithelial cells during X. laevis gastrulation.

**Xbves Plays a Role in Proper Cell Fate Decisions.** Clonal disruption of Xbves 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 Xbves 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 Xbves-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 Xbves function is not incompatible with mesoderm production (4), we cannot exclude the regenerative 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 Xbves-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 Xbves 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 Xbves sequences isolated after screening 10⁶ independent clones and were

![Fig. 7. Xbves 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 Xbves MO injection. Note the absence of labeled cells from the head region with Xbves MO injection. (D) A cross section through the head region of control embryos reveals blue cells in various cranial structures and tissue types. (E) Sections through Xbves 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.](image-url)
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 Xbves antisera designed to specifically recognize the Xbves protein (Ab1, CENWREIHHLVAT; Ab2, KLYSLNDPTLGKKRKLDT) were generated in rabbits and were used. 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 and the protein concentration determined. Fivefold serial dilutions of extracts beginning at 50 μg 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, Immunohistochemical, and Histological Analyses.** Three separate Xbves probes (bp 70–670, 870-1210, and full-length 1–1738), Xbves (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 μm from the surface, and significance was determined by χ² analysis.

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Supplementary Data Figure 1

A. Xbves

B. control

C. L-cell Assay

D
Supplementary Data Figure 2

A. Xbves
B. E-cadherin
C. overlay

D. vector
E. xbes
Supplementary Data Figure 4
Graph I

Morphometric analysis of clone shape and movement

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Graph II

Number of cells in zones

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