CHARACTERIZATION OF BVES FUNCTION IN EPITHELIAL INTEGRITY

by

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CHAPTER I

INTRODUCTION

Scientific Discovery

In the world of basic biological investigation, scientists often take one of two approaches to explore their research focus. They may choose to study a *process* and attempt to unravel the intricacies of that process by understanding which molecules are involved and how they behave. For example, they try to answer a question such as "how does the heart develop?" as our laboratory has done in years past. To answer such a question, one must address many topics, which brings about even more questions. When does heart development begin and thus, when can you detect the earliest traces of cardiogenic cells? Next, how and when do the necessary cell progenitors arise to form the outer covering of the heart, the heart wall muscle, and the endocardial lining? Importantly, how do the coronary vessels form during development so they can sufficiently supply the thick myocardium with blood in the adult? Finally, how does the heart make the proper connections to the lungs and the giant vessels so it can function to supply a growing organism with oxygen? Thus, many avenues exist to address the main question and as one might expect, there are hundreds if not thousands of molecules intricately involved in the processes that ultimately result in a fully functioning adult heart.

Contrarily, a researcher will examine an *element* or *factor*, without necessarily knowing how or what biological processes to which it contributes. There exist a wide

range of genetic screens designed to isolate previously unidentified molecules. Scientists can use the results of these screens to provide new direction to a study that is already underway, but often the outcome initiates a new focus. The work encompassed in this dissertation describes a molecule identified through a subtractive screen that we named Bves, an acronym for **b**lood **v**essel **e**picardial **s**ubstance. We hoped that our type of screen, originally performed to discover heart-enriched genes, would guide our study of Bves and contribute to our research on heart development. However, the path that has been taken to uncover its role proves quite the contrary. This investigation of Bves rather involves a detailed examination and understanding of epithelial cell types, epithelial interactions, and epithelial cell adhesion. Here, I have not defined a mechanism to answer a fundamental question such as "how does the heart develop?" but I have contributed to our understanding of how Bves may function during heart morphogenesis because of the extensive knowledge gained about Bves in, surprisingly, epithelia.

Fundamental Functions of Epithelia

The word epithelium, introduced in 1670 by Frederik Ruysch, came from the Greek *epi* (overlying) and *thele* (nipple) to describe the skin of the lips (George, 2003). This archaic reference provides a description of just one epithelial cell type found at the edge the human mouth, but does not begin to encompass the vast numbers of cell layers qualified as epithelia. Epithelia are tightly bound sheets of cells that cover or line surfaces, cavities, and tubes and function to protect the cells or organs they overlay. Epithelial tissues can be found in most, if not all multicellular organisms, and are widely

diverse, but share significant similarities in function. Epithelia can be simple (single layer) or stratified (multilayered) and the cells can be classified into squamous (flat), cuboidal (square) or columnar (rectangular) shape, among others, by cellular morphology.

Examples from across the biological kingdoms demonstrate the versatility of this cell type. The tunicate, an evolutionarily primitive chordate, has two simple squamous epithelial monolayers found internally, the peribranchial and branchial epithelia, which form the blood compartment. The integrity of these two layers is essential to form the tight protective barrier needed between the internal and external environment of this organism (Georges, 1979). In higher chordates, many organisms such as the chick begin as a single-layered epithelium that gives rise to every tissue in the embryo. This occurs through the process of gastrulation, which initiates the formation of the three germ layers, the ectoderm, mesoderm and endoderm, which are all epithelial in nature (Duband et al., 1987). The epithelial ectoderm protects the organism while the internal structures develop following cell proliferation and differentiation.

In the adult, the columnar epithelium lining the intestinal wall provides an example of a highly evolved and specialized cell layer. The gastric mucosa serves as the primary interface between the outside world and our internal organs. This epithelium secretes digestive enzymes and defense factors to ward off ingested antigens, and regulates fluid uptake into the body (Gebbers and Laissue, 1989). Importantly, the gastrointestinal tract undergoes rapid self-renewal to supply the gut lining with healthy, properly functioning cells, which is important in maintaining its barrier function (de Santa Barbara et al., 2003). In another example, the cornea of the adult eye is a stratified

squamous epithelium. Because this epithelium provides an important external barrier, it also must demonstrate an efficient self-renewal system in order to maintain layers of healthy, properly functioning cells (Sun and Lavker, 2004). Finally, in an example from the plant kingdom, epidermal cells cover the entire stem, leaf and root of most if not all plants. This epithelium provides protection, support, and reduces water loss. As demonstrated by this wide range of examples, epithelia are an essential tissue type and serve specialized but similar functions in many different organisms.

Properties of Epithelial Cell Adhesion

Epithelial cells are maintained as structured sheets by cell junctions, which are the multi-protein complexes found at cell-cell boundaries and function to attach neighboring cells together. These junctions provide two key features of this versatile cell type: cell-cell adhesion and cell polarity. Epithelial cell-cell adhesion provides the structural integrity that is critical for proper development of the embryo and for maintenance of tissues in the adult, as well as the remodeling and differentiation of many distinct tissue layers (Kobielak and Fuchs, 2004; Thiery et al., 1985; Tucker et al., 1988). During embryonic development, the epithelial germ layers undergo dynamic morphogenetic movements in order to complete the processes of gastrulation and neurulation (Gerhart and Keller, 1986; Jamora and Fuchs, 2002; Marsden and DeSimone, 2003; Wallingford et al., 2002). Throughout these processes, the adhesive nature and integrity of the epithelia are regulated such that proper tissue rearrangements occur. For example, in the chick, cell contacts of the epiblast are dramatically loosened and then compromised entirely when specified mesoderm

undergoes an epithelial-to-mesenchymal transition (EMT) during gastrulation (Duband et al., 1987). Furthermore, the strength of cell-cell adhesion must be tightly controlled during development. Upon subsequent reorganization of mesenchymal tissue into epithelial somites, the expression of adhesion proteins such as N-CAM and N-cadherin is again observed at the cell surface after being previously down-regulated upon EMT (Bellairs et al., 1978; Cheney and Lash, 1984). In another example, a precise regulation of cell adhesion proteins is essential for normal optic placode development in *Drosophila*. In the absence of a specific adhesion protein DE-cadherin, the placode undergoes apoptosis, but overexpression results in the failure of optic placode cells to invaginate, suggesting that a titrated level of the cell adhesive forces is critical (Dumstrei et al., 2002).

Adhesive interactions between cells are also regulated by various internal cellular activities, including that of the cytoskeleton, different signaling pathways, as well as external requirements like sorting of specific cell populations (Jamora and Fuchs, 2002; Tepass, 2002; Wheelock and Johnson, 2003). The cytoskeleton establishes the cellular architecture of the epithelial sheet and the mechanical forces generated by the actin network influence cell shape and motility (Jamora and Fuchs, 2002). Filopodial projections from epithelial cells have been reported to facilitate fusion of epithelial sheets during development (Martin-Blanco et al., 2000; Raich et al., 1999; Tanaka-Matakatsu et al., 1996). The importance of epithelial cell adhesion for cell signaling is highlighted by involvement of the tightly regulated β-catenin/Wnt pathway in epithelial morphogenesis and maintenance (Bienz, 2005). Wnt-1 is an extracellular matrix-associated factor that binds the cell junction-associated protein β-catenin and stimulates

cell growth upon activation (Cadigan and Nusse, 1997; Hinck et al., 1994). As mentioned previously, EMT relies on a loosening of cell contacts, and thus a precise control of adhesive interactions. This process is accomplished by Wnt-induced uncoupling of β-catenin in the adhesive complex (Conacci-Sorrell et al., 2002; Wheelock and Johnson, 2003). Dysregulation of this pathway can result in aberrant cell adhesion and/or cancer (Birchmeier et al., 1995). In fact, the requirement for the regulation of cell adhesion in the maintenance of tissue organization has been implicated in many types of cancers, where cells have lost adhesive contacts within the tissue and become metastatic (Guilford et al., 1998). Also, the types of adhesive contacts formed between cells can establish distinct margins between morphologically homogeneous cell populations (Kullander and Klein, 2002; Perez-Moreno et al., 2003). In the developing *Xenopus*, zebrafish, and mouse embryos, an adhesion protein called PAPC is expressed in a specific pattern that regulates the epithelial organization of cells at the segmental borders between somites (Kim et al., 2000; Rhee et al., 2003; Yamamoto et al., 1998). This suggests that cell adhesion can play a role in the establishment of differential cell affinities that orchestrate the formation of tissue boundaries.

Another key feature of epithelial tissues is the establishment of apical-basal polarity. The apical surface faces the lumen, the basal surface faces the basal lamina and extracellular matrix, and the lateral surface of each cell opposes that of another cell. Cell polarity establishes the orientation of the epithelial layer, which can be critical for proper function of the tissue, and is maintained by the proper sorting of apical and basolateral proteins, which is accomplished by proper targeting of membrane vesicles from the Golgi (Wheelock and Johnson, 2003). Epithelial polarity is instituted in the

earliest stages of development. For example, in the *Drosophila* embryo, mutations in polarity genes induce overproliferation in the cell layers and exhibit malignant-like characteristics (Bilder, 2004). Similarly, when polarization is lost specifically in the neuroepithelium of zebrafish mutants, severe defects in the developing retina occur, resulting in disorganization and apoptosis of cells (Pujic and Malicki, 2001). The importance of proper cell polarity in the function of a mature epithelium is also demonstrated by studies of human autosomal dominant polycystic kidney disease. This disorder results from inappropriate membrane localization of two proteins, the NaK-ATPase and the EGF receptor, in the kidney epithelium due to a mutation in the junctional protein polycystin-1 that regulates cell polarity (Charron et al., 2000; Wilson, 1997).

Interestingly, the ultimate developmental fate of a cell may be determined by the state of cell polarity. The cell junction proteins that provide cell-cell adhesion and polarity depend on the cytoskeleton for mechanical support. The cytoskeletal network, in turn, controls the internal architecture of the cell, including the orientation of the mitotic spindle. Thus, a decrease in expression levels of cell adhesion molecules could change current state of cell polarity and architecture and may favor, for example, an asymmetric cell division. This may, in turn, result in the differentiation into a new cell type (Bilder et al., 2003; Kraut et al., 1996; Lu et al., 2001; Schober et al., 1999). Thus, state of cell polarity is a driving force in asymmetric cell divisions, which is critical for controlling cell fate, such as stem cell determination (Jan and Jan, 2001).

Clearly, the proper function of epithelial tissues relies heavily on strong cell-cell adhesion and establishment of polarity in all organisms. The players that regulate not

only the strength of cell adhesion, but establish cell polarity are the molecular components of the cellular junctional complexes. These complexes permit an epithelium to alter its intercellular relationships to meet the functional requirements of the organism as a whole (Garrod et al., 1996; Provost and Rimm, 1999; Runswick et al., 2001; Schneeberger and Lynch, 2004). Understanding the role of each type of junctional complex in the regulation of tissue integrity is critical to unraveling epithelial function, since each complex carries out a specialized role to allow proper epithelial sheet formation and to maintain the integrity of the cell layer. These cell junctions, which localize to the basolateral compartment, are divided into two categories: cell-cell junctions and cell-matrix junctions. The discussion of junctions will be limited to cell-cell junctions, which provide the adhesive interactions between neighboring cells.

Epithelial Junctional Complexes

In polarized epithelial cells, cell adhesion is mediated by junctional complexes located within the terminal bar, an archaic term describing the dense structure of proteins at an apical location within the lateral membrane. As described above, these junctions help maintain the integrity of the epithelial tissue and regulate the strength of adhesion between cells. The expression of junctional molecules often correlates with the adhesive requirements of the epithelium. For example, adhesion proteins are downregulated during developmental stages when dynamic epithelial movements are required (Duband et al., 1987). Then, upon maturation and stabilization, epithelial sheets highly express junction proteins in order to maintain a strong intercellular interaction. Four types of cell-cell junctional complexes exist along the lateral membrane

of polarized epithelia: the tight junction (TJ), the adherens junction (AJ), the desmosome, and the gap junction (GJ) (Gumbiner, 1996). The first three junctions are referred to as the epithelial or apical junctional complex (AJC) and contribute to cell-cell adhesion (Balda and Matter, 2000b; Garrod et al., 1996; Matter and Balda, 2003; Vogelmann et al., 2004). A detailed discussion of these junctions follows. The primary function of the GJ is to electrically couple cells, not cell-cell adhesion, and thus a description of GJ will not be included in this chapter.

Proteins at the TJ - The TJ, or zonula occludens, is the apical-most junction restricted to the lateral compartment of an epithelial cell layer. Under freeze-fracture microscopy, the TJ appears as a belt-like network of "sealing strands" that circumscribes the cell (Gonzalez-Mariscal et al., 2003). The TJ serves two primary functions to maintain the integrity of an epithelial sheet. First, proteins of the TJ establish apical-basal polarity and restrict the passage of molecules between the apical and basolateral lateral compartments. Second, the TJ provides a diffusion barrier, and in some cases, a water-tight seal, to prevent the undesired passage of ions or fluid across the cell layer (Balda and Matter, 2000b). For example, the TJ is critical for proper functioning of the gut epithelium. Columnar intestinal cells are the standard prototype for demonstrating the structure of these junctions, since their well-established polarization exhibits a functional significance as a selectively permeable barrier. TJs permit the gastric mucosa to retain contents in the lumen, but allow nutrients to pass through the intestinal wall (Collins, 2002). The primary transmembrane components of TJs are occludin (Furuse et al., 1993), the claudins (Furuse et al., 1998), and junctional adhesion molecule (JAM) (Martin-Padura et al., 1998). Occludin is a 4-pass

transmembrane protein and contributes to the barrier and fence functions of the TJ (Balda et al., 1996; Furuse et al., 1993; McCarthy et al., 1996; Wong and Gumbiner, 1997). However, surprisingly, occludin-null ES cells formed fully functioning TJs (Saitou et al., 2000), although the protein appeared to be a component of the TJ strands (Fujimoto, 1995). Recent data suggests that occludin may act through the Rho signaling pathway (discussed in detail below) to reorganize the actin cytoskeleton (Yu et al., 2005). The discovery of claudin proteins several years following was significant since, to this point, the protein(s) responsible for TJ barrier function had not been identified. Claudins were shown induce the formation of TJ strands, visible by freeze fracture EM (Furuse et al., 1998; Morita et al., 1999). Thus, claudins were accepted as the major structural and functional components forming the TJ barrier. Claudins are also responsible for generating cell adhesion at the TJ, although not solely, via calcium-independent adhesion properties interactions (Kubota et al., 1999). Alterations of claudin proteins result in a defective permeability barrier (Hoevel et al., 2002; Simon et al., 1999; Turksen and Troy, 2002). Currently, over 20 claudins have been discovered (Gonzalez-Mariscal et al., 2003). The third transmembrane component of TJ, JAM, is a glycoprotein with two extracellular Ig domains that exhibits roles in junction assembly and adhesion, leukocyte transmigration, platelet activation (Bazzoni, 2003; Martin-Padura et al., 1998).

In addition to the transmembrane components, a myriad of other proteins form the cytoplasmic plaque of the TJ including the ZO (zonula occludens) family of peripheral membrane proteins, ZO-1 (Stevenson et al., 1986), ZO-2 (Gumbiner et al., 1991), and ZO-3 (Haskins et al., 1998), as well as various adaptor proteins including

cingulin, symplekin, ZONAB, and Rab proteins (Balda et al., 2003; Citi et al., 1988; Marzesco et al., 2002; Sunshine et al., 2000; Takagaki and Manley, 2000). The ZO proteins have three PDZ protein-protein interaction domains, a SH3 domain, and a MAGUK domain (Gonzalez-Mariscal et al., 2000). ZO-1, the most well-characterized of the three, is a ~220kD protein (Anderson et al., 1988) and is concentrated at TJ through direct interaction with occludin (Fanning et al., 1998; Furuse et al., 1994), claudins (Itoh et al., 1999) and JAM (Bazzoni et al., 2000; Itoh et al., 2001). ZO-1 also interacts with ZO-2 and ZO-3 (Haskins et al., 1998; Wittchen et al., 1999), actin (Itoh et al., 1997; Ryeom et al., 2000), among many other proteins whose interactions are not well defined.

Although ZO-1 was the first molecule to be identified at the TJ complex (Stevenson et al., 1986), its homology to other proteins and localization patterns suggested a multifaceted role for the protein. Anderson and colleagues recognized its homology to the *Drosophila legal discs-large* (*dlg*) gene and the PSD-95/SAP-90 protein isolated from rat brain tissue (Willott et al., 1993) and added ZO-1 proteins to the family of membrane-associated guanylate kinase proteins (MAGUK) (note: the guanylate kinase domain in MAGUK proteins is thought to be inactive) (Haskins et al., 1998; Kistner et al., 1995). MAGUK proteins have functions at epithelia TJ, septate junctions in insects (Woods and Bryant, 1991) and neuronal synapses (Cho et al., 1992; Kistner et al., 1993). Like other MAGUK proteins, ZO-1 is present in several cell types where TJ are not formed and TJ proteins such as occludin are not expressed. In non-epithelial fibroblast cells, ZO-1 functions as a cross-linker between the actin cytoskeleton and the cadherin-based adhesion complex through its direct interaction with α-cat and actin

filaments (Itoh et al., 1997; Mandai et al., 1999; Yamamoto et al., 1997). It has been suggested that, in epithelial cells, a competition between TJ proteins and α-catenin exists for binding of the ZO-1 amino tail (Gonzalez-Mariscal et al., 2000). Also, at the cardiac intercalated disc, ZO-1 complexes with both N-cadherin and connexin-43, a gap junction protein (Barker et al., 2002; Gutstein et al., 2003; Toyofuku et al., 1998). Currently, ZO proteins are accepted to be scaffolding proteins within the junctional regions, due to their interaction with so many molecules. The ZO-1 C-terminal tail contains two nuclear sorting signals and interacts with a Y-box transcription factor ZONAB through its SH3 domain at the TJ to modulate TJ barrier function and in the nucleus where it may play a role in cell cycle progression (Balda and Matter, 2000a). Thus, while ZO proteins exhibit functions to organize proteins at the TJ, they may serve a dual role to transduce signals (Gonzalez-Mariscal et al., 2000).

Elucidating the endogenous function of ZO-1 has been challenging, although intensive efforts have been put forth (Fanning et al., 1998; Itoh et al., 1997; Ryeom et al., 2000). Recently, Umeda and colleages demonstrated that TJ formation was significantly delayed following a calcium switch in mouse epithelial cell clones lacking ZO-1 expression (Umeda et al., 2004). Functional redundancy between ZO family members is thought to be a primary reason for the difficulties in assessing the role of these proteins (Gonzalez-Mariscal et al., 2000).

In the past decade, the number of proteins identified that localize at or near the TJ complex has grown considerably. The Pals1/PATJ/Crumbs ternary complex localizes to the TJ and functions as an apical polarity determinant (Roh et al., 2002). The PAR-3/PAR-6/aPKC complex also localizes to TJ where it interacts directly to JAM via the

PAR-3 PDZ domain (Itoh et al., 2001). This complex also regulates cell polarity (Bowerman et al., 1997; Ebnet et al., 2001; Guo and Kemphues, 1996). In addition, MAGI/BAP-1 (Ide et al., 1999), MUPP-1(Hamazaki et al., 2002), and AF-6/Afadin (Yamamoto et al., 1997), among many others have been localizes to the TJ (Gonzalez-Mariscal et al., 2003). MAGI/BAP-1 is a tumor suppressor that co-localizes with ZO-1 and interacts with GEP, a GDP/GTP exchange protein (Mino et al., 2000). MUPP-1 interacts at TJ with PDZ claudins and JAM and has been shown to recruit Pals1 to the TJ (Roh et al., 2002). AF-6/Afadin localizes to adherens junctions (Asakura et al., 1999), but also binds JAM (Ebnet et al., 2001) and cingulin (Cordenonsi et al., 1999). Unraveling how these TJ complexes cooperate will be critical to understanding TJ biogenesis and maintenance.

Proteins at cadherin-based adhesive junctions- The AJ, also referred to as the zonula adherens, is localized basal to the TJ along the lateral compartment. The primary function of the adherens junction is the establishment of strong adhesive interaction between neighbors by interconnecting the actin network (Nagafuchi, 2001). E-cadherin, a transmembrane component of the AJ, maintains cell-cell adhesion through calcium-dependent homophilic binding (Adams et al., 1998). Intracellularly, E-cadherin is complexed with α- and β-catenin, which in turn, connect E-cadherin to the actin network (Provost and Rimm, 1999; Yap et al., 1998). Cadherin-mediated adhesion stimulates rearrangement of the cortical actin, via catenins, p120 (Reynolds et al., 1994), and other actin-binding proteins like vinculin, α-actinin, plakoglobin (Adams et al., 1996; Vasioukhin and Fuchs, 2001). In turn, cadherin-based adhesive events can also trigger various signaling pathways (Gottardi and Gumbiner, 2001; Noren et al., 2001).

This highly complex network of interactions suggests that cell adhesion is a finely tuned process and is malleable, given the requirements of the epithelial cell layer.

Recently, a new set of proteins has been placed at cadherin-based cell-cell adherens junctions called the nectin-afadin-ponsin (NAP) complex (Asakura et al., 1999; Mandai et al., 1999; Takahashi et al., 1999). Nectin is a calcium-independent immunoglobulin-like cell adhesion molecule and which binds to afadin, an actin-binding protein (Tachibana et al., 2000; Takahashi et al., 1999). Ponsin binds afadin and vinculin, and this complex localizes to forming junctions with AJ proteins (Asakura et al., 1999). AF-6/Afadin possesses a PDZ domain and provides a link to the plasma membrane of AJ (Mandai et al., 1999) through interaction with α-catenin, which also recruits ZO-1 to nectin-based adhesion sites via afadin (Pokutta et al., 2002). The importance of this adhesion system is underscored by the loss of neuroepithelial polarity in AF-6/afadin mutants, suggesting this protein provides a critical junctional element to apical/basolateral asymmetry (Zhadanov et al., 1999). Thus, nectin-based adhesion participates in epithelial morphogenesis, independently but cooperatively with both the TJ and AJ complexes (Miyoshi and Takai, 2005).

Desmosomes, a second type of adherent junction, reside more basally than the TJ and AJ along the lateral surface. Desmosomes provide additional, and more robust, mechanical strength to the cell structure by serving as anchoring sites for the intermediate filament network spanning the epithelial sheet (Garrod et al., 1996). The transmembrane linker proteins of desmosomes are desmocollin and desmoglein, which belong to the cadherin family and exhibit calcium-dependent adherence (Koch and Franke, 1994). Desmoplakin and plakoglobin interact with the transmembrane proteins

to link them to the intermediate filaments (North et al., 1999). Understanding proper desmosome formation is critical to the investigation of epithelial disease states. For example, in some forms of pemphigus, which results in blistering and epithelial edema, antibodies attack desmosomes of skin that causes a loss in cell-cell adhesion (Amagai et al., 1991).

Junction-related proteins- The Rho protein family of small GTPases, which includes Rho, Rac, and Cdc42, regulate processes like cell shape change, cell motility and cytokinesis and thus have the ability to modulate cell adhesion (Braga, 1999; Takaishi et al., 1997; Vaezi et al., 2002). When inactive, these proteins exist in a GDP-bound state and are found in the cytoplasm. Following GTP exchange, they translocate to the membrane, a process regulated by GEF and GAP proteins (Moon and Zheng, 2003; Rossman et al., 2005; Siderovski and Willard, 2005). When activated by GTP, these proteins interact with effector proteins at the membrane and trigger signaling pathways that regulate cell adhesion, cell proliferation, and tumorigenesis (Hall, 1998; Mackay and Hall, 1998; Van Aelst and D'Souza-Schorey, 1997; Yuan et al., 1998). The Rho family of GTP-binding proteins preferentially regulates actin organization (Eaton, 1997; Vasiliev et al., 2004). Specifically, Rho activates stress fiber formation, Rac1 stimulates lamellipodial formation, and Cdc42 induces filopodial extensions (Ridley, 2001; Ridley and Hall, 1992). These proteins have been shown to be necessary for formation and maintenance of cadherin cell contacts, potentially through mobilization of the actin cytoskeleton (Braga, 2002). Activation of pathways involving these proteins disrupts cadherin-mediated adhesion, altering a balance in cell-cell interaction. Also, the Rho GTPases can influence TJ barrier function (Nusrat et al., 1995). Not surprisingly,

dysregulation of these proteins has been implicated in steps of cellular transformation and alterations of adhesion status. Rho proteins can cooperate with pathways linked to EMT and even cancer, where cellular de-differentiation is accompanied by reduced cell-cell adhesion (Lozano et al., 2003).

While the broad functions of each of these three epithelial junctional complexes and associated proteins are understood, questions do remain concerning the relationships that the complexes share with one another. Also, the continual identification of new molecules both simplifies and complicates what we know about junctional formation, integrity and function. Clearly, additional investigation will be required to elucidate the associations that orchestrate junction formation and regulate epithelial development and maintenance in the adult.

Xenopus laevis as a Model for Examining Epithelial Function

Following the initial cleavage events of the fertilized egg, the cells of an embryo quickly rearrange into an epithelium. This mass of cells continues to divide and undergo significant morphogenetic movements ultimately resulting in gastrulation, the developmental event from which the ectodermal, mesodermal and endodermal germ layers arise (Leptin, 2005). The three germ layers are also epithelial in nature and give rise to specific tissues and organs of varying types. Thus, animal model systems of development offer excellent ways to examine epithelial behavior in an in vivo environment. In particular, the South African clawed frog *Xenopus laevis* (*X. laevis*) provides an important experimental tool in the work of this dissertation. *X. laevis* was chosen for a variety of reasons. The large size and external development of the oocytes

and embryos make this organism highly amenable to manipulation. The morphology of the early embryo has been well-studied and stages of development have been precisely defined by Nieuwkoop and Faber (Hubrecht-Laboratorium (Embryologisch Instituut) et al., 1967). Typically, embryos are plentiful in number and very hardy and thus, present a good model system for microinjection of tracer molecules or RNAs for loss or gain of function studies. A detailed description of epithelial movements and morphogenesis is provided in Chapter IV.

Discovery of Bves

To facilitate further investigation of heart development, which is the broad focus of study in our laboratory, we performed a subtractive hybridization screen for genes enriched in the developing chick heart at Hamburger-Hamilton (HH) stage 18 (Reese et al., 1999). *bves (**b**lood **v**essel **e**picardial **s**ubstance)* is a novel message identified through this screen. *bves*, a 1.8kb message detected by Northern blot, is expressed at high levels in both the developing and adult heart and is first seen at stage 10 of the chick embryo using this method (Reese et al., 1999). *bves* was identified independently as one of three members of the *popeye* family (*pop1a*) (Andree et al., 2000). This gene family is comprised of transmembrane proteins Pop1-3 (ranging from 292-359 a.a. in chick) that possess a C-terminal Popeye domain, the function of which is currently unknown (Figure 1) (Andree et al., 2000). *bves* shows no significant homology to any other known gene families and is highly conserved across species in which it is expressed. At present, there have been three family members identified in chick, human and mouse, and only one in *X. laevis, Danio* and *Drosophila* (Andree et al.,

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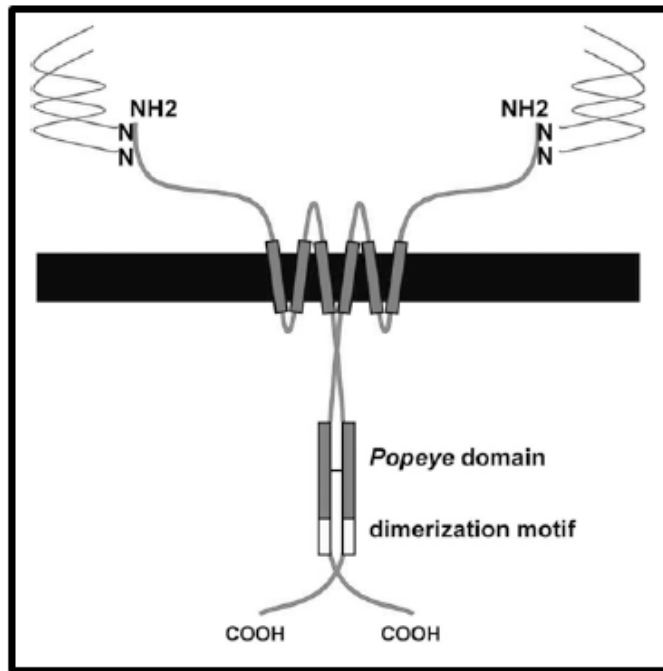
Figure 1. Predicted structure of Bves.
Bves has three transmembrane domains. The amino terminus is extracellular and has two N-linked glycosylation sites. The intracellular C-terminal tail possesses an oligomerization domain and a *Popeye* domain, shared by other members of the Bves/Pop family.

2002b; Andree et al., 2000; Hitz et al., 2002; Reese and Bader, 1999; Reese et al., 1999). Brand and colleagues have analyzed the mRNA expression patterns for members of *popeye* gene family in a variety of species including mouse, chick and *X. laevis* (Andree et al., 2002a; Andree et al., 2002b; Andree et al., 2000; Breher et al., 2004; Hitz et al., 2002). Whole mount in situ and Northern blot data from their work confirm *bves/pop1a* expression in the heart and identifies gene expression in other tissues such as skeletal muscle. The function of this gene in muscle is unknown and a null mutation in *bves/pop1a* does not result in embryonic lethality, although animals appear to have impaired muscle regeneration (Andree et al., 2002a). Meanwhile, a third group, Duncan and colleagues, independently identified Bves/Pop1a protein in heart using a monoclonal antibody (DiAngelo et al., 2001). Publications from both laboratories stressed that the transcript and protein were not expressed in the epicardium or any other epithelia. Assessment of any function for this gene in epithelia is lacking prior to the current studies.

Generation of two Bves-specific polyclonal antisera, D033 and B846, against the C-terminus of chick Bves permitted the study of the cellular distribution of the protein product (Reese et al., 1999; Wada et al., 2001). Immunohistochemical analysis of embryonic chick hearts using D033 detected Bves in the proepicardial organ (PEO) and several progenitor cell types (Figure 2A; Reese et al., 1999). The PEO is a cluster of cells that migrates as an epithelium around the heart, forming the epicardium. Select cells from the epicardium will undergo epithelial-to-mesenchymal transition and delaminate, becoming mesenchymal in nature. These cells move through the subepicardial space into the myocardial tissue and ultimately arrive at sites of coronary
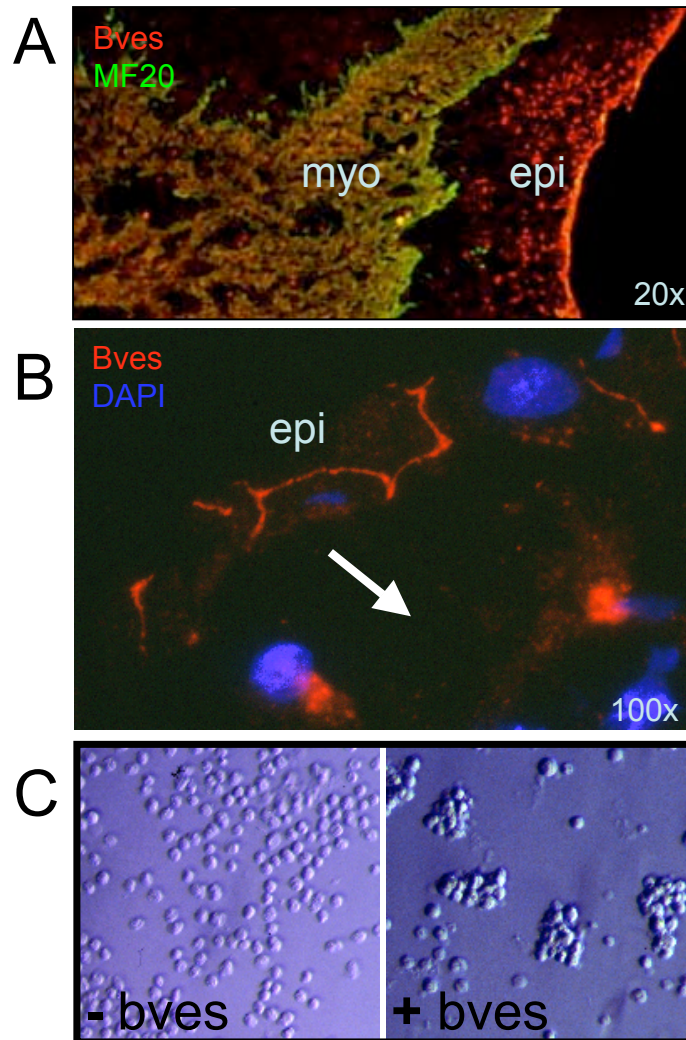
Figure 2. Initial characterization of Bves. Bves protein localization studies were performed on 9.5 day chick heart sections. D033 antisera detects Bves in the epithelial epicardium and delaminated mesenchymal cells (A). Higher power shows Bves at cell-cell contacts of the epicardium and at a perinuclear spot in cells undergoing EMT (B). Arrow represents the direction of cellular EMT movements into the subepicardial space. Transfection of Bves confers adhesive activity to non-adherent mouse L-cells (C).

vessel formation. Bves is expressed in both the PEO and epithelial epicardium, as well as migrating mesenchymal cells derived from the epicardium, and the cells that also express smooth muscle markers, indicating a forming vessel (Figure 2A, figures within Reese et al., 1999; Wada et al., 2001). This distribution pattern suggested a role for Bves in the development of coronary vasculature. A detailed localization analysis reveals a dynamic subcellular distribution of Bves from the epicardium to the single mesenchymal cells. Bves localizes to cell-cell borders in the epithelial epicardium, but is internalized and appears as a perinuclear spot in the delaminated mesenchymal cells (Figure 2B). This trafficking pattern is suggestive of a role in cell adhesion, since it resembles the characteristic expression patterns of several well-known adhesion molecules, such as E-cadherin (Adams et al., 1998; Gumbiner et al., 1988).

The B846 antisera revealed a slightly different staining pattern in the heart. Not only was Bves protein expression in the epicardium observed, the myocardium of the heart was highly positive for Bves. The finding that Bves was expressed in heart muscle correlated with the findings from the Brand and Duncan groups (Andree et al., 2002a; Andree et al., 2000; DiAngelo et al., 2001; Vasavada et al., 2004). Upon the discovery that B846 reacted robustly with the epicardial-mesothelial cell (EMC) line, an in vitro model of the epicardium (Eid et al., 1994), it became a tool widely used in our laboratory for examination of epithelia. Also, while D033 only reacts with chick, B846 reacts with all species tested thus far.

Computer-based modeling predicts that Bves possesses three hydrophobic domains. Immunocytochemical and biochemical data suggest that the N-linked glycosylated N-terminus is extracellular and the C-terminus is intracellular (Knight et al.,

2003). However, Bves lacks known motifs, such as a PDZ, SH3, or other protein-protein interaction domains, that would suggest a function. In vitro transcription/translation reactions show that Bves can embed in microsomes and is post-translationally modified (Wada et al., 2001), further supporting its existence as a transmembrane protein. Functional assays suggest that Bves exhibits homophilic binding activity. When non-adherent mouse L-fibroblasts are transfected with a full-length wtBves construct and subjected to a hanging-drop aggregation assay (Kubota et al., 1999), cells are then able to adhere to one another (Figure 2C). These initial studies reveal that Bves is a novel membrane protein that localizes to cell-cell borders in the epicardial epithelia and confers adhesiveness in non-adherent cells. These properties are suggestive of a role in adhesion and led to the subsequent characterization of Bves in other epithelial cell types. In early cell-cell interactions of sub-confluent EMCs, Bves is detected prior to E-cadherin at points of cell-cell contact (Wada et al., 2001). These findings strongly suggest a role for Bves in the initial stages of cell-cell interaction and provide further evidence that Bves may influence epithelial cell adhesion.

Preliminary data from our laboratory suggested a role in cell adhesion, a phenomenon critical to epithelial cell types, and a corresponding localization pattern in the epithelial epicardium. However, the Brand and Duncan groups disagreed that Bves and related Popeye family members were expressed in epithelial cell types. Thus, a difficult task lay ahead to prove that Bves was not only expressed in the epicardium and additional epithelia but that its expression within this cell type correlated with a function.

Summary

Although formation of adhesive contacts has been closely examined and the known adhesion molecules fulfill many of the requirements to coordinate cell adhesion, gaps in understanding of cell-cell adhesion are prevalent and cannot be addressed by current roles attributed to known adhesion molecules such as E-cadherin. Clearly, it is highly possible that a molecule possessing adhesive properties and an appropriate spatio-temporal distribution such as Bves could satisfy specific aspects of the remaining uncertainties. Thus, it is critical to determine the role of Bves during early cell-cell interaction and throughout epithelial sheet formation. My proposal attempts to integrate the understanding of epithelial contact formation in vitro with the generation, movement and maintenance of epithelia in the developing embryo. The preliminary findings that Bves is expressed in epithelia at early cell contacts leads to my central hypothesis that *Bves plays an integral role at cell junctions and in the fundamental processes of epithelial cell-cell interaction.* In Chapter II, I present an expression analysis of Bves during chick gastrulation and germ layer formation. The data show that Bves is expressed in epithelia of all three germ layers early in development. Furthermore, Bves protein is observed in epithelial tissues during organogenesis, specifically the developing epidermis, the gut endoderm, and the epicardium of the heart. These data support the hypothesis that Bves may play a role in cell adhesion and movement of epithelia during early embryogenesis. The work described in Chapter III is the first to identify a function for Bves in epithelia and supports the hypothesis that Bves contributes to establishment and/or maintenance of an epithelial integrity. I show that Bves localizes to the TJ, interacts with the ZO-1 protein complex, and is essential for TJ

integrity. In Chapter IV, I present studies illustrating that Bves proteins are critical for epithelial movements and morphogenesis in vivo. The amphibian *X. laevis* provides an excellent model system for these studies. Using morpholino oligonucleotides to deplete the *X. laevis* Bves homolog, we demonstrate the essential nature of this protein in the large-scale epithelial rearrangements that occur during early development. Together, the work I present in this dissertation provides a comprehensive examination of Bves/Pop1a proteins. My work spans a stage when doubt still existed as to whether Bves was expressed in epithelia to a point where Bves has been assigned to a specific junction and clearly has functional roles in vitro and in vivo. The implications of this work are discussed in Chapter V.

CHAPTER II

**AN EXPRESSION ANALYSIS OF BVES
IN DEVELOPING AND MATURE EPITHELIA**

Introduction

This chapter reports a broad expression analysis of Bves in epithelial cells and tissues of varied origin and cell type. Following identification of *bves* by our laboratory, the gene was independently discovered by the Brand group, who published that *pop1a* (*bves* is the accepted nomenclature for this gene) is specifically expressed in cardiac and skeletal muscle. Thus, although we demonstrated Bves protein expression in the epicardium in our first publication describing Bves, controversy arose as to whether the gene product Bves was expressed in epithelia, or whether that finding was incorrect. Based on preliminary data, we hypothesized that Bves plays an integral role at cell junctions and in the fundamental processes of epithelial cell-cell interaction. The data presented in this chapter clearly establish that the *bves/pop1a* gene product is highly expressed in epithelia derived from a wide range of tissues and cell lines. Also, expression of both transcript and gene product are demonstrated in the early embryo prior to the differentiation of cardiac or skeletal tissue. Thus, this work undoubtedly clarifies any uncertainty that Bves message or protein may be restricted to striated muscle types. Importantly, these findings have permitted the initiation of functional studies of Bves in epithelia in order to further examine the central hypothesis. These studies are discussed in Chapters III and VI.

In the initial phases of Bves characterization, protein expression was observed in the epicardium, the epithelial layer that surrounds the heart during development. Throughout dynamic phases of growth and differentiation of the embryonic heart, cells from this layer are thought to give rise to a variety of progenitors, including those with vasculogenic potential. As heart development proceeds, cells of the epicardium delaminate into the sub-epicardial space and migrate deep into the trabeculating myocardium. These cells are thought to ultimately take up residence within the endothelial lining of forming coronary vessels. To reiterate, epicardial cells begin as part of an epithelium, break free to migrate as single cells through the developing myocardial wall, and finally resume an identity as part of an epithelium. During this process Bves undergoes dynamic changes in subcellular distribution. Bves is observed at the lateral boundary between epicardial cells, but upon delamination into the sub-epicardial space, Bves is translocated perinuclearly to the Golgi (Wada et al., 2001). Upon arrival of epicardial cells to primordial coronary vessels, Bves again localizes at cell boundaries. A cell culture model of the epicardium was identified (Eid et al., 1994) and subsequently used in our laboratory for study of Bves. These cells, EMCs, are derived from a rat mesothelioma and appear to possess both the epithelial and differentiative capacities as its source (Wada et al., 2003). In a confluent monolayer of EMCs, antibodies label Bves at the lateral compartment around the cell circumference. In concert with the finding that transfected Bves confers adhesion to fibroblast cells, we developed a hypothesis that Bves not only played a role in epithelial integrity, but that its function could be cell adhesive in nature.

Our original report on this protein in the developing chick embryo demonstrated antibody reactivity to Bves in the epicardium, epicardium-derived cardiac mesenchyme, and smooth muscle of coronary arteries (Reese et al., 1999). Andree et al. reported high levels of *bves/pop1a* mRNA expression by RT-PCR and in situ hybridization in developing mouse embryos in cardiac and skeletal muscle (2000), and thus introduced the transcript as specific to striated muscle. Subsequent generation of mono- and polyclonal antibodies in our laboratory detected mouse and chicken Bves in cardiac and skeletal muscle as well as additional epithelial cells types (T. Smith, unpublished data, Wada et al., 2003). In addition, close examination of data from this publication reveals that the transcript is also present in the epithelium of the gut (Andree et al., 2000). Furthermore, Andree and co-authors detected *bves/pop1* mRNA by PCR analyses in several tissue types during embryogenesis, which did not match the presented RNA in situ hybridization analyses (Andree et al., 2000). At this point, both our group and the Brand laboratory had reported Bves message or protein expression in tissues in addition to striated muscle. Finally, Duncan and colleges independently generated an antibody to Bves/Pop1a. In their initial report, DiAngelo et al. showed high levels of expression in cardiac muscle using a monoclonal antibody to chicken Bves, but could not detect the protein in the epicardium (2001). However, in a second publication, the Duncan group demonstrated expression of Bves/Pop1a in the epicardium of a mouse (Vasavada et al., 2004). Clearly, a consensus as to the true expression pattern of this gene and gene product had not been established. As a result, any progress toward determination of its basic function was impaired. Thus, a comprehensive analysis of Bves expression in epithelial was both warranted and necessary.

Included in this chapter is a detailed investigation of Bves expression in epithelia. The analysis was performed on a variety of cell lines and on early stages of embryonic development of the chick. It is important to note that development of the entire avian embryo begins with the epithelial epiblast. Thus, all cells within the organism, whether they are adhesive or freely migratory at later stages, are derived from this epithelium and each of the three germ layers, the ectoderm, mesoderm and endoderm, begin as epithelia (Duband et al., 1988). Differentiated cardiac and skeletal muscle cells are not present at these early stages. Thus, these studies show that Bves message and protein are expressed during early development and, importantly, are present in cells other than myocytes. The distribution of Bves at the lateral membrane is conserved in all epithelia analyzed, which suggests a conserved function in this cell type. Taken together, the distribution of Bves demonstrated in this investigation indicates that this gene product not only has a function in striated muscle, but also in epithelial morphogenesis.

Materials and Methods

Antibodies and immunohistochemical analyses

Two antisera against chick Bves have been previously reported (Reese et al., 1999; Wada et al., 2001; Wada et al., 2003). Antiserum DO33 reacts with only with avian species (Reese et al., 1999). Antiserum B846 reacts with avian and mammalian Bves. While the specificity of these antisera has been reported, further characterization is given here to document their reactivities. We have recently developed a bank of monoclonal antibodies against mouse Bves that do not cross-react with the chicken

protein. These antibodies are currently being characterized (T. Smith, unpublished data). Other monoclonal antibodies used include: ZO-1, 1:250 (Zymed), Desmin, 1:100 (Sigma); Flag 1:500, (Sigma); MF20 hybridoma supernatant, 1:4 (Hybridoma Bank). ZO-1, a well-characterized component of the TJ, is marker of epithelial junctions to which the localization of Bves is compared. Secondary antibodies were conjugated to Alexa 488 and Alexa 568, 1:3000 (Molecular Probes). Samples were counter-stained with DAPI (Roche) to visualize nuclei. Standard methods for mono- and polyclonal antibodies were used for tissue sections and cultured cells (Bader et al., 1982; Reese et al., 1999; Wada et al., 2003). Secondary antibodies (Alexa) were used at manufacturer's specifications. Negative controls included peptide competition, no primary antibody application, and non-immune antibody treatment. All of these preparations revealed no reactivity and are not shown. Western blotting experiments were conducted using standard protocols (Ausubel et al., 2002). α-mouse and α-rabbit alkaline-phosphatase conjugated secondary antibodies were purchased from Sigma and used at a 1:10,000 dilution. Tissue sections and cell culture slides were fixed 10 minutes in 70% methanol for α-Bves antibodies. Reactivity is reduced with PFA or formalin fixation.

Embryos and tissue preparation

Fertilized white leghorn eggs were obtained from Spafus/Trelow Farms and all animal protocols have been approved by Vanderbilt University. Chick embryos were staged using the standard staging protocols (Hamburger, 1951). Whole embryo or tissue samples (gut) for both chick and mouse were dissected, washed with PBS, and

embedded in Tissue TEK OCT compound (Sakura). Tissue freezing molds were frozen in a dry ice bath and stored at –80ºC. Blocks of frozen tissue were cryosectioned at 8μm thickness on a Jung CM 3000 cryostat (Leica) and placed on slides, which were stored at –20ºC until processing. Immunostaining was performed as described above.

Cell culture

Epicardial-mesothelial cells (EMC) were obtained from Hoda Eid (Eid et al., 1994) and grown as previously described (Wada et al., 2001; Wada et al., 2003). HCA-7 cells, a human intestinal carcinoma line, and Caco-2 colon epithelial cells were obtained from Dr. Robert Coffey (Vanderbilt University) and HEK293, HeLa, and 4T-1 mammary cells were a gift from Dr. L. Matrisian, (Vanderbilt University). Human corneal epithelial (HCE) cells were kindly provided by Dr. K. Araki-Sasaki (Araki-Sasaki et al., 1995). NRK cells were a gift from the Nephrology Department of Vanderbilt University. Madin-Darby Canine Kidney (MDCK) epithelial cells, CHO cells, and COS cells were purchased from ATCC. All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM-Biowhittaker) supplemented with 10% FBS and penicillin/streptomycin cocktail. Cell lines were passaged upon reaching confluency as previously described.

Transient transfection of chick *bves*

Chick *bves* was previously cloned as a cDNA product of a subtractive library screen (Reese et al., 1999). A full-length chick *bves* with a 3' Flag tag was generated using PCR and cloned in frame into pCIneo at the *SalI/NotI* sites (Promega). Primer sequences are as follows:

5'SalI - AGAGCTAGCGTCGACTTCAAGATGGACACTACGGCA and 3' NotI/flag TACATATGCGGCCGCCTACTTGTCATCGTCGTCCTTGTAGTCAGGCAGCCGCTGC AGCTC. COS fibroblast cells were transiently transfected with pCIneo/*bves* (or positive and negative control plasmids) using the Fugene6 Transfection reagent (Roche).

Generation of chick epidermal cultures

Epidermal cultures were generated as follows: skin (epidermis and dermis) was removed from chick embryos staged to HH 30-32 and rinsed in PBS. The skin was incubated in Dispase II (Invitrogen) for 5 min. at 37ºC, and transferred to media for recovery. The epidermis was separated from the dermis, transferred to chamber slides (Lab-Tek), and fragmented with forceps to create small clumps. Epidermal patches were grown for 24-48 hours in M199 media (Cellgro) with the following additives: 10% Tryptose (Invitrogen), Penicillin/Streptomycin (Cellgro), 5% FBS, and 1% chick serum. Epidermal patches were subsequently processed for immunofluorescence as described below.

RT-PCR analyses

Human epithelial cells grown to confluency were washed 1x with serum-free media and harvested in Trizol Reagent (Invitrogen) using 1 ml per 2 plates of cells. RNA was extracted following the standard protocol (Invitrogen). Whole chick embryos and tissues were isolated under sterile conditions, and RNA was extracted with the Trizol Reagent system. Approximately 50ng of RNA template was used for each reaction. RT-PCR was performed using the Titanium One-Step kit (BD Biosciences

Clontech). The RT step was performed for 60 minutes at 50ºC. PCR was performed

using the following conditions: 94ºC for 5 minutes, 40 cycles of 94ºC for 30 sec, an

annealing step for 30 sec at primer-specific temperatures (listed below), and 68ºC for 3

minutes. Final extension was 68ºC for 2 minutes. Chick Bves primers are specific to the

bves/pop1a message and do not amplify other splice variants. The primer sets used for

RT-PCR reactions are listed as follows:

Gene product	5' sequence	3' sequence	Size(bp)	T_{opt} (ºC)
Chick Bves	TTTCAACGAGACTGCATGTG	GCCTGTCATCAACTGATGTT	434	52.3
Chick GAPDH	TATTGGCCGCCTGGTCACC	CACGATGCATTGCTGACA	419	57.5
Chick Cytokeratin	GTGCTCAGCATCTGCCTGCA	TGTTTCCTTCTCGCCAGCCG	322	63.3
Human Bves	TGTGAAAACTGGAGAGAGAT	TTCTCTTGACCAGCATAAAA	600	58.0

Results

Characterization of α-Bves antisera

Two polyclonal antisera (DO33 and B846) were previously generated against the

C-terminus of chicken Bves but a detailed characterization of their binding activities has

not been performed. The following experiments establish the reactivity of these antisera

for Bves. Recognition of chicken Bves protein by B846 was established using Western

Blot analysis of Flag-tagged chicken *bves* transfected into COS-7 cells (Figure 3A, lane

2). B846 binds a protein with the same mobility as α-Flag and does not react with non-

transfected cells. Previously, Bves was detected by D033 by Western Blot and

published by our laboratory (Reese et al., 1999). Specificity of B846 and D033 was also

demonstrated by immunofluorescence microscopy as both antisera colocalize with α-

Flag in COS cells transfected with Flag-tagged *bves* (Figure 3B,C). Peptide competition

Figure 3. Characterization of α-Bves polyclonal antisera.

Specificity of the B846 antisera is determined by Western Blot. B846 recognizes a ~52kD band from COS cell lysate transfected with a Flag-tagged chicken Bves construct (lane 2). Minor bands are unglycosylated forms of Bves (Knight et al., 2003). No reactivity is detected with untransfected COS lysate (lane 1). a-Flag antibody recognizes a band of similar mobility (lane 3). Specificity of B846 (B) and D033 (C) is demonstrated by detection of transfected chicken Bves in COS cells processed for immunofluorescence. Bves antisera is labeled using Alexa 488 (green) secondary antibody and a-Flag antibody staining is represented by Alexa 568 (red) secondary antibody. A merge shows colocalization between Bves and Flag. Cell nuclei are stained blue with DAPI. The Bves protein sequence depicts the epitopes of D033 (red) and B846 (blue) antisera that are found within the C-terminus (D). Putative hydrophobic/transmembrane domains are represented in green text.

D

MDTTAISPLTPLGVIPDLKNATSVPFNETACENWKEIHIHLVFEVANICFAAGLVIPTTLNLHMIFLRGLL
TVGCALFIWATLYRCALDIMIWNBVFLVVNLLHFTYLVYKRRPIKIEKELSSLYKRMFEPLHVPPELFQ
RLTGQFCNIQTLKTGQAYAAEDKTSVDDRLSILLKGKMKVSYRGHFLHNIYPCAFIDSPEFRSTQMNRs
GEKFQVTIADDNCKFLCWSRERLTYFLETEPFLYEIFKYLIGKDITNKLYSLNDPTTLNDKASKIDRQP
8LCSQLSVMQMRNSMARsSSDSEDGLQMFLRGTSSSSSLRPGRTSPYLRTSAKMKPIEESVEDDVFEA
PSAEKLELQRLP

33

studies extinguish antiserum reactivity, further establishing the specificity of these reagents (Reese et al., 1999). These results demonstrate that the B846 and D033 polyclonal antibodies identify the Bves protein and are useful tools for studying the expression patterns of Bves.

The location of the antibody epitopes, D033 and B846, within the full-length chick Bves protein sequence is shown in Figure 3D. All experimental evidence thus far suggests that these epitopes are specific to the Bves/Pop1a isoform. Extensive blast searches do not reveal these epitopes in other *pop* gene products. However, an analysis of antisera reactivity with different vertebrates determined that D033 detects Bves in the avian class, while B846 recognizes the protein in a variety of species including avian, amphibian, and mammalian classes.

Differences in Bves localization with DO33 and B846

While the protein expression patterns of Bves revealed by DO33 and B846 predominantly overlap, specific variations in antigen labeling exist. These differences are best exemplified by the reaction of these two sera with sections through the developing heart. As previously reported (Reese et al., 1999), DO33 labels Bves in the proepicardial organ (PEO), epicardium, and delaminated mesenchyme of the heart at stage 18 (Figure 4A,B). Reaction of D033 antisera with the myocardium is minimal or reduced. In contrast, B846 detects Bves in the PEO, epicardium, and delaminated mesenchyme but also stains myocardial cells, as visualized by colocalization with α-desmin (Figure 4C). A view at higher magnification demonstrates that Bves localizes to cell-cell borders of the epicardial epithelium (Figure 4D, see arrow).
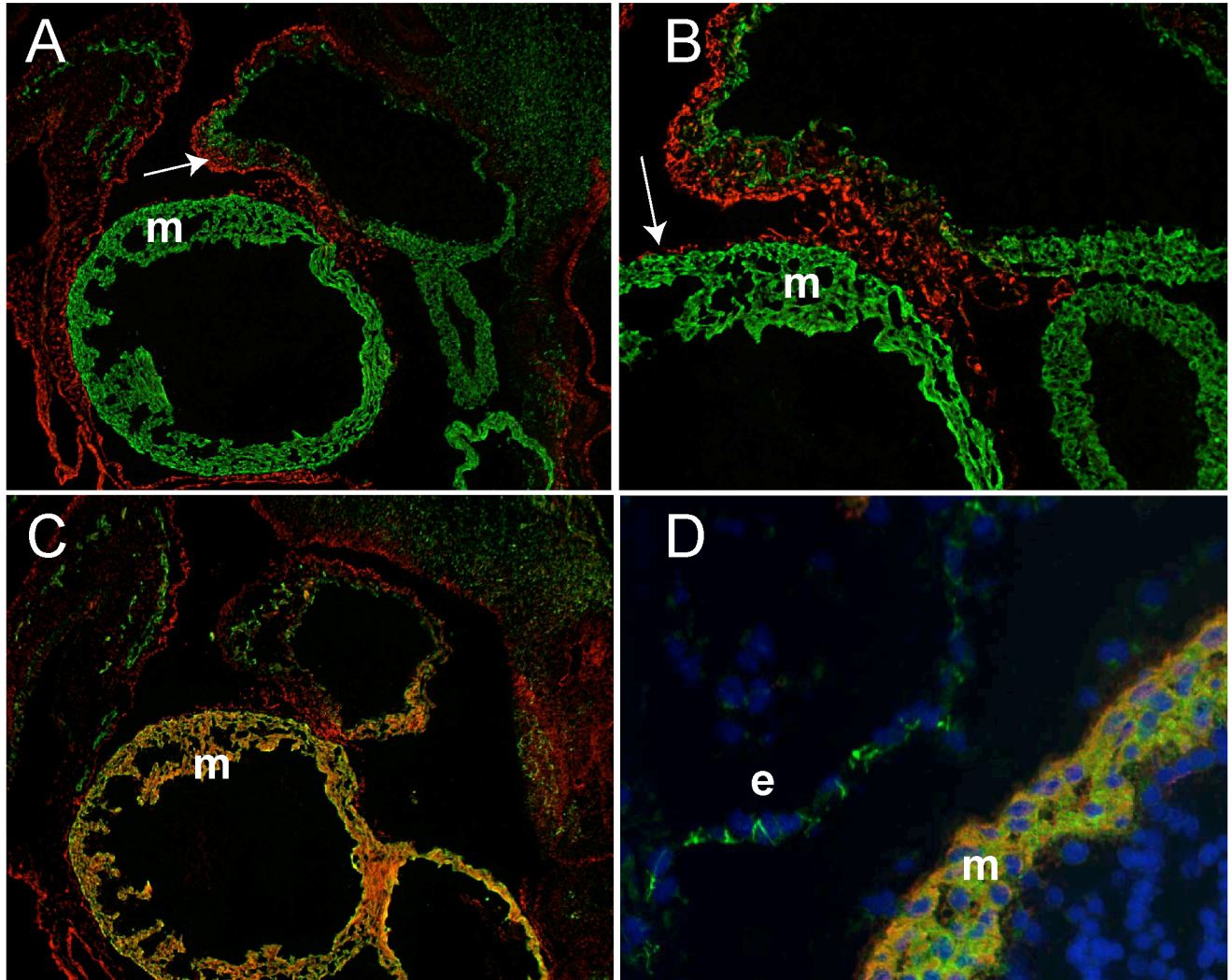
Figure 4. Patterns of B846 and D033 antisera in the developing chick heart.
Transverse sections of HH18 chick embryos show the proepicardial organ (PEO) migration over the heart. The PEO (arrow) is positive for Bves, as detected in red by D033, while anti-desmin labels the myocardium (m) in green (A). The epicardium is formed (arrow) as the Bves-positive PEO migrates from the liver primordium over the surface of the myocardium, a process observed at higher power (B). Labeling of a serial section with B846 detects Bves protein (red) in the migrating PEO/epicardium as well as the heart tube, which is also labeled with anti-desmin in green (C). A view of a section through a HH31 heart at high magnification is consistent with a previous report that Bves protein localizes to the cell-cell borders of the epicardium (e), as labeled with B846 in green (D). Bves is expressed in the epicardium and colocalizes with MF20 (red) in cardiac myocytes (m).

Transverse sections of HH 31 embryos also reveal subtle differences between the two antisera. Both B846 and D033 label the epidermis (Figure 5A,C). D033 strongly labels somites but is not reactive with the developing neural tube (Figure 5A,B), whereas B846 selectively labels the ependyma (Figure 5C,D). B846 does not label somites at this stage, because at this point in development, somites are no longer epithelial in nature, and have begun to migrate as individual cells (Duband et al., 1987). Additionally, in chick epithelial cells that are reactive with both antisera, such as the epicardium (Figure 4) and the epidermis (see Figure 10), B846 has a more distinct labeling pattern at cell membranes while D033 has a more diffuse signal. There are several explanations for this variation. First, it is possible that antigen presentation in different cell types varies slightly for the two sera. This could be the result of the interaction of Bves with accessory proteins in a tissue-specific manner. In addition, multiple splice variations of these genes have been identified. Thus, it is possible that immunochemical reagents, whether they are mono- or polyclonal antibodies, recognize variant isoforms of a gene family to different degrees. For example, we have generated monoclonal antibodies for mouse Bves that recognize variant subcellular distribution patterns. Also, we are currently screening antibodies to related family members, Bves-2 (Pop2) and Bves-3 (Pop3) (T. Smith, unpublished data). While the present study has clearly demonstrated the expression during the early phases of embryogenesis and suggest a possible function at this time, further analyses with these additional tools will aid in delineating the expression of all gene family members.
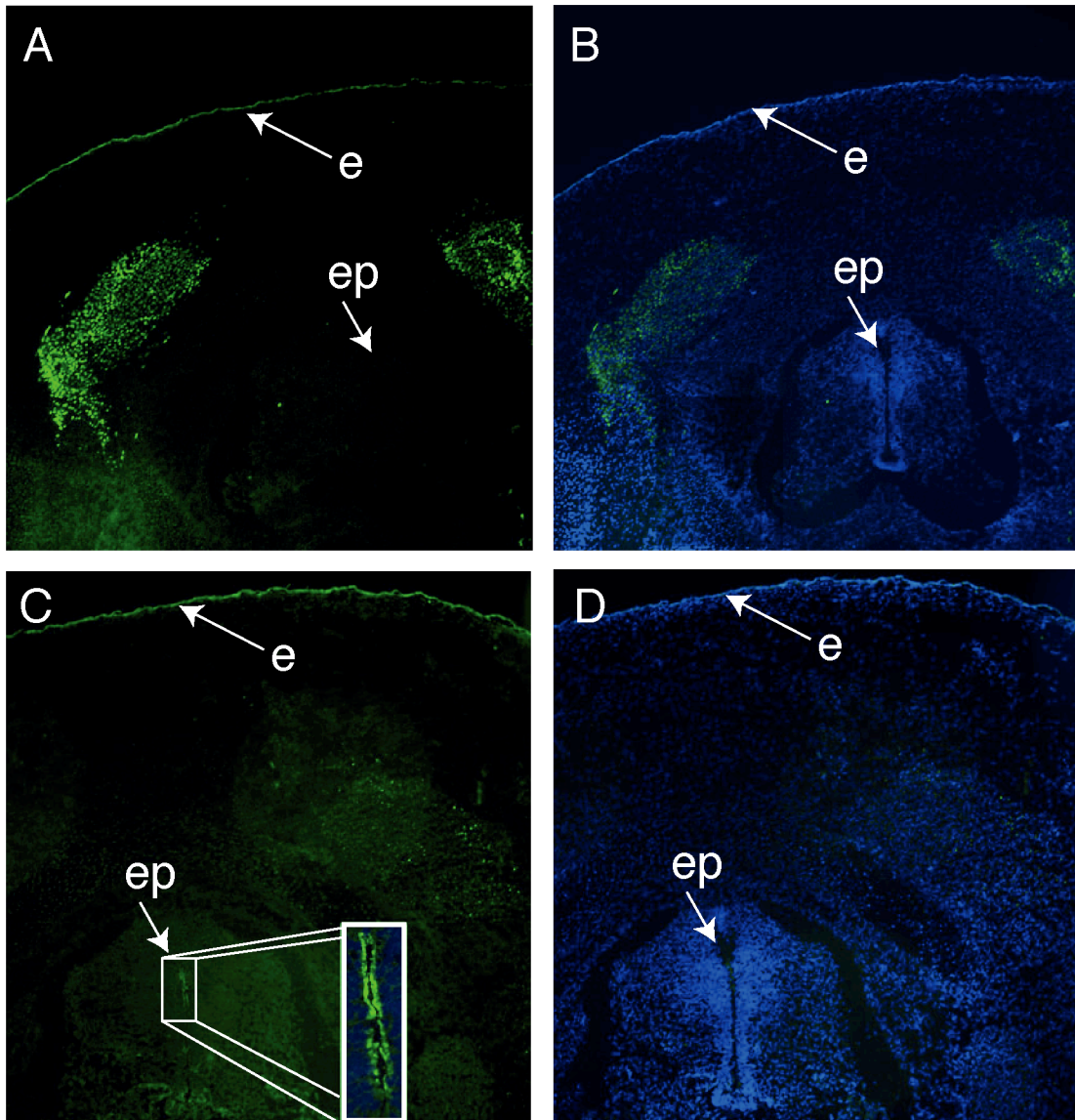
Figure 5. Patterns of Bves antisera in chick embryos.
B846 and D033 affinity differences are observed in cross-sections of developing chick (HH31). D033 labels epidermis (e) and developing somites (A) whereas B846 detects Bves in the epidermis and the developing ependyma (ep) (C). An inset shows a magnification of the ependymal staining in C. Images in B and D show DAPI-labeled nuclei.

Bves membrane localization pattern in various epithelial cells

At present, the expression of *bves* in epithelial cells is controversial. Here, antibody labeling with the B846 reagent, which recognizes Bves in a wide range of species, demonstrates α-Bves reactivity in various epithelial cell lines. Not only are many clonal epithelial cell lines widely available, clonal lines permit analysis of pure cell populations without contaminating cells from primary tissue sources. To determine whether Bves is a conserved component of epithelia, we analyzed its expression in several epithelial cell lines of varying origin, including several cancer lines. We detected Bves in many epithelial cell types and observed a pattern that paralleled what was seen in the EMCs, an epicardially-derived rat cell line (Figure 6A). Labeling of Bves at the cell membrane was observed in epithelial lines including MDCK, a simple cuboidal kidney epithelium (Figure 6B), columnar Caco-2 human colon adenocarcinoma and HCA-7 cells (Figure 6C, G), cuboidal 4T-1 mouse mammary tumor cells (Figure 6D), and human corneal cells (HCE), a stratified squamous cell line (Figure 6E). Expression was not restricted exclusively to heart tissue or a specific type of epithelia unlike, for example, TJ protein JEAP, which is only endogenously expressed in various exocrine cells (Nishimura et al., 2002). While Bves appears to be expressed at cell-cell contacts in fibroblastic NRK cells (Figure 6H), it is not expressed in all fibroblast cell lines, as shown in CHO cells and HEK cells (Figure 6F,I). These data demonstrate that Bves is a conserved component of divergent epithelial cell types. It is important to note that several new monoclonal antibodies directed against mouse Bves react with mouse epithelial tissue and various mammalian cell lines, yielding a similar localization pattern
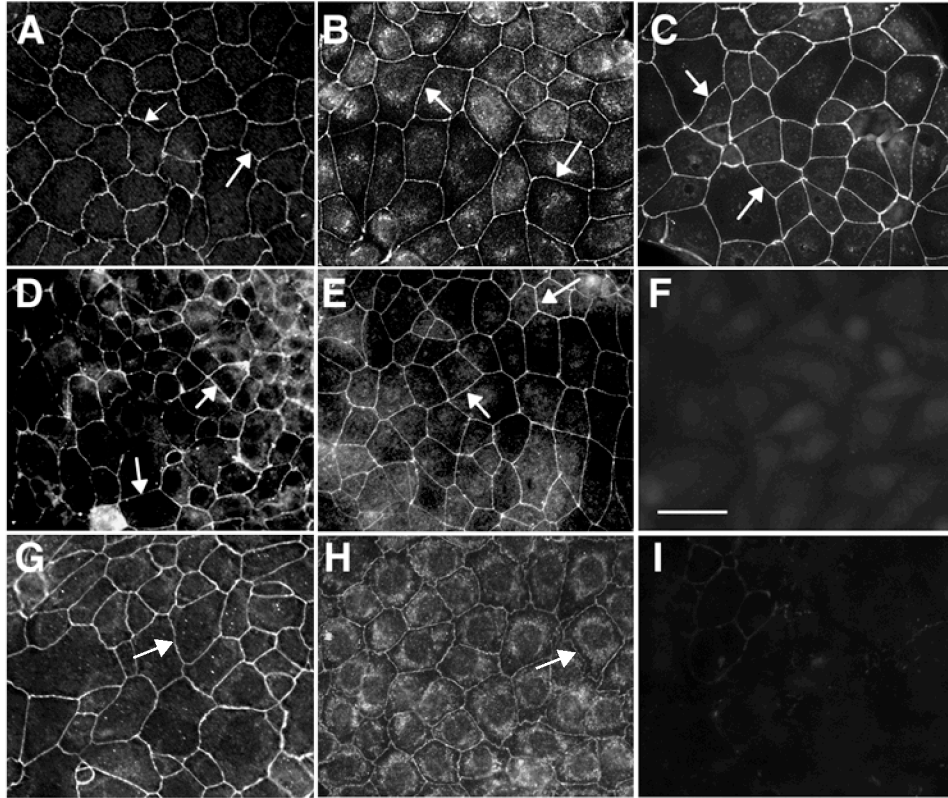
Fig 6. Bves expression in confluent epithelial cell lines.
The B846 polyclonal α-Bves antibody labels Bves around the cell periphery of confluent monolayers of different epithelial cell lines. (A) Epicardial Mesothelial Cells (EMCs). (B) MDCK cells. (C) Caco-2 cells. (D) Mouse 4T-1 mammary cells. (E) Human corneal epithelial (HCE) cells. (F) CHO cells. (G) HCA-7 cells. (H) NRK cells. (I) HEK cells. Arrows denote membrane labeling. Intracellular labeling of Bves in the Golgi occurs in several cell lines. Scale bar, 50 μm.

at cell borders (T. Smith, unpublished data). Detection of the Bves/Pop1a protein in epithelia has been confirmed by Vasavada et al. (2004).

bves mRNA expression developing and mature epithelia

RT-PCR analyses confirmed the presence of *bves* mRNA in epithelial cell lines and tissues. Human epithelial lines HCA-7 (Figure 7A), Caco-2, and HCE express *bves*, while HEK cells are negative (not shown), which correlates with the results of B846 immunostaining (Figure 6). In embryos, *bves* is most highly expressed in cardiac myocytes after the formation of a definitive heart tube has fully developed (Andree et al., 2000; Reese and Bader, 1999). In a recent study, Andree et al. *(*2000) generated RT-PCR products from several epithelia in addition to the heart, and numerous ESTs from non-muscle sources were previously reported in a variety of organisms (NCBI). However, *bves* mRNA was not detected at high levels in developing chick embryos (HH 11-18) using whole mount in situ hybridization (Andree et al., 2000). Therefore, we used RT-PCR analysis to determine whether *bves* mRNA is detectable in the early chick embryo prior to heart tube formation. As seen in Figure 7B, a *bves* RT-PCR product was observed in HH 6 whole embryo preparations at the predicted mobility. At this time, germ layer differentiation is occurring anteriorly while gastrulation continues posteriorly. As expected, the Bves transcript was also detected in HH 24 embryos that have developed a primordial heart tube (Figure 7B).

Additional RT-PCR analyses were conducted to determine whether *bves* could also be detected in extracardiac cell types after the heart is formed, specifically in the ectoderm/skin and gut, as we were particularly interested in the development of
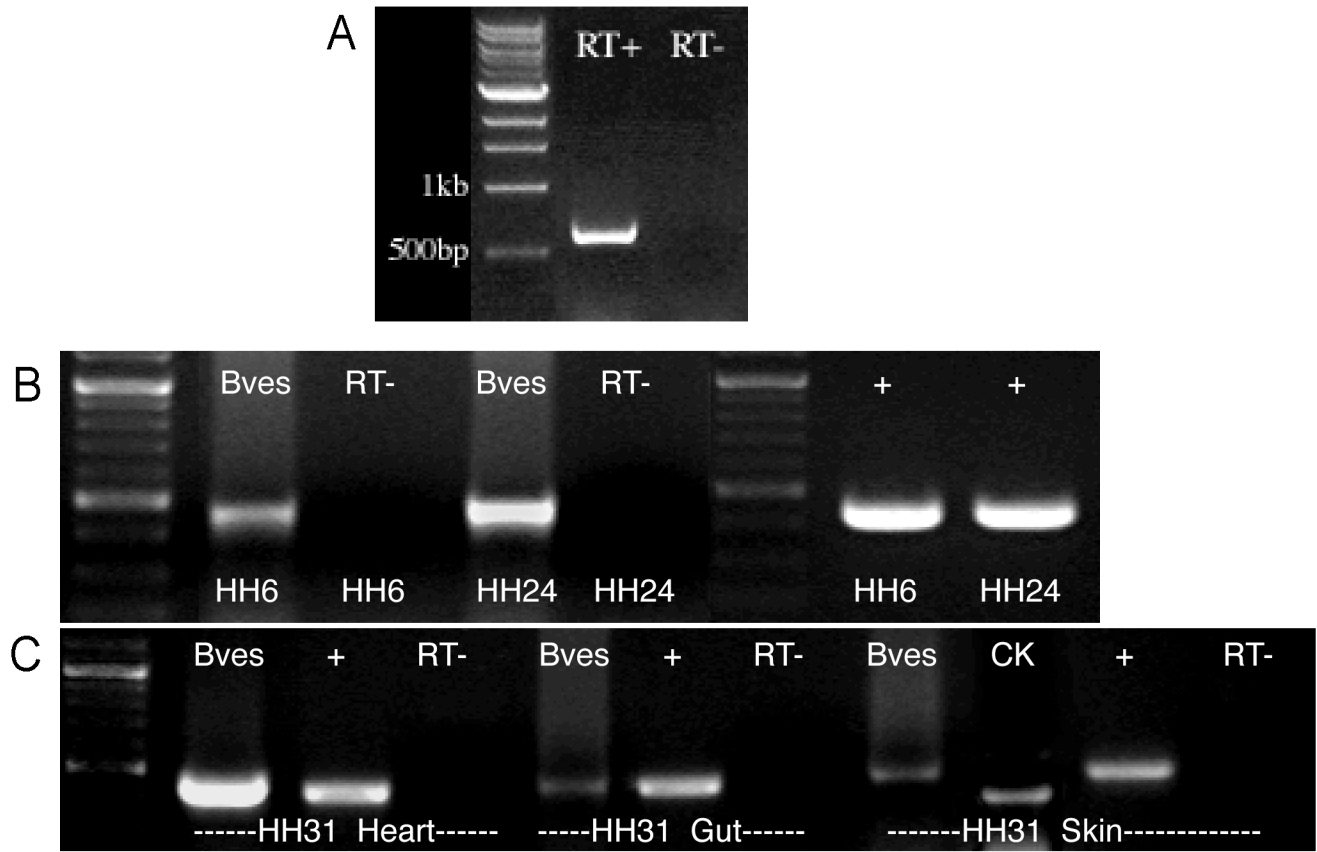
Figure 7. *bves* **expression in epithelial cells and chick embryos.**
Bves mRNA is detected in the HCA-7 clonal intestinal epithelial cell line (A). Total RNA was extracted from whole chick embryos staged at HH6, HH24, and HH31 using Trizol reagent. RT-PCR was performed using primers specific to chicken Bves. Bves mRNA was detected in both HH6 and HH24 (B). RT- reactions for HH6 and HH24 show no contamination of genomic DNA. GAPDH controls (+) for HH6 and HH24 verify input of RNA. RT-PCR on HH31 embryonic tissue detects chicken Bves in total RNA from heart, gut, and skin (C). GAPDH primers serve as a positive control (+) and the negative control RT- reaction shows no product. A cytokeratin (CK) transcript was detected in the skin as expected (C).

epithelial structures in these two organ systems. Expression of a *bves* transcript was observed in the ectoderm/skin and the developing gut (small intestine) at HH 31 (Figure 7C). As expected, *bves* was detected in the developing heart (Figure 7C). These data indicate that *bves* message is present in a variety of embryonic cell types, in addition to the heart, and that investigation of the protein expression at the cell and tissue levels is warranted.

Localization of Bves protein during gastrulation

The gastrulating embryo is a complex structure comprised of both epithelial and mesenchymal elements. Following initial cleavage stages, the avian embryo becomes a two-layered blastoderm with a dorsal epiblast and a ventral hypoblast. The embryo proper is derived entirely from the epithelial epiblast/definitive ectoderm. Primitive streak formation occurs upon the thickening of the epiblast, marking the initial ingression of mesendodermal precursors. The cells migrating into the blastocoel undergo an epithelial-to-mesenchymal transition. A specific population of these ingressing cells is programmed to become endoderm and revert to an epithelial phenotype as they intercalate with hypoblast cells (Lawson and Schoenwolf, 2003). Some of the remaining mesodermal precursors will remain mesenchymal, while others will revert to epithelia upon formation of specific structures, such as somites (Pourquie et al., 1995). Immunocytochemical methods using both antisera were employed to localize Bves protein in the gastrulating embryo at stage 4. At this time, Bves is detected in the epiblast/forming ectoderm (Figure 8A, B, see arrows). At higher magnification, Bves is seen at the cell membrane in the epithelium of the
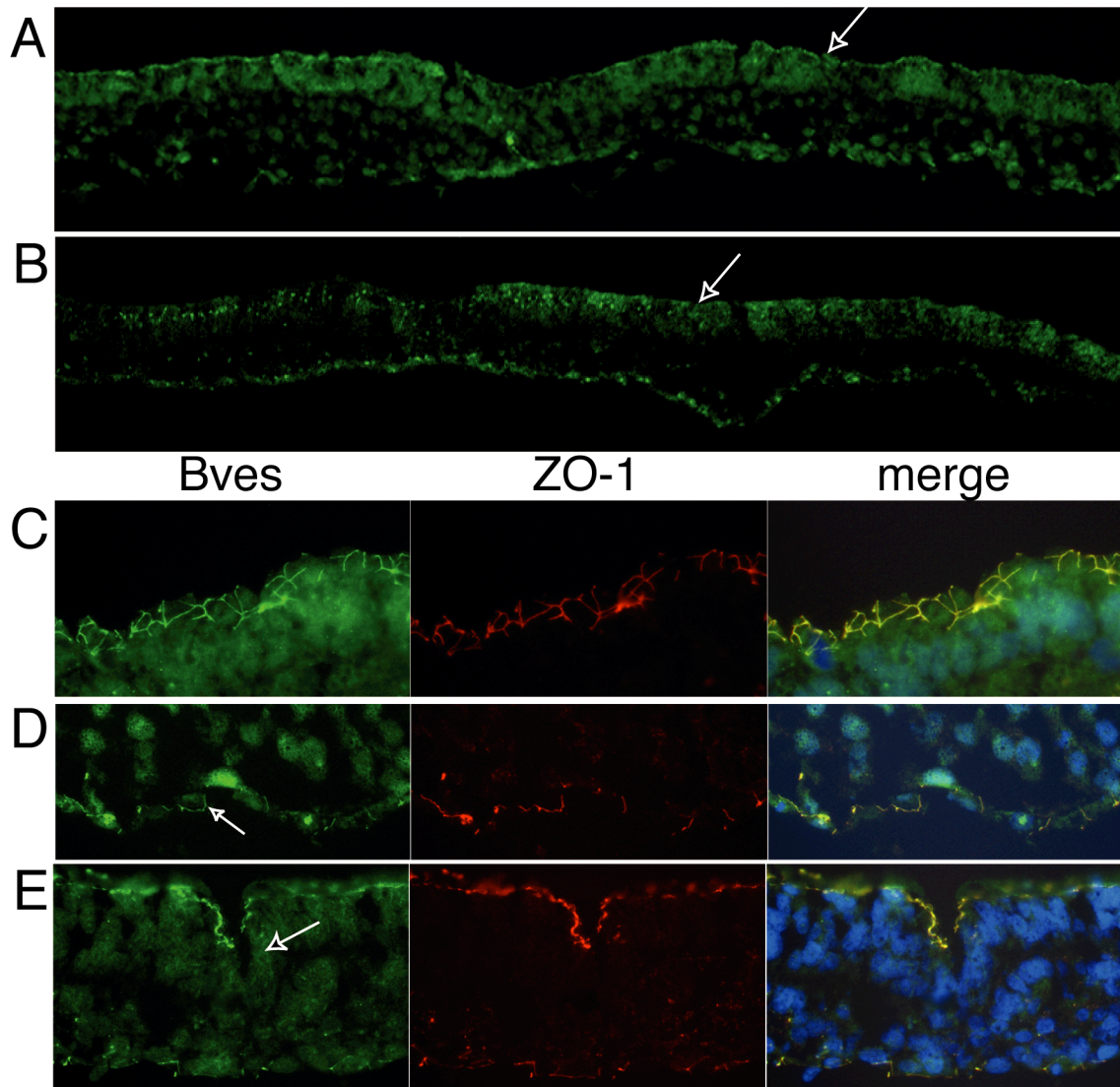
Figure 8. Expression of Bves prior to and during chick gastrulation.
HH 4 embryos labeled with B846 show Bves expression in the epiblast (A, arrow). This posterior section of the embryo has not yet undergone gastrulation. D033 labeling of the same HH 4 embryo reveals a similar pattern of staining in the epiblast (B, arrow). Higher power images of an HH 6 embryo undergoing gastrulation allow the observation of distinct Bves localization. B846 labels Bves in the epiblast/definitive ectoderm at cell-cell borders, as indicated by colocalization with ZO-1 (C). Regions of the hypoblast/developing endoderm also express Bves at cell membranes, as detected by B846 antisera (D, arrow). At the primitive streak, B846 detects Bves in cells that are involuting, a pattern similar to that of ZO-1 (E). Arrow denotes loss of membrane staining of ingressing cells.

epiblast/ectoderm (Figure 8C). Colocalization with ZO-1, a TJ marker, demonstrated cell membrane deposition of Bves in the epithelial epiblast/ectoderm in these specimens. Bves is also detected at cell-cell borders in the ventral region of the gastrulating chick embryo (Figure 8D, see arrow). At present, we cannot determine whether this staining is hypoblast or newly forming endoderm. Interestingly, Bves protein is distributed apically/laterally in cells of the primitive streak and it colocalizes with ZO-1 (a section posterior to Henson's node in a stage 6 embryo is shown, Figure 8E). As cells ingress either as individuals or as groups to form endoderm and mesoderm, Bves protein is eliminated or greatly reduced at the cell surface (Figure 8E, see arrow). The reactivity seen in these cells is above background staining. Still, it should be noted that newly gastrulated cells do not traffic Bves protein to their cell surface when they are in a mesenchymal state. This reduction of membrane staining of Bves is similar to that observed during epithelial/mesenchymal transition in vivo (Reese et al., 1999; Wada et al., 2001) or in vitro (Wada et al., 2003).

Localization of Bves during early germ layer differentiation

Development of the chick continues with the differentiation of the three definitive germ layers. Ectoderm and endoderm primarily remain epithelia throughout development with specific exceptions, such as neural crest cells (Selleck and Bronner-Fraser, 1996). The mesoderm can, however, undergo various epithelial and mesenchymal phases depending on the structure to be generated. Because one of our central hypotheses is that Bves plays a general role in cell/cell adhesion in epithelia, we sought to determine whether Bves is present in epithelial elements of the three germ

layers. As seen in Figure 9, Bves is clearly present at membranes in cells of the definitive ectoderm (A and B) of a HH 8 embryo. After gastrulation is initiated, the formation and closure of the neural tube proceeds. Bves is expressed at the luminal surface of the developing neural tube, which was once continuous with the apical surface of the ectoderm. It is also interesting to note that the connection between the surface ectoderm and the developing neural tube remains positive for Bves and ZO-1 for a brief period before complete loss of contact between these structures (Figure 9B, see arrow). Bves staining is also observed in the endoderm of the open foregut (not shown). As previously noted, Bves protein was not detected at high levels in newly gastrulated mesoderm, especially at the cell membrane (Figure 9E, see arrow). Since avian cells gastrulate individually or in small groups, elimination of Bves from the cell surface was not unexpected.

We next examined the expression of Bves protein in a HH 11 embryo as mesodermal cells form various epithelial structures. Findings in Figure 8 exemplify a recurring pattern of Bves deposition observed during the differentiation of mesoderm. Specifically, Bves is present at the apical/lateral regions of mesodermally-derived epithelia, similar to the ZO-1 pattern. After neurulation is underway, the paraxial mesoderm begins to organize into epithelial somites. During this process of mesodermal epithelialization, B846 detects Bves along the inner margin of the somites (Figure 9D,E), which is the apical surface of this epithelium. Also, the epithelial cells of the somites become attached by TJ (Bellairs, 1979), which can be observed by ZO-1 staining (Figure 9D). D033 labels Bves in the somite also, but the distribution within the epithelium is not as highly localized (Figure 9C). The intermediate and lateral plate

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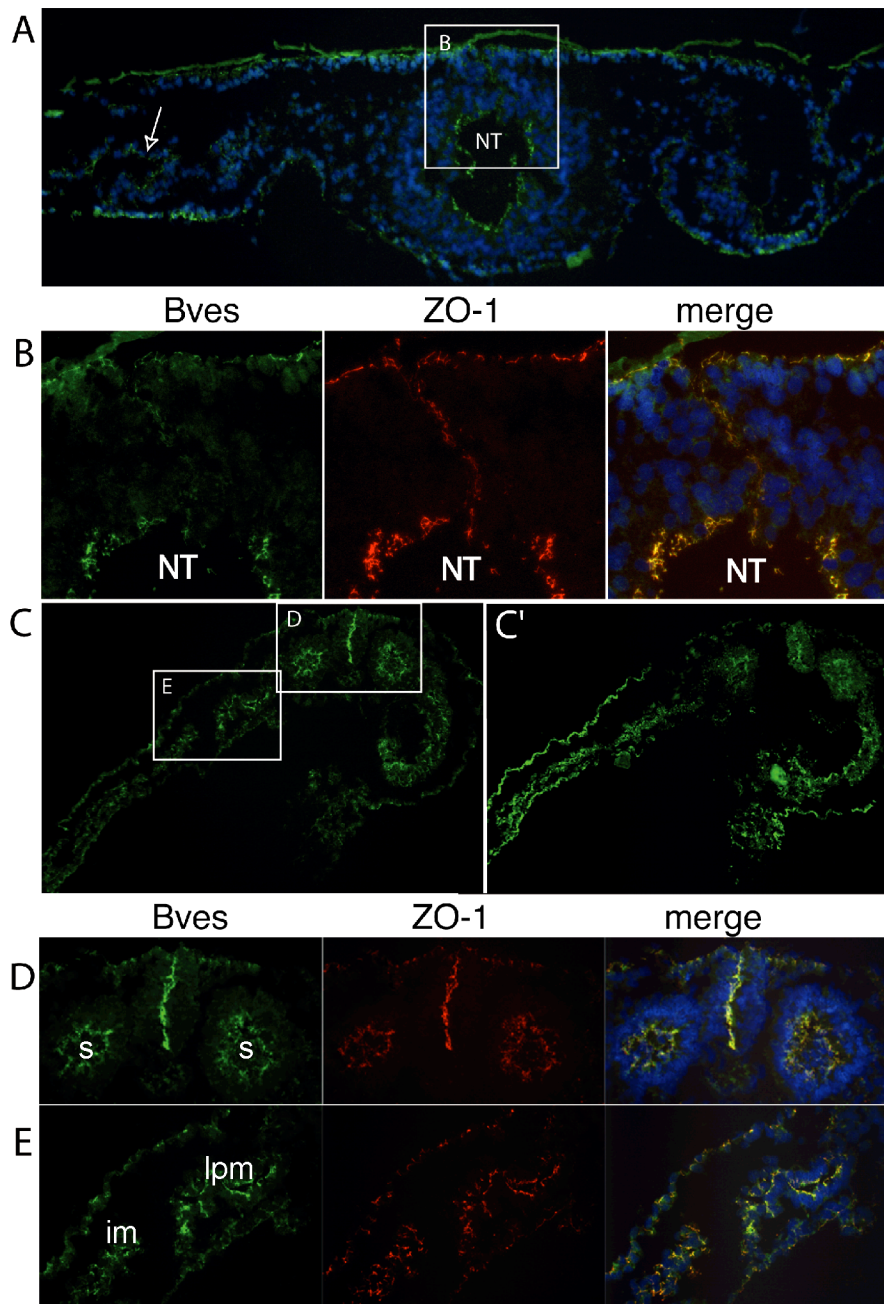
Figure 9. Bves protein expression during germ layer formation.
An embryo staged at HH 8 is labeled with B846 (A-C). At the termination of neural tube closure, Bves protein is expressed in the ectoderm, endoderm, and the epithelium that forms the lumen of the neural tube (NT). Mesodermal labeling by α-Bves antisera at this stage is observed (see arrow). A higher power of the NT (B) illustrates the apical/lateral distribution of Bves, like ZO-1, in the epithelial structures of the ectoderm. B846 labeling of an HH11 embryo undergoing somitogenesis reveals Bves protein expression at the ectoderm/endoderm layers, the neural tube, the epithelial somites, and epithelialized intermediate and lateral mesoderm (C). D033 labeling of a serial section is also shown (C'). Higher magnification of epithelial somites (s) reveal a colocalization of B846 staining with that of ZO-1 (D). Both antibodies label the apical region of the NT epithelia and the somitic epithelia and colocalize as observed in the merge (D, far right). The intermediate (im) and lateral plate mesoderm (lpm) are both epithelial in nature and express Bves, as detected by B846. Colocalization with ZO-1 is observed with a merged image (E)

mesoderm are also formed at this time by the clustering of cells into epithelial structures. Interestingly, when these mesodermal structures were examined with α-Bves antisera, the protein was observed at the apical/lateral regions (facing inward toward the coelomic cavity) (Figure 9E). Bves shares the same labeling pattern as TJ protein ZO-1 in the intermediate and lateral plate mesoderm (Figure 9E). Additionally, the notochord is positive for Bves (data not shown). Thus, the initial epithelial structures formed by the partitioning of mesoderm are positive for Bves. However, B846 antisera do not detect Bves protein at high levels in mesodermally-derived mesenchyme. As development proceeds, mesodermal derivatives such as cardiac, smooth, and skeletal muscle cells are positive for α-Bves antisera.

Bves is expressed in the epithelia of the developing skin and gut

From the data presented above, it is apparent that Bves, like other adhesive proteins, is expressed in epithelial structures of the developing organism. Because Bves is initially detected in both ectoderm and endoderm, we wished to determine the pattern of Bves expression in differentiating skin, an ectodermal derivative, and gut epithelium, which is originates as endoderm. The outermost surface cell layer of the developing embryo, the periderm, arises from an initial single layer of ectoderm. This one cell layer quickly becomes two layers, the outer being the temporary periderm, and the inner being the cuboidal germinal layer that gives rise to the stratified epidermis. The cells of the periderm die, keratinize, and are eventually exfoliated from the underlying stratified epidermis several days prior to hatching (Isokawa et al., 1996). In the developing skin, the epidermis that surrounds the embryo expresses Bves and the protein appears to be

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restricted to cell-cell borders (Figure 10A-C). The developing dermis is negative when labeled with α-Bves antibodies and remains so throughout development.

We have established an epidermal in vitro culture system in order to study Bves protein expression and function in more detail. After growing patches of epidermis for several days in culture, antibodies to Bves detect the protein at the cell membranes, similar to the observed pattern in vivo (Figure 10D). These data suggest that Bves protein retains its expression in the ectoderm as it differentiates to form the epidermis.

The endoderm of the chick embryo forms the mucosa/epithelium of the gut tube and undergoes dynamic changes throughout gut morphogenesis (Roberts, 2000). Since Bves expression in the early stage endoderm was pronounced, we sought to determine whether Bves protein could be detected in the gut epithelium. As shown in Figure 11, Bves is expressed throughout gut development. Closure of the gut tube occurs around HH 20-22 and at this stage, the gut epithelium forms a simple tube (Figure 11A). The developing gut of the chick embryo undergoes rapid proliferation and morphogenesis. As the primordial villi are begin to take shape, the resulting gut epithelium is more undulated and the epithelial surface area is enlarged (Figure 11B-D). Protein expression analysis of Bves in a HH 41 gut reveals that Bves is restricted to the gut epithelial cells, specifically to the apical/lateral region of cell-cell borders (Figure 11E). Mesenchyme derived from lateral splanchnic mesoderm that surrounds the gastric mucosa is negative. Sections through adult mouse gut demonstrate Bves distribution in a mature and developed intestine (Figure 11F,G). Bves is highly expressed epithelium in the crypts and villi, and localizes to an apical-lateral spot between neighboring columnar cells, reminiscent of a cell junction protein (Figure 11G,
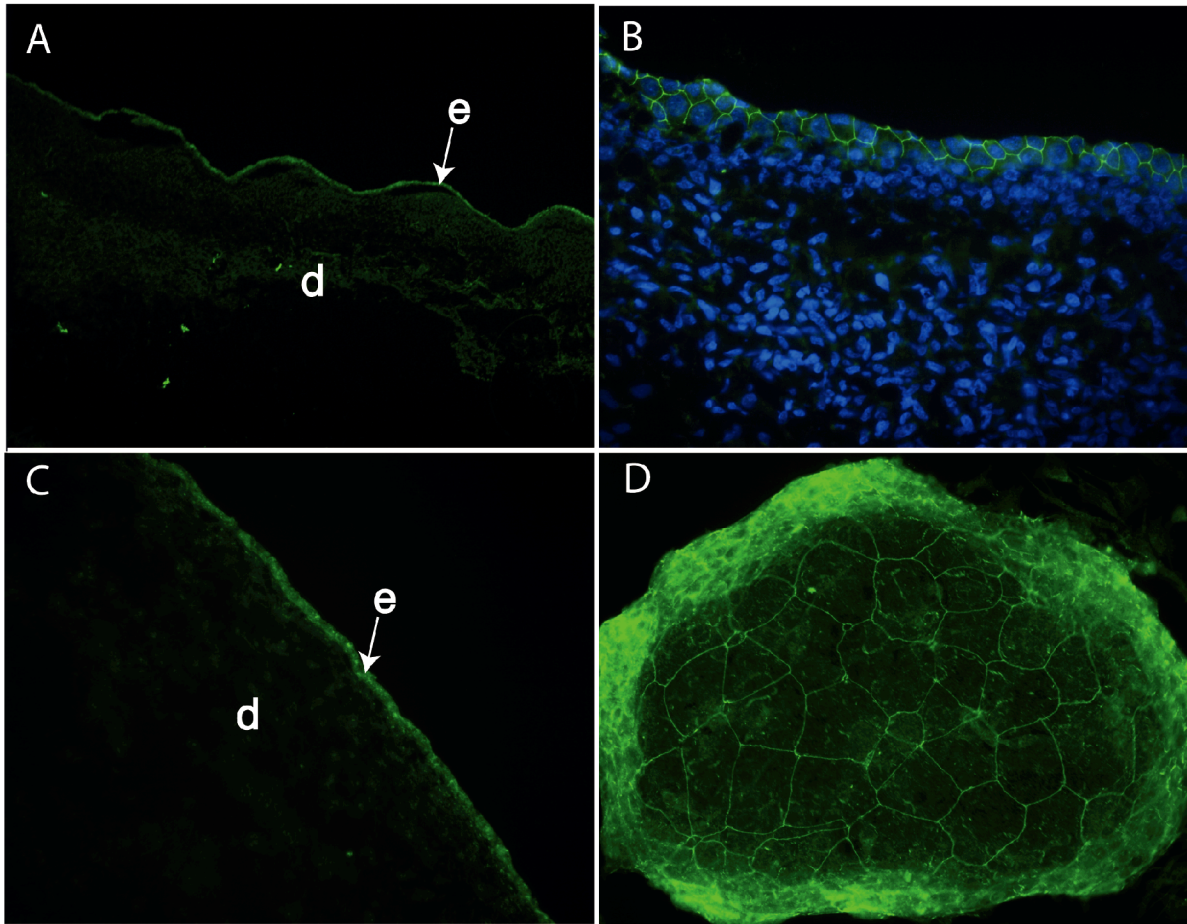
Figure 10. Bves distribution in the chick epidermis.
B846 (A) and D033 (C) α-Bves antisera exclusively label the epidermis (e) of HH 31 chick embryos in cross sections. The dermis (d) is negative. At higher power, epidermis labeled with B846 detects Bves protein at cell borders of the epidermal epithelium (B). An epidermal culture, extracted from HH 31 embryo and grown on a glass slide, was fixed and labeled with B846 antisera (D). This image shows Bves at cell membranes in vitro, a pattern consistent with the pattern observed in vivo (B).
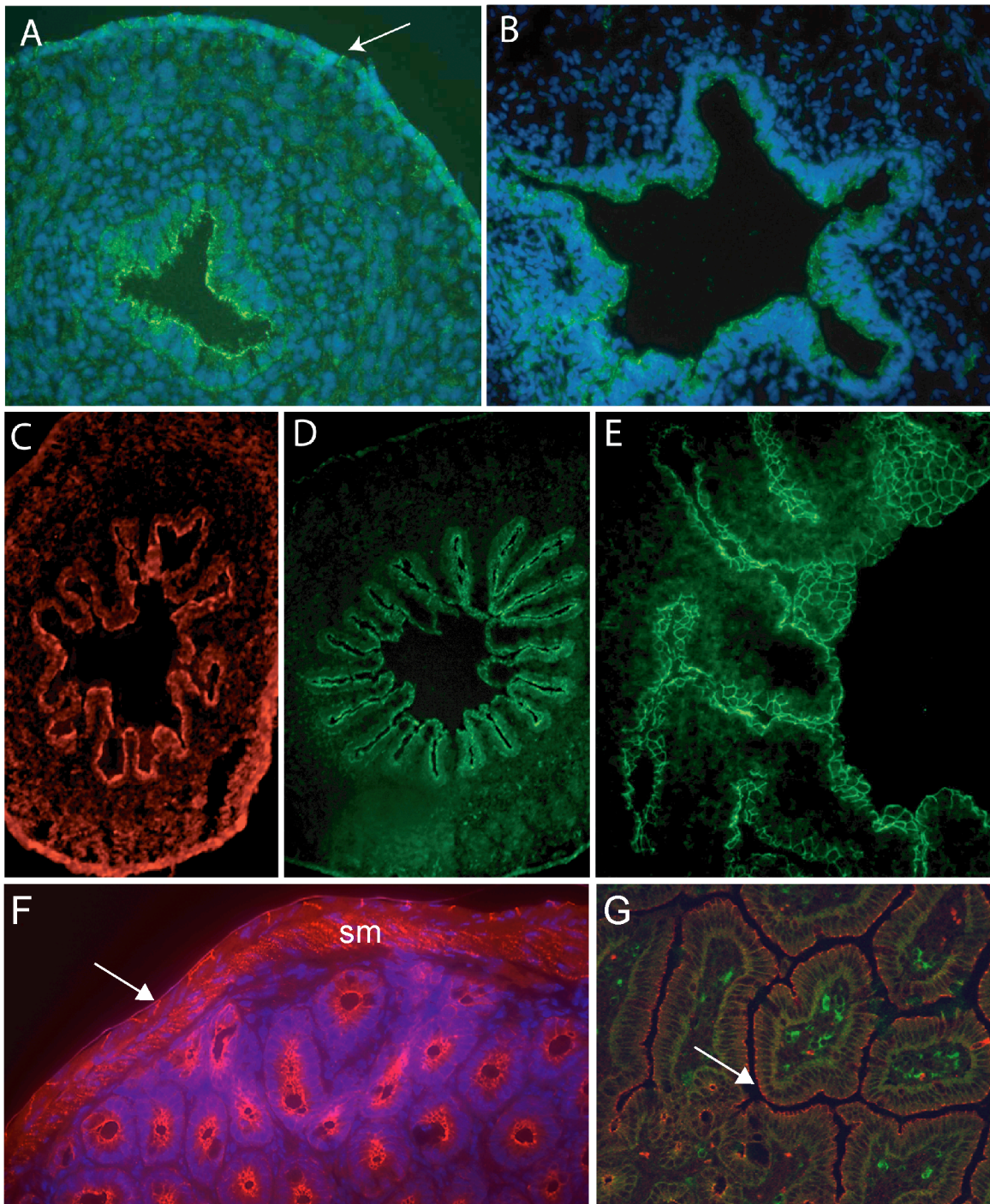
Figure 11. Bves expression throughout gut development.
Chick embryos were labeled with B846 at various stages of gut development. (A) Day 7 (HH 30-31). (B) Day 8 (HH 33-34). (C) Day 11 (HH 37). (D) Day 13 (HH 39). (E) Day 15 (HH 41). B846 antisera labels the gastric epithelium throughout gut morphogenesis and villi extension. The serosa, the outer epithelial covering of the gut, is also positive for Bves (A, arrow). Labeled sections through adult mouse gastric mucosa show Bves is highly expressed in the serosa (arrow), smooth muscle (sm), and crypts (F). DAPI labels cell nuclei. Cross sections through villi demonstrate Bves is located at a punctate apical/lateral spot between cells of the columnar epithelium (G, red). Sections were co-labeled with E-cadherin (G, green).

arrow). Bves is expressed in the smooth muscle layer surrounding the gut tube (Figure 11F) In addition, Bves is expressed in the serosal epithelium (Figure 11A,F, see arrows), the mesothelial covering of the gut. This layer is similar to the epicardium that surrounds the developing heart. D033 staining of the gut is much weaker and not shown.

Discussion

bves is a novel message identified through a subtractive hybridization screen for genes enriched in the heart (Reese et al., 1999). *bves* encodes a transmembrane protein that localizes to cell borders of the epicardium and is postulated to play a role in cell adhesion. While it is clear that Bves and related gene products are also expressed in adult striated and smooth muscle cells, the distribution of these proteins in epithelia had not been critically examined. This chapter presents a broad expression analysis of Bves in a variety of epithelial cells and tissues. We demonstrate that Bves is expressed in epithelia of various type and origin, and that the distribution at cell borders is conserved. A specific focus is placed on Bves expression during stages of chick gastrulation and germ layer formation, since these processes involve dynamic movements and morphogenesis of epithelial layers. Taken together, these data clearly demonstrate that Bves protein is a component of epithelial cell types and support the hypothesis that Bves may play a role in cell adhesion and movement of epithelia during early embryogenesis.

Previous studies have demonstrated that members of the *popeye* gene family are highly expressed in cardiac and skeletal myocytes during embryogenesis (Andree et

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al., 2000; Breher et al., 2004; DiAngelo et al., 2001; Vasavada et al., 2004). While our initial findings were consistent with these publications, we detected Bves in epicardial cells in vivo and in vitro in contrast to what was reported by the other two laboratories (Reese et al., 1999; Wada et al., 2001; Wada et al., 2003). Over the next few years, however, several pieces of evidence emerged, suggesting that Bves was more widely expressed than we had expected (Andree et al., 2000; Breher et al., 2004; Vasavada et al., 2004; Wada et al., 2001). We detected *bves* transcripts in many mammalian epithelial cell lines and during early chick embryogenesis (Wada et al., 2003; Osler and Bader, 2004; Ripley et al., 2004). In addition, EST database analyses reveal that transcripts of the *popeye* gene family are widely distributed in various cell types at different stages of vertebrate development. Interestingly, the whole mount studies of Andree et al. also demonstrate *popeye* gene expression in the gut epithelium, a cell type highly reactive with our antisera (2000). However, the group stressed that *bves/pop1a* expression in the epicardium was never observed. Thus, a consensus of Bves expression in epithelia was still lacking. Discrepancy in expression patterns between data from Andree et al. (2000) and the findings from our laboratory (Wada et al., 2003) could be due to a low level of *bves* gene expression in those epithelia that produce the transcripts. In addition, the lifetime of the mRNA message in epithelia is unknown and could potentially be short-lived, impairing its detection. Thus, variable sensitivity of assay systems may account for the apparent variation in mRNA. The present analysis demonstrates that Bves message and protein are indeed present in a wide range of epithelial cell lines and tissues. Importantly, Bves is spatio-temporally

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localized during early embryogenesis when epithelial movements are critical for the initial differentiative events.

Assessment of the Bves expression pattern from published work along with the present study suggests important roles during development and in the adult. Since the observation that Bves is an epithelial component (Vasavada et al., 2004; Wada et al., 2001), we have demonstrated that Bves is expressed in a wide range of epithelial cell lines, various other tissues, the epicardium of the developing heart, the primordial endoderm, mesoderm and ectoderm in chick (Osler and Bader, 2004) and frog embryos as well as the epithelial components of the developing eye (Ripley et al., 2004) and gut (Osler and Bader, 2004). Given the broad distribution of Bves in developing and mature epithelia, the present data suggest a conserved, significant and wide-ranging function of this novel gene family. The presence of Bves in the early embryo suggests that this protein may have a conserved function in epithelial structures, specifically during gastrulation and germ layer differentiation. Our expression studies demonstrate that Bves is present in epithelia undergoing dynamic rearrangements such as migration of epicardium, movement of the epiblast, cavitation of the lateral plate, formation of the epithelial somite, and tubulogenesis in the neuroectoderm and intermediate mesoderm. Later, after gross movements and reshaping of these embryonic structures is completed, Bves expression may be downregulated in epithelia but remain highly expressed in the heart and skeletal muscles. If Bves indeed functions as an adhesion molecule between actively moving cells, its expression in embryonic epithelia and contracting muscle supports a common role in two different and highly specialized cell types. Also, Bves is localized to the lateral regions of epithelial cells in culture as well as

epithelial components of the developing embryo, including the epiblast, neural tube, somites, and newly formed intermediate, somatic, and splanchnic mesoderms. Bves in these regions suggests that the protein plays a role in the generation or maintenance of cell polarity. For example, while these embryonic epithelial structures are highly dynamic during development, the necessity of cell adhesion during morphogenesis is critical for proper shaping of these layers. This is consistent with previous data suggesting an adhesive role for this protein (Wada et al., 2001). From this analysis, it is not possible to determine whether Bves is restricted to a certain type of epithelia or is universally expressed. Clearly, Bves expression was explored in a wide range of epithelia and the present data is consistent with the hypothesis that Bves is involved in cell-cell adhesion in various types of epithelia. Also, the spatio-temporal localization of Bves mimics the pattern of proteins with roles in cell interaction and cell polarity. Furthermore, this work, which is published (Osler and Bader, 2004), has generated a foundation for future investigation, which will be geared toward unraveling the molecular mechanism of Bves during development.

CHAPTER III

BVES MODULATES EPITHELIAL INTEGRITY
THROUGH AN INTERACTION AT THE TIGHT JUNCTION

Introduction

We first identified Bves as a transmembrane protein that localized to the lateral compartment of the epithelial epicardium. Bves traffics to sites of cell-cell contact in cultured epicardial cells and promotes adhesion following transfection into non-adherent fibroblastic L-cells, reminiscent of a cell adhesion molecule. Currently, no function for Bves in relation to epithelial cell adhesion has been identified. We hypothesize that Bves plays a role at cell junctions to establish and/or modulate cell adhesion or cell-cell interactions in epithelial cell types. In this study, we establish that Bves regulates epithelial integrity and that this function may be associated with a role at the TJ. We report that Bves localizes with ZO-1 and occludin, markers of the TJ, in polarized epithelial cell lines and in vivo. We find that the behavior of Bves following low calcium challenge or TPA treatment mimics that observed for ZO-1 and is distinct from AJ proteins such as E-cadherin. Furthermore, GST pull-down experiments show an interaction between ZO-1 and the intracellular C-terminal tail of Bves. Finally, we demonstrate that Bves modulates TJ integrity, as indicated by the loss of transepithelial resistance and junction protein localization at the membrane following Bves knock-down in cultured cells. This study is the first to identify a function for Bves in epithelia and supports the hypothesis that Bves contributes to the establishment and/or maintenance of epithelial cell interaction.

As discussed in Chapter I, epithelial cells are defined by cell-cell adhesion and polarity, which are established by the AJC found on the lateral surface between neighboring cells (Gumbiner, 1996). TJ, AJ, and desmosomes provide structural support via a connection to the internal cytoskeletal networks (Garrod et al., 1996; Gonzalez-Mariscal et al., 2003; Nagafuchi, 2001). The two key functions of the TJ are the establishment of the apical-basal polarity to retain complexes and/or receptors within the proper membrane domain, and the creation of a water-tight seal to prevent the undesired passage of ions or fluid through the cell layer (Balda and Matter, 2000b). Primary components of TJs are transmembrane elements such as occludin (Furuse et al., 1993) and the claudins (Furuse et al., 1998) and peripheral membrane proteins ZO-1 (Stevenson et al., 1986), ZO-2 (Gumbiner et al., 1991), and ZO-3 (Haskins et al., 1998). The AJ, found immediately below the TJ, provides the core adhesive interaction between neighbors by interconnecting the actin network (Nagafuchi, 2001). E-cadherin, a transmembrane component of the AJ, maintains cell-cell adhesion through calcium-dependent homophilic binding (Adams et al., 1998). Desmosomes provide additional mechanical strength to the cell structure by serving as anchoring sites for the intermediate filament network spanning the epithelial sheet (Getsios et al., 2004). These three junctional networks regulate the strength of adhesion between cells, thus allowing epithelia to modulate their integrity depending on the functional requirements of the cell layer (Garrod et al., 1996; Provost and Rimm, 1999; Runswick et al., 2001; Schneeberger and Lynch, 2004). For example, during embryonic development, the epithelial germ layers undergo dynamic morphogenetic movements in order to complete the processes of gastrulation and neurulation (Gerhart and Keller, 1986; Marsden and

DeSimone, 2003; Wallingford et al., 2002). During these processes, the adhesive nature and integrity of the epithelia are regulated such that proper tissue rearrangements occur. Understanding the contributory role of each junctional complex in the regulation of tissue integrity is critical to unraveling the intricacies of epithelial cell-cell interactions.

The function of each junctional complex is generally understood and numerous components have been identified (Balda and Matter, 2000b; Garrod et al., 1996; Gonzalez-Mariscal et al., 2003; Gumbiner, 1996). However, additional roles for and interactions between junctional proteins are continuously revealed and thus, the discovery of novel regulators and components of cell junctions is essential for gaining insight into the mechanism by which junctions are established during formation and establishment of epithelial integrity. Chapter II localizes Bves to the lateral border of epithelial cells where the cell junctions reside. Because the current literature fails to demonstrate any function for Bves in epithelia, a critical step toward a comprehensive understanding of Bves is assignment to a specific junction complex within the lateral compartment. Since each junction is known for a particular role, the placement of Bves at a certain junction will drive the direction of future experiments and will facilitate in narrowing the potential function of the protein. For example, if Bves were found to be a desmosomal component, a next experiment might involve a determination of how Bves might influence the binding strength of the complex. Likewise, if Bves were found to be a TJ component, subsequent investigations would assess potential roles in barrier function or establishment of polarity. Clearly, identification of Bves within a specific junction is necessary to the forward progress of its characterization as a whole. The

current chapter describes work that designates Bves as a functional component of the TJ.

Localizing a putative junction protein to a distinct molecular complex can be accomplished through several means, including co-localization microscopy studies, cell culture challenges to junction integrity, and interaction screens. The use of available immunoreagents and microscopy can quickly and easily colocalize a protein of interest with known junctional markers. Ultrastructural analysis can be used to confirm light and confocal microscopic data. Secondly, a wide variety of cell culture methods and treatments have been used to identify components of certain junctions and/or draw similarities between proteins. For example, performing assays in calcium-depleted media can address the calcium dependency of a protein's function. Alternatively, treatment with pharmacological agents or detergents can be used to assess the membrane properties of the protein in question as compared with defined junctional markers. Experiments such as these are useful in assigning proteins to a particular junction based on the response to various treatments. Finally, a most convincing method to assign a protein at a specific junction is demonstrating a physical interaction with a known junctional component. Yeast two-hybrid analysis, co-immunoprecipitations, and GST pull-down experiments are among the variety of methods commonly used to identify or verify protein-protein interactions. Identifying interactions provides a pathway for further experimentation, such as isolating the exact binding sequences, and allows the generation of additional tools, such as deletion constructs, for the next phase of discovery.

Following these analyses, functional studies can then be a means to address whether a protein is critical in the overall role of the junction complex in epithelial integrity. The methods used to explore the functional nature of the protein will clearly be dependent on the role of the particular junction. A common method to assay function is the elimination of the endogenous protein from an experimental system. This can be accomplished in cell culture models by commercially available reagents such as siRNA or antisense oligonucleotides. Overexpression studies are also widely used as functional determinants. Next, readouts to assess potential phenotypes must also be selected. For example, uncovering the changes in junction protein distribution or cell morphology can indicate a loss of proper epithelial and cell junction function. Furthermore, a critical readout is an assay that challenges the overall function of the junctional complex within the context of the epithelial layer. For example, the TJ exhibits a barrier function by regulating passage of solutes and molecules across the monolayer. The transepithelial resistance (TER) is a quantitative readout of TJ integrity. Should the molecular interactions of the TJ be manipulated following knockdown of a putative component, the distribution of TJ components ZO-1 and occludin, as well as the TER value, may be altered.

Work from this chapter demonstrates that Bves localizes closely with TJ components such as ZO-1 and occludin in mature epithelia in vitro and in vivo. Additionally, the timeframe of junction formation/maturation during which Bves is trafficked to the membrane has been defined. Striking similarities to ZO-1 were observed following physiological challenges to cell adhesion. Also, GST pull-down experiments demonstrate an interaction with ZO-1. Thus, we predict that Bves function

is coupled to its localization of the TJ. Finally, using an antisense morpholino oligonucleotide (MO) knock-down/rescue approach, we reveal that Bves is essential in the regulation of epithelial integrity of an intact monolayer, as indicated by decrease of TER, disruption of the epithelial sheet, and a loss of membrane-localized ZO-1 protein. Taken together, our results demonstrate that Bves is a fundamental component of TJs and exhibit a pivotal role for Bves in the maintenance of epithelial cell integrity.

Materials and Methods

Cells and Antibodies

EMCs were obtained from Dr. H. Eid and have been described previously (Eid et al., 1994; Wada et al., 2003). MDCK epithelial cells (ATCC) were maintained in Dulbecco's Modified Eagle Medium (DMEM-Biowhittaker) supplemented with 10% FBS and penicillin-streptomycin cocktail. The SV40-transformed human corneal epithelial (HCE) cells, kindly provided by Dr. K. Araki-Sasaki (Araki-Sasaki et al., 1995) were grown in the serum-free keratinocyte growth medium (Invitrogen-Gibco). Protocols used for cell culture and tissue sections were standard (Osler and Bader, 2004; Wada et al., 2001). The rabbit α-Bves antisera (B846 at 1:200) used in this study has been described previously (Wada et al., 2001) and detects an intracellular C-terminal epitope of Bves in all species tested. Antibodies were used according to published methods and manufacturer's recommendations and were purchased as follows: E-cadherin monoclonal (Transduction Labs), ZO-1 and occludin monoclonal antibodies (Zymed), desmosome monoclonal (Sigma), Alexa 488 and Alexa 568-conjugated secondary

antibodies, Phalloidin and DAPI (Molecular Probes). Antibody concentrations used for Western Blot analysis are as follows: B846 at 1:200, monoclonal β-actin at 1:5000 (Sigma), and ZO-1 at 1:200. HRP-conjugated secondary antibodies were used per manufacturers suggestions.

Immunofluorescent and Electron Microscopy

Confocal image capture using a Zeiss LM-410 or LM-510 was performed in part through the use of the VUMC Cell Imaging Shared Resource, and processed using MetaMorph 6.1 (Universal Imaging Corp.) To generate electron micrographic samples, an adult mouse was starved overnight, the small intestine was dissected, washed with PBS, and perfused with 4% PFA in PBS for 30 minutes at RT. Following fixation, the intestine was dehydrated through a graded series of alcohols and embedded in Lowicryl Resin. Nickel grids with thin sections were blocked in 1% BSA in PBS, incubated with B846 antisera at 1:100 at 4 C overnight, and bathed with α-rabbit 5 nm immunogold conjugate secondary antibody (Sigma) for 1 hour at RT. Grids were fixed with 2.5% gluteraldehyde for 15 minutes and counterstained with 2% uranyl acetate for 5 minutes. PBS washes were performed between each step. It is important to note that a post-fixation antibody labeling technique, as used here, may result in less reactivity when compared to pre-fixation antibody incubations (Dr. W. Gray Jerome, personal communication). Quantification of colloidal gold bead binding was performed on sections perpendicular to the cell surface. The distance of each bead from the cell surface was determined and grouped in increments of 100 nm. Controls using no

primary antibody were performed and no bead labeling was detected. Experiments were performed in part through the use of the VUMC Research EM Resource.

Immunoblotting

Western blotting on HCE cell lysate were performed using standard techniques as previously reported (Knight et al., 2003). Briefly, cells were harvested by trypsin treatment and resuspended in 1 ml TBS with protein inhibitor (0.5%, Roche Diagnostics, Catalog # 1836170). Cells were disrupted by sonciation and followed by centrifugation to collect the pellet. The pellet was resuspended in sample buffer (60 mM Tris, 10 mM glycine, 2% SDS, pH 6.8) with 0.5% protein inhibitors and further sonicated followed by centrifugation. The supernatant was collected, and 20 µg total protein separated by polyacrylamide gel electrophoresis. The samples were transferred to a polyvinylidene fluoride membrane (Immobilon-P membrane, Millipore). The membranes were probed with antibodies against Bves, β-actin, and ZO-1, followed by appropriate species HRP-conjugated secondary antibodies (Pierce, Rockford, IL).

Glutathione bead preparation

GST fusion proteins were generated by PCR from the C-terminal tail of Bves (a.a.115-347) and the N-terminal tail (a.a.1-36) by A. Wada and cloned into the pGEX bacterial expression vector. GST-N terminal Bves, a 34kD protein, consists of the GST tag 5' of the extracellular N-terminal region of Bves. GST-C terminal Bves, which migrates at 66kD, contains the GST tag followed by the intracellular C-terminal tail. Constructs were transformed into BL21 E. coli bacterial strain and protein was induced

with IPTG, per standard methods (Amersham). Bacterial lysate was stored at –80°C until use. Preparation of GST beads for pull-down was performed as follows. A 50% slurry of Glutathione Sepharose 4B was prepared from a commercially available 75% slurry (Amersham). An aliquot of 1 ml of bacterial lysate expressing the GST fusion proteins was cleared by centrifugation (14,000g) prior to the addition of 40 μl of 50% slurry. Cleared lysate was incubated with beads for at least 2 hours or overnight, beads were washed 3 times with 100 μl of PBS, and resuspended in 100 μl of PBS. Samples from all three fractions were subject to PAGE and colloidal blue staining and amount of GST-bound protein used for pull-downs was equilibrated.

GST pull-down

MDCK cell lysate used for GST pull-down experiments were performed using the methods of Fanning et al. (1998). Briefly, cells were grown on 60 mm plates to confluency, placed on ice, and washed twice with PBS. Protein was extracted with 1ml of extraction buffer (20 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton-X, 0.05% SDS, 1 mg/ml BSA, 1 mM DTT) and 100 μl of protease inhibitor (Sigma, P8340). Cells were incubated on ice for 30 minutes, scraped off the plate and centrifuged for 30 minutes at 18,000 g at 4°C. Cell lysate was removed from the pellet and retained. Lysate was precleared by incubation with 20 μl bed volume of beads for 2 hours at 4°C after which beads were spun down and lysate was removed. Glutathione beads bound with GST constructs were added to the lysate and incubated overnight at 4°C. Beads were washed 5 times with 100 μl PBS and bound protein was eluted with 20 μl of 1x SDS sample buffer, boiled for 3 minutes, and loaded onto a 8-10% polyacrylamide gel.

Western blotting was performed using standard methods and antibody concentrations were used as listed above.

Cell culture treatments

Low calcium culture conditions were achieved by growing EMCs in MEM (Sigma) with 8% serum (Atlanta Biosciences) and penicillin-streptomycin cocktail. A small volume of $CaCl_2$ is added, in addition to the calcium contributed by the serum, giving a final calcium concentration in the culture medium of <10 μM. We have determined that the EMC line can support the development of a primordial monolayer even in the persistent absence of exogenous calcium. Cells continue to grow, although more slowly, when maintained for several weeks in calcium-depleted media. Experiments were performed following passages three or four in low calcium media. For TPA treatment of cells, MDCK or EMC cells were plated at high density (5 x10^5 cells/well) on 4-well chamber slides (Lab-Tek) in complete DMEM. The following day, cells were incubated in serum-free DMEM for 1 hour. Cells were switched to serum-free DMEM with 5 mM EGTA for 2 hours. Control wells were switched back to serum-free DMEM with normal calcium levels. Experimental wells were treated with 100 nM TPA in DMEM for 1 hour. Cells were fixed and processed by standard methods (as referenced above). For the actin-depolymerizing studies, cells were treated with 2 μM cytochalasin D (Sigma) for varying times upon which cells are fixed and processed. The α-*bves* MO used in this study has been previously described (Ripley et al., 2004). HCE cells were treated with nonspecific control or α-human *bves* MOs at 3 and 5 days after cells reached full confluence, at which TER measurements were recorded.

Measure of transepithelial resistance

HCE lines were seeded onto clear polycarbonate (0.4 μm) membrane cell culture inserts (Falcon, #35-3090) at a density of 10^4 cells/cm^2. The TER was measured at 14 days using an epithelial voltometer (EVOMX-A, World Precision Instruments, Sarasota, FL). After the TER was obtained, polycarbonate membranes were cut away from the plastic insert and immunofluorescence staining was performed.

Generation of chicken Bves HCE cells for rescue experiments

In order to assign specific phenotypes to the MO knock-down of Bves, a rescue strategy was applied. A "rescue" plasmid was generated as follows: chick Bves cDNA (Reese et al., 1999), which encodes the full length Bves protein (358 a.a.) and does not contain the MO target sequence, was cloned in frame into a neomycin-resistant expression plasmid with CMV promoter and FLAG epitope. HCE cells were transfected using Lipofectamine 2000 (Invitrogen) and selected in 20 μg/ml G418 antibiotic (Sigma). Five clones were selected based on FLAG labeling at the cell surface. The HCE cell line reported here, which stably expresses the MO-resistant chicken Bves rescue construct, is referred to as HCE-R. Two-tailed Student's t-test statistical analyses were performed to determine p values (Microsoft Excel).

Results

Bves colocalization with TJ markers in epithelial monolayers

We have defined Bves as an integral membrane protein distributed at cell borders, potentially regulating cell adhesion (Wada et al., 2001). However, it is not known whether Bves localizes to a particular junction complex within the lateral compartment. In order to assign Bves to a specific domain at the cell membrane, Bves subcellular distribution was compared to that of defined components of the TJ, AJ, and desmosome. For this confocal microscopic investigation, we used the MDCK cell line, since it has been extensively studied and because a wide range of marker antibodies is available for analysis. Findings revealed that Bves is highly colocalized with TJ proteins occludin and ZO-1 in an apical-lateral position within the Z axis (Figure 12A, B, arrows). Bves did not exhibit marked overlap with AJ-associated proteins E-cadherin (Figure 12C, arrows) and β-catenin (12D, arrows), or with desmosomal proteins (Figure 12E, arrows). A similar pattern of colocalization between Bves and TJ proteins was observed in other epithelial lines including squamous HCE cells (Figure 20A) and EMCs, as well as Caco-2 and HCA-7 cells, which both form a columnar epithelium (data not shown).

To extend these findings, we next determined the distribution of Bves in vivo at the cellular and ultrastructural levels using the murine intestinal absorptive epithelium as a model. This highly polarized columnar cell layer is ideal for localizing potential epithelial junction proteins because the TJ and AJ are well separated (Itoh et al., 1993). Cross sections through intestinal villi revealed a precise overlap of Bves, occludin, and ZO-1 that was not observed with E-cadherin (Figure 13A). Ultrastructural analysis using
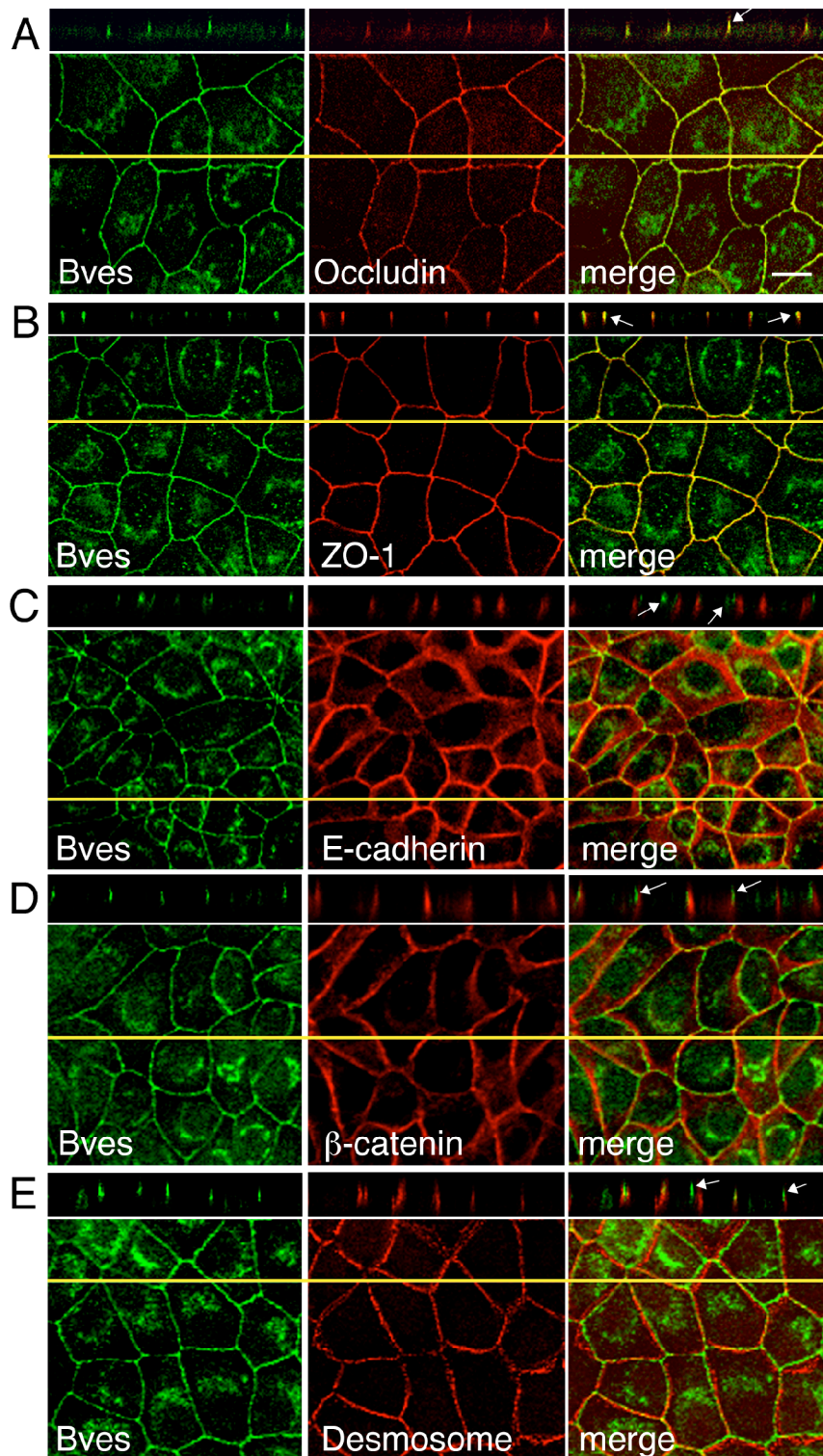
Figure 12. Bves localization at the TJ in confluent epithelial cells.
Confluent MDCK cells were labeled for Bves and junctional proteins. Confocal images show colocalization of Bves with TJ proteins, Occludin (A) and ZO-1 (B) in the XY and Z planes (arrows). Overlap of Bves with E-cadherin (C) b-catenin (D) or Desmosomal proteins (E) is not as pronounced (see arrows). The Z plane view shown above each XY panel corresponds to the yellow line transecting the XY image. Scale bar, 20 μm.
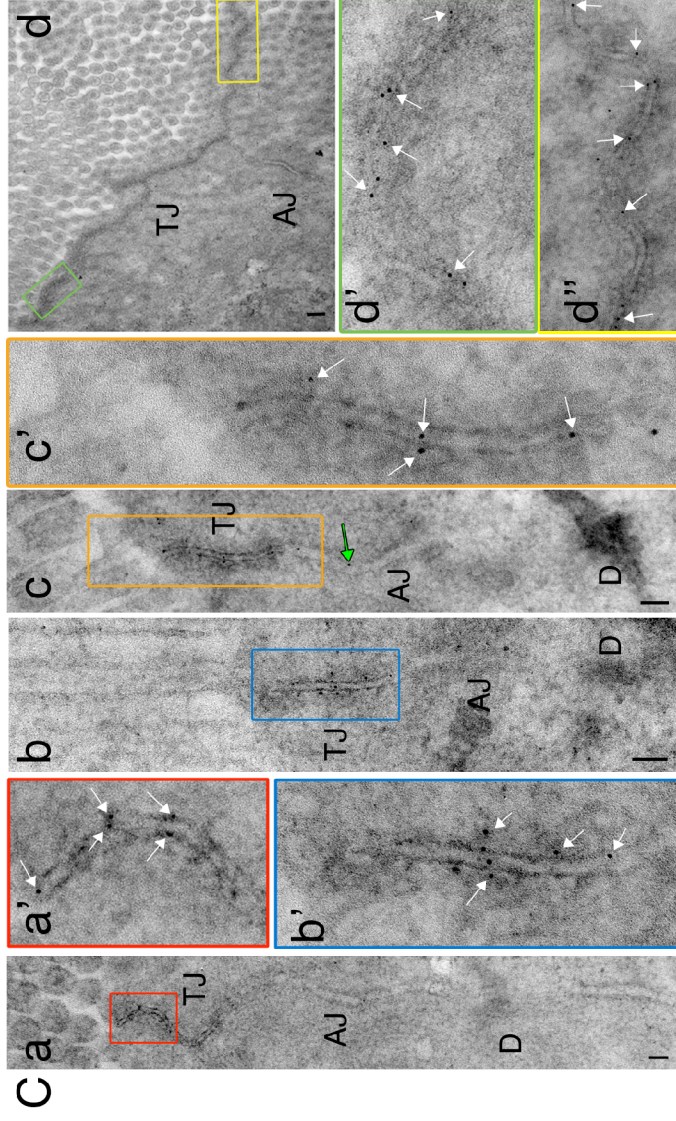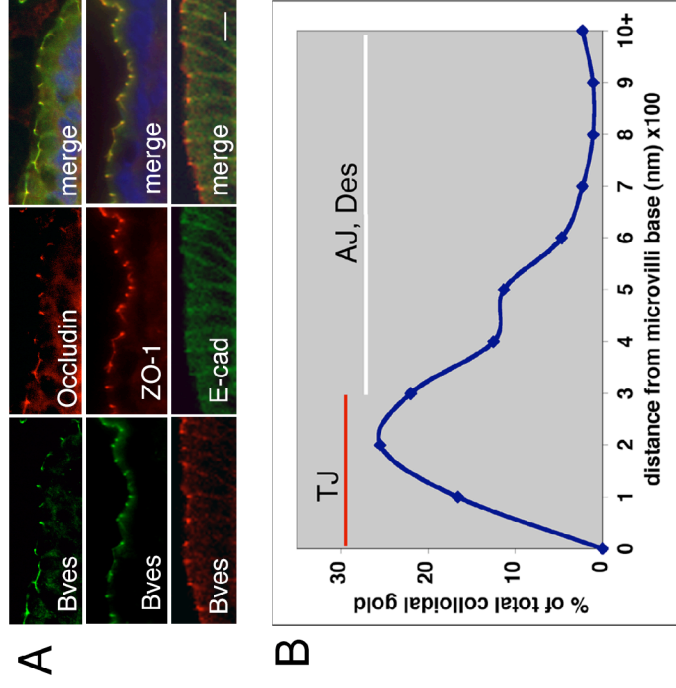
Figure 13. Bves localization at the TJ in vivo.

Cross sections through adult mouse small intestine were labeled with antibodies to Bves and Occludin (A), ZO-1 (B), or E-cadherin (C). Merged images show a high degree of overlap of Bves with TJ proteins at an apico-lateral position. Scale bar, 10 μm. Ultrathin sections were labeled with Bves and detected by a 5 nm colloidal gold-conjugated antibody. Quantitative analysis of immunogold location shows the percent of total beads that lie within a specific distance from the apical surface (B). The greatest number of beads is found in the domain where the TJ is located (B, red bar). Cross sections of gastric epithelium show junctional components TJ, AJ and Desmosomes vertically oriented along the lateral membrane (Ca, Cb, and Cc). As seen in higher magnification, colloidal gold beads are clustered within 300 nm of the cell surface in the TJ domain (white arrows, Ca', Cb', Cc'). Occasionally, beads are detected below the TJ (Cc, green arrow) in regions where AJ and desmosomes are typically located. *En face* sections show tangentially sectioned microvilli, with a structure reminiscent of a TJ strand in a region within 300 nm from the cell membrane (Cd). An AJ is clearly visible below the circumferential TJ strand. At high power, colloidal gold beads are easily visible along these TJ structures (Cd' and Cd''). Scale bars, 100 nm.

immunogold labeling of Bves on the gastric epithelium correlates with this finding (Figure 13B,C). TJ strands are primarily located in the region from the base of the microvilli to 300 nm below the apical surface (Bloom and Fawcett, 1975). In images of perpendicular sections, gold beads were clustered in the apical TJ region (Figure 13Ca-c). An *en face* view of TJ strands also demonstrates the concentration of gold bead labeling at or very near the TJ domain (Figure 13Cd). Graphical representation of colloidal gold distribution demonstrates that the majority of beads (64%) are located within the TJ domain (Figure 13B, red line). Still, Bves is not entirely restricted to the TJ, as suggested by detection of Bves outside of the predicted domain (Figure 13Cc, arrow). Taken together, both in vitro and in vivo localization studies described here predict a role for Bves at the TJ**.**

Bves localizes early to points of cell-cell contact

Trafficking of proteins during early contact development has been critical in establishing the function of adhesive proteins in epithelial sheet formation. If Bves were involved in establishing cell contacts and/or epithelial integrity, we postulate that Bves will behave like known junctional components (Adams et al., 1998; Adams et al., 1996; McNeill et al., 1993; Vasioukhin et al., 2000). Thus, in an effort to determine how Bves is mobilized during epithelial maturation, we assessed how Bves protein localization changes during the formation, expansion, and compaction of contacts, relative to the dynamic remodeling of the actin network. The EMC line was used for this study because it is a representative epithelial model of epicardial development where Bves was first identified. We determined that Bves is confined to an intracellular

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compartment in single cells (Figure 14A, arrow), that colocalized with markers of the Golgi (Wada et al., 2001), while the actin network existed as a cortical ring at the cell periphery (Figure 14A, arrowhead). Upon apposition of neighboring cells, Bves traffics to positions outside of the cortical actin network (Figure 14B, arrow), similar to the pattern observed with E-cadherin at forming contacts (Adams et al., 1998; Ando-Akatsuka et al., 1999). As contacts expand, the cortical actin network collapses and increased Bves labeling is observed between apposing cells (Figure 14C, D, arrows). Finally, as contacts compact and adhesions with additional cells are generated, Bves labeling is confined to regions of cell-cell contact and ultimately will encompass the entire cell circumference (Figure 12). Bves was never observed at the free surface of cells (Figure 14, arrowheads).

Bves incorporates into the cell membrane coincidently with AJ proteins

Investigation of the dynamic processes of cell-cell interaction with regards to specific junctional proteins is critical to the understanding of epithelial formation. Past studies have proposed a specific sequence in which junction proteins translocate to points of cell-cell contact (Adams et al., 1998; Adams et al., 1996; Vasioukhin et al., 2000). Within the first ten minutes of initial cell-cell contact, actin filaments and actin binding proteins extend from the membrane in an exploratory manner but a stable contact is not made (Adams et al., 1996). In a second phase of contact formation, E-cadherin-containing "puncta" localize to forming contacts in Triton-X insoluble structures. The distribution of other proteins such as the catenins and ZO-1 mirrors this early translocation to developing contacts (Adams et al., 1998) and precedes assembly

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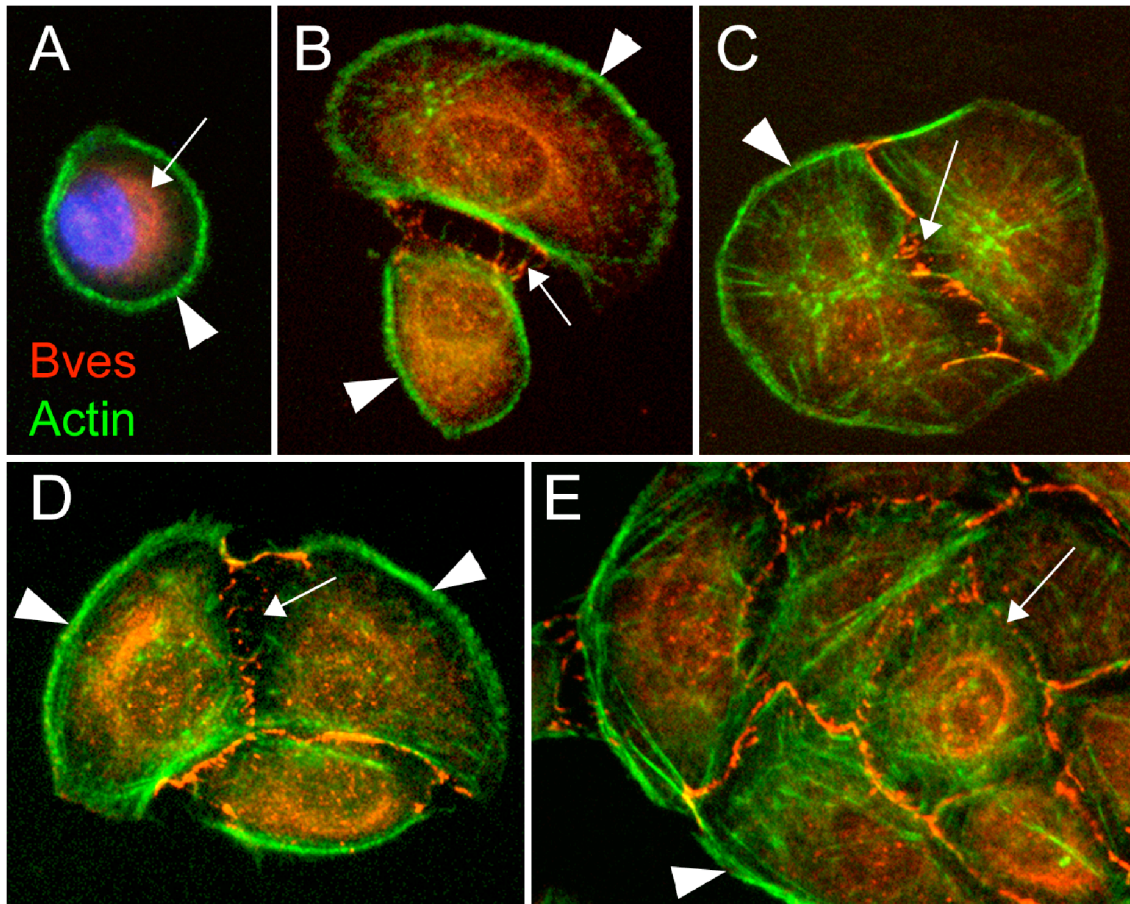
Figure 14. Bves distribution at forming epithelial cell contacts.
Subconfluent EMCs were labeled with Bves (red) and phalloidin, detecting the actin cytoskeleton (green). DAPI stains the nucleus blue in A. Progressive stages of cell contact are represented. A single cell shows intracellular Bves (A, arrow). Bves is observed between neighboring cells (B-D, arrows) and at the cell periphery in groups of cells (E, arrow). Bves is not observed at free edges with the cortical actin ring (arrowheads). Scale bar, 10 μm.

of the AJ (Yonemura et al., 1995). Finally, condensation of adhesive plaques result from reorganization of the actin network as neighboring cells connect together to ultimately form a polarized monolayer. During this process following the establishment of contacts, desmosomes assemble (Getsios et al., 2004; Vasioukhin et al., 2000) and TJ proteins like occludin and claudins translocate to flanking membranes contributing to polarization (Ando-Akatsuka et al., 1999). Importantly, ZO-1 specifically concentrated at TJ in polarized cells with occludin and claudins (Itoh et al., 1993; Stevenson et al., 1986). To understand the dynamic redistribution of Bves during cell-cell contact, we expanded our analysis of Bves in relation to this spatio-temporal framework of junction assembly. Cells have been co-labeled with antibodies to Bves and junctional proteins to correlate Bves appearance at the membrane with the established time course. MDCK cells have served as a model in previous studies and were used in this examination of Bves. The extension of actin filaments and localization of cytoskeletal binding proteins at cell contacts define exploratory/immediate events of cell adhesion (McNeill et al., 1993; Vasioukhin et al., 2000). Bves arrives at membrane contact points following actin and vinculin, as demonstrated by the absence of Bves at points of contact (Figure 15A,B, arrows). Arrival of Bves at cell contacts is concurrent with E-cadherin, ZO-1, (Figure 15C,D, arrows) and β-catenin (not shown), proteins that localize to contacts at an early stage soon after an initial contact is formed (Adams et al., 1998; Ando-Akatsuka et al., 1999; McNeill et al., 1993; Rajasekaran et al., 1996; Vasioukhin et al., 2000). Bves arrives at points of cell-cell contact before desmosomal components (Figure 15E) and occludin (not shown) were translocated to the cell contact site. Subsequently, segregation of junctional molecules into discrete junctional subdomains

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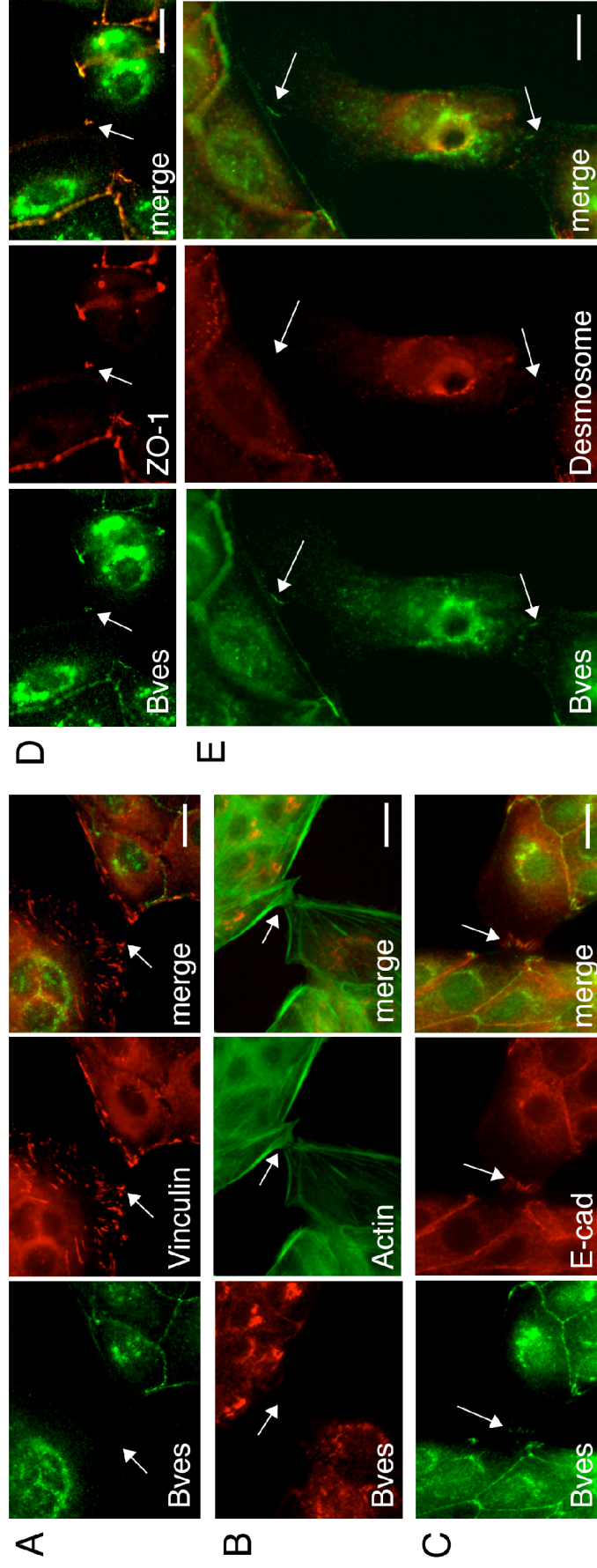
Figure 15. Bves localizes to cell-cell contacts coincidently with ZO-1 and AJ proteins.
Subconfluent MDCK cells were labeled with Bves and antibodies or stains detecting vinculin (A), actin (B), E-cadherin (C), ZO-1 (D) and desmosomes (E). Bves is absent from immediate contacts as represented by actin and vinculin (A, B, arrows). Bves arrives at the cell membrane with AJ proteins and ZO-1 (C, D, arrows). Bves arrives prior to desmosomal proteins (E, arrows) to sites of contact. Scale bars A-C, 10 μm. Scale bars D-E, 20 μm.

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occurs (Ando-Akatsuka et al., 1999; Farshori and Kachar, 1999; Vasioukhin et al., 2000). Thus, Bves is recruited to membrane protrusions at an early stage and localizes to forming contacts with ZO-1 and AJ components, but ultimately segregates with the TJ components ZO-1 and occludin as contacts mature (Figure 12A,B) (Ando-Akatsuka et al., 1999; Rajasekaran et al., 1996). This dynamic distribution parallels the pattern observed with ZO-1. Furthermore, our findings demonstrate that Bves traffics to cell-cell contact points in a spatio-temporal manner appropriate for a protein involved in cell adhesion and/or maintenance of junctional complexes (Adams et al., 1996; Ando-Akatsuka et al., 1999; McNeill et al., 1993).

Bves response following challenges to epithelial integrity

Low calcium and phorbol ester challenges to cell-cell adhesion and monolayer integrity have been used to explore junctional protein behavior and, in some cases, assign proteins to specific junctional complexes (Farshori and Kachar, 1999; Nishimura et al., 2002; Stevenson and Begg, 1994; Yamada et al., 2004). These culture manipulations were performed to elucidate Bves membrane properties.

Calcium switch assays challenge the integrity of an epithelial monolayer because many adhesion proteins, such as cadherin, rely on calcium for their function. When calcium is transiently depleted from a confluent, polarized monolayer of cells, the adhesive junctions disassemble and the cohesive nature of the epithelial sheet is compromised. This is represented by the loss of membrane-localized junctional proteins (Gumbiner et al., 1988; Nagafuchi et al., 1987). We found that EMCs can form a monolayer with primordial adhesive contacts following persistent absence of exogenous

calcium, as has been observed with various other cells (Chaproniere and McKeehan, 1986; Ochieng et al., 1990; Shirakawa et al., 1986). Under these conditions, Bves was observed at contact points between the cortical actin networks of apposing cells (Figure 16A, arrows) and was distributed around the cell circumference at confluence (Figure 16B, arrows). This demonstrates that Bves localization and function at the membrane is calcium-independent, which correlates with our previous finding that exogenously expressed Bves induces L-cell aggregation in a calcium-independent manner (Wada et al., 2001). Interestingly, ZO-1 mimicked this pattern at the cell surface after persistent calcium depletion, and colocalized precisely with Bves (Figure 16B, merge). Conversely, E-cadherin was absent from the cell membrane, as expected, (Figure 16C, middle panel arrow) and was only observed at the cell periphery in the presence of calcium (Figure 16C, middle panel insert).

While many studies have shown TJ assembly to be dependent on AJ assembly (Rothen-Rutishauser et al., 2002) and exogenous calcium (Wilson, 1997), others have reported that ZO-1 is retained at the membrane of specific epithelial lines under various low calcium culture manipulations (Fukuhara et al., 2002; Ide et al., 1999; Kartenbeck et al., 1991; Nishimura et al., 2002). Occludin was also absent from the cell membrane (data not shown) signifying that, although ZO-1 is retained at the membrane, intact TJ cannot form under these conditions. These findings reiterate the fact that Bves and ZO-1 are regulated differently than E-cadherin and the well-established calcium-dependent cadherin protein complex. These findings highlight the similarity of Bves and ZO-1 action under these conditions as well as underscore the calcium-independent nature of Bves function in epithelia. We demonstrate that Bves resides at the TJ with ZO-1 and
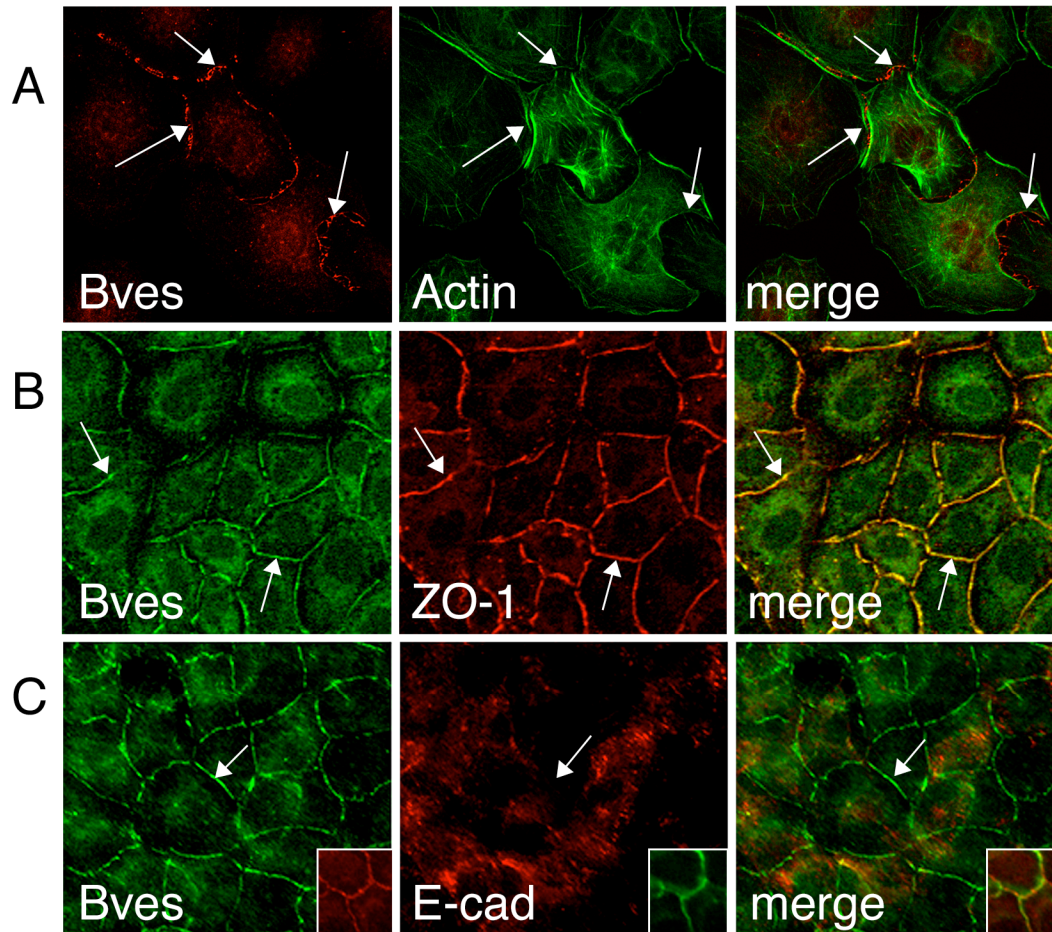
Figure 16. Bves response to persistent low calcium.
EMCs were passaged in low calcium media (A-C). Subconfluent cells, labeled with Bves and actin-staining phalloidin (A) show that Bves is found at the cell periphery only where two cells appose (arrows). Confluent EMCs are labeled with Bves and ZO-1 (B) or E-cadherin (C). Note that E-cadherin is lost from the membrane in low calcium, but shares the same membrane pattern as Bves in cells cultured in complete media (C, inserts).

occludin (Figures 12,13). Here, we again find a striking similarity between Bves and ZO-1 response, further suggesting a role for Bves at the TJ.

The phorbol ester TPA activates protein kinase C, which regulates both TJ biogenesis and calcium-induced polarization of epithelia (Balda et al., 1993; Stuart and Nigam, 1995). The addition of TPA to confluent epithelial cells following a calcium switch is thought to bypass calcium-dependent cell-cell adhesion and initiate the formation of "TJ-like" structures at the cell periphery (Balda et al., 1993; Farshori and Kachar, 1999; Nishimura et al., 2002; Ohsugi et al., 1997). Proteins such as JEAP and MAGI have been designated as TJ components because they localize to TPA-induced structures with ZO-1 and occludin (Ide et al., 1999; Nishimura et al., 2002). Therefore, we used this method to determine whether Bves would also aggregate at the membrane with known TJ components. This study was performed on MDCK (Figure 17) and EMC (not shown) lines, which both produced similar results. In untreated cells, Bves, ZO-1, occludin, and E-cadherin localize around the cell circumference (Figure 17A,C,E). Following TPA treatment, Bves membrane localization was redistributed with TJ components, occludin (Figure 17B, arrows) and ZO-1 (Figure 17D, arrows), in "TJ-like" structures. This localization occurred in the absence of cadherin trafficking (Figure 17F, arrow). Again, Bves behaves in a manner predicted for a TJ protein, providing accumulating evidence for its involvement at the TJ.

Bves localization following actin disruption

Cytochalasin-D (CytD) is a pharmacological agent used to disrupt F-actin. To determine how Bves responds to actin disruption, EMCs were grown in CytD containing
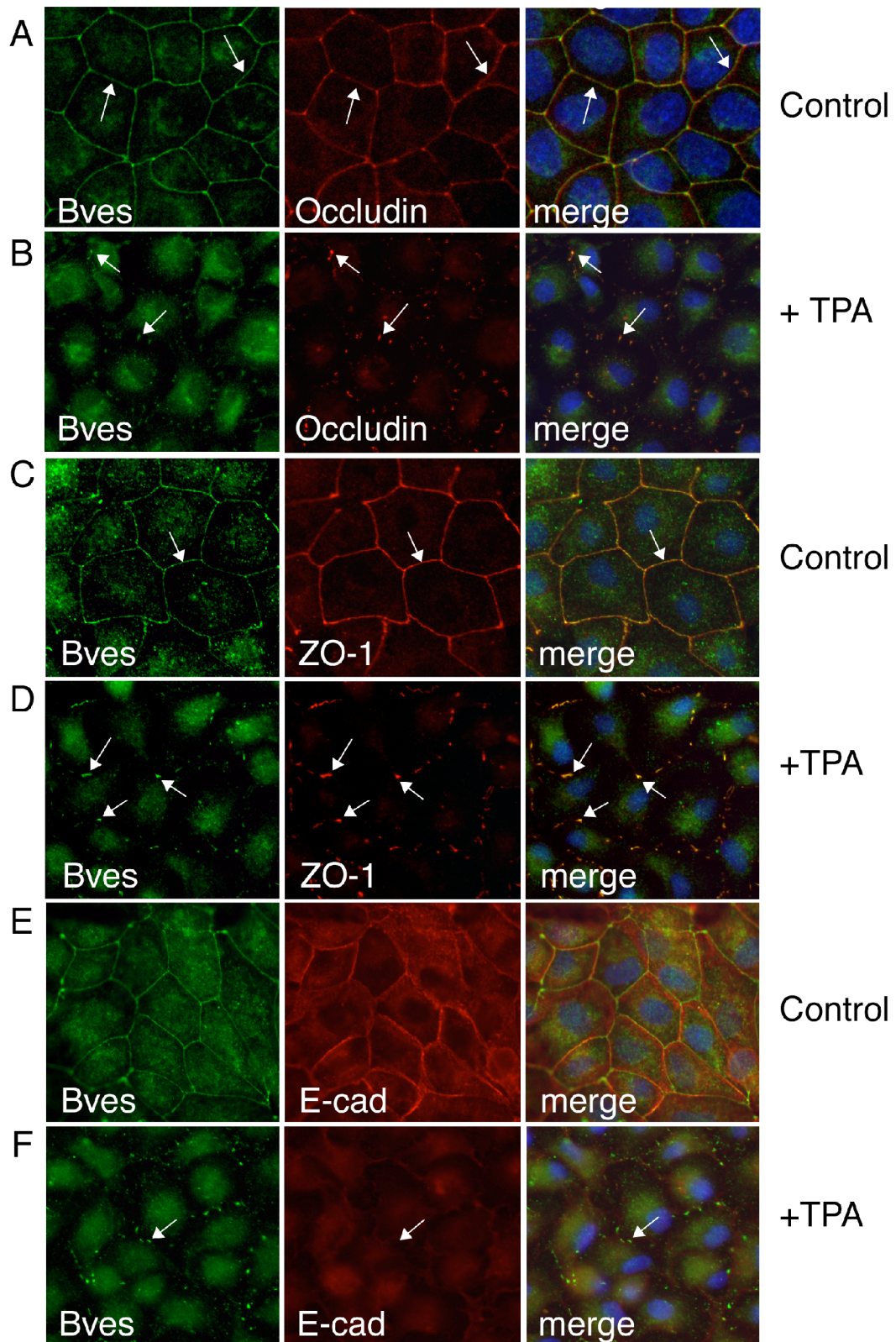
Figure 17. Bves incorporation into TPA-induced membrane structures.
MDCK cells were cultured in serum-free media, switched to EGTA-containing media, and either returned to serum-free media (A,C,E) or treated with TPA (B,D,F). Following TPA treatment, Bves, occludin, and ZO-1 are found in TPA-induced TJ-like structures at the cell membrane (B,D, arrows). E-cadherin labeling becomes diffuse and does not localize to these structures (F, arrows). Scale bar, 10 μm.

media. Bves and E-cadherin label the entire cell periphery of untreated cells, while actin filaments form a network from cell to cell (Figure 18A). As shown in Figure 18B, Bves and E-cadherin are still deposited at points of membrane contact after 10 minutes of exposure to CytD. As time of treatment increases, the cytoskeleton is further dismantled. As a result, less Bves and E-cadherin staining is observed at the cell membrane, but remaining punctate spots appear to colocalize with clusters of actin (Figure 18C,D). This finding suggests that Bves localization is directly or indirectly dependent on the actin network for stabilization at the lateral membrane.

Bves C-terminus interaction with a ZO-1 protein complex

To show that Bves is a component of an epithelial junction, providing evidence of physical association of Bves and known components is critical. The present data suggest that Bves functions at the TJ and thus, could interact with TJ proteins. A GST pull-down assay was performed to determine whether Bves binds, either directly or indirectly to components of the TJ. GST, GST N-terminal Bves, and GST C-terminal Bves constructs were used to probe for interaction with candidate TJ proteins (Figure 19A). ZO-1 is detected in the complex retained on beads bound with GST-C terminal Bves (Figure 19B). Precipitated ZO-1 was not detected in the GST control fraction or, importantly, in the GST N-terminal Bves fraction, as this portion of the molecule has an extracellular distribution (Knight et al., 2003). However, an interaction between occludin and GST-Bves was not detected (Figure 19C). This indicates that Bves may interact with the TJ directly or indirectly through the peripheral membrane protein ZO-1 and not membrane-bound occludin. This finding provides additional strength to the hypothesis
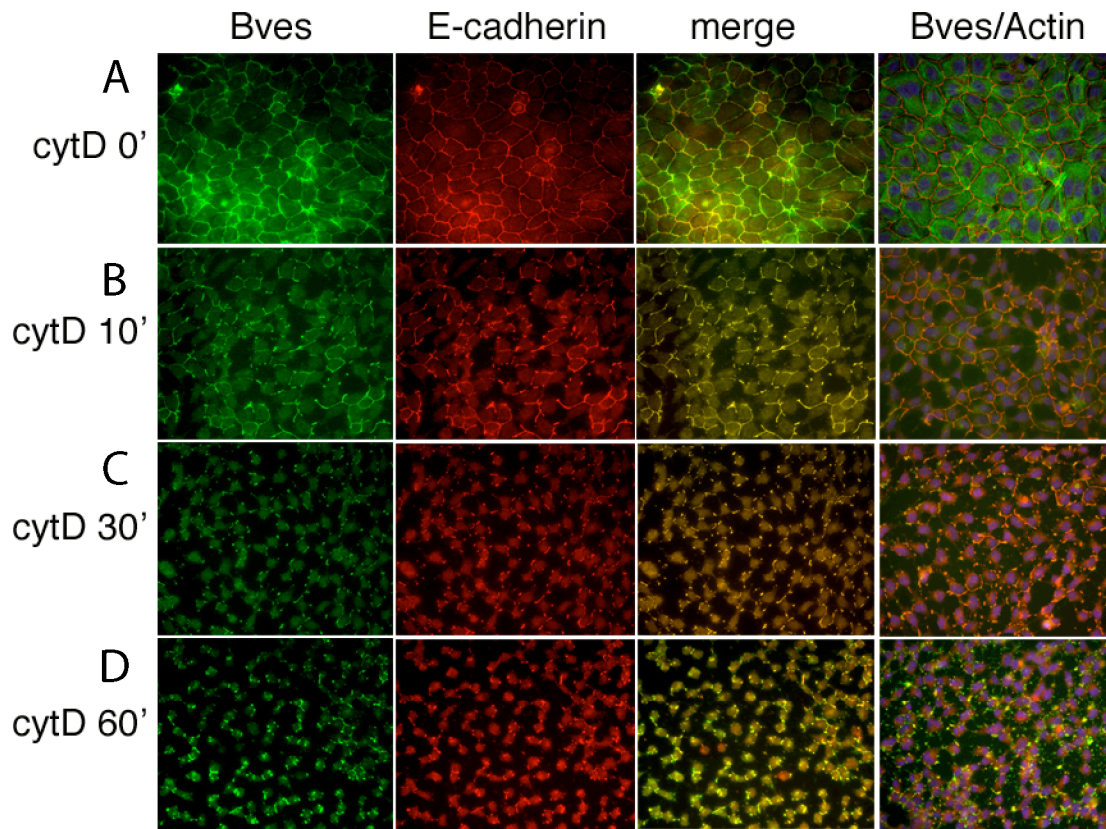
Figure 18. Bves response following actin depolymerization.
Treatment of confluent MDCK cells with Cytochalasin D (CytD) causes a redistribution of junction molecules into actin-rich rich structures. Untreated cells at confluency exhibit Bves and E-cadherin around the cell periphery, and the actin network is intact (A). The far right panels show merged images of Bves (red) and actin (green). After CytD treatment for 10 minutes, the actin network begins to dismantle and membrane labeling of Bves and E-cadherin has been compromised (B). Longer exposure to CytD results in an increased loss of Bves and E-cadherin at cell borders and an accumulation of both proteins into punctate structures (C,D). Actin conglomerates remain at these punctate spots with Bves after disruption by CytD for 30-60 minutes.
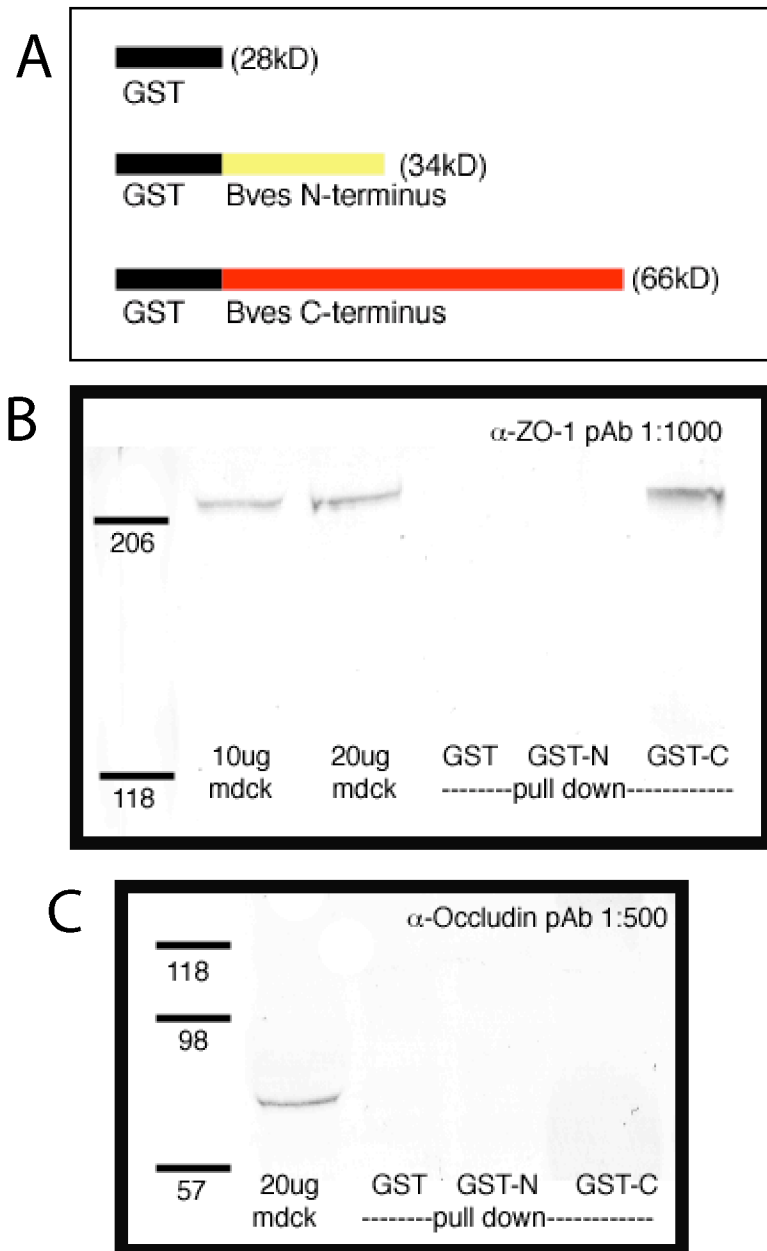
Figure 19. The C-terminus of Bves interacts with a complex containing ZO-1.
GST constructs used for a GST pull-down assay are diagrammed (A). Constructs were generated that express the GST tag fused 5' to either the N-terminus or C-terminus of chicken Bves. In the endogenous conformation of Bves, the N-terminus is extracellular while the C-terminal portion of the protein is intracellular. Western Blot analysis indicates an interaction between the ZO-1 protein complex and GST-Bves C-terminus recombinant protein following GST pull-down (B). An antibody to ZO-1 detects a ~225kD band in 10 and 20 μg of MDCK cell lysate. The presence of a similar band is detected in the protein fraction eluted from beads bound with GST-C-term Bves. Occludin was not detected in the protein fraction eluted from any glutathione beads (C).

that Bves is a functional component of the TJ, as these GST pull-down experiments demonstrate an association between Bves and the multimolecular complex containing ZO-1.

Knock-down of Bves function disrupts TJ integrity

To analyze Bves function, a method to eliminate and rescue Bves activity in an epithelial cell system was generated. We accomplished this by treating HCE cells with α-human *bves* MO and rescuing with transfected exogenous chicken Bves. HCE cells were used in this assay for several reasons. HCE parental cells are of human origin and display the same membrane distribution of Bves and TJ proteins (Figure 20A) as observed with other cells, like MDCK (Figure 12). In addition, the cells more readily took up transfected DNA and MO than other lines tested, making HCE cells ideal for this study. Importantly, after examining numerous cell lines, HCE-R cells that stably express a "MO-rescuing" chicken Bves construct appropriately traffic the FLAG-tagged Bves to the membrane (Figure 20B). This is highly significant, as this is the first cell culture system where expression and trafficking of exogenous Bves mirrors the endogenous pattern. Previously, we and others (Andree et al., 2000) have made several attempts to generate stable cell lines expressing Bves constructs and found that other cells did not properly traffic to the cell membrane upon transfection. However, this cell line affords, for the first time, an opportunity to identify Bves function and manipulate Bves in an in vitro environment. Furthermore, MO treatment of HCE cells results in a decrease of detectable membrane-localized Bves (Figure 20C).

TER and localization of TJ markers provided readouts for epithelial integrity. Previously reported TER measurements for polarized HCE cells are typically between 200-800 Ω cm^2 (Toropainen et al., 2001; Wang et al., 2004; Yi et al., 2000). Our culture conditions used in this study were designed to promote monolayer growth and the average measured resistance of the parental line was ~ 390 Ω cm^2. As demonstrated in Figure 20D, *bves* MO-treated HCE cells exhibit a significant loss of TER (asterisk, $p < 0.05$) as compared with untreated or control MO-treated cells. HCE-R cells display nearly a 100% increase in TER over the parental control cell line. The TER of the HCE-R cell line overexpressing FLAG-tagged chicken Bves is only slightly decreased by *bves* MO treatment (Figure 20D, double asterisk), indicating that exogenous Bves rescues the MO-induced drop in TER observed in the parental line. Immunolabeling of ZO-1 in MO-treated HCE cells demonstrates a visual compromise of epithelial integrity, which correlates with the TER data. The MO-induced phenotype is characterized by loss ZO-1 staining around the entire cell periphery and gaps in the epithelial sheet, while untreated and control MO cells show circumferential ZO-1 labeling (Figure 20E). HCE-R cells exhibit retention of ZO-1 at the surface following *bves* MO treatment similar to controls (Figure 20E, lower panel). As demonstrated by Western Blot analysis, MO treatment of HCE cells results in a decrease of Bves and ZO-1 (Figure 20F) while transfection of chicken Bves into HCE cells results in an increase of Bves, as expected, as well as an increase of membrane-associated ZO-1. In addition, occludin, E-cadherin, and β-catenin localization at the membrane is disrupted by MO treatment, although the disturbance of membrane labeling of the AJ proteins appears less severe than ZO-1 and occludin (Figure 20G).
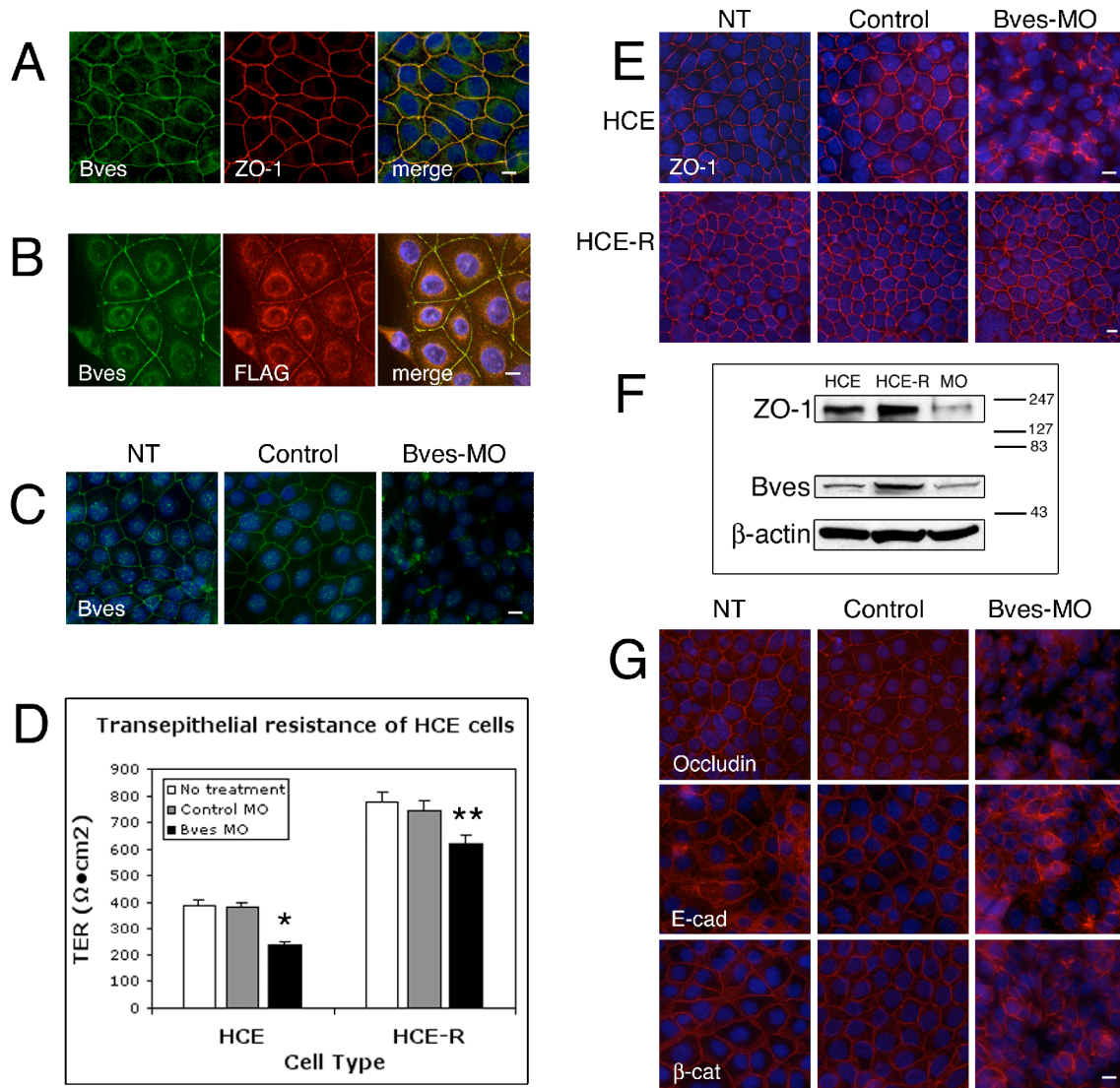
Fig 20. Compromise of TJ integrity following knock-down and rescue of Bves in HCE cells. Expression of Bves and ZO-1 at the cell periphery in parental HCE cells is shown (A). HCE-R cells stably expressing the "rescue" chicken Bves-FLAG construct exhibit labeling of the membrane by both FLAG and Bves antibodies (B). Note the intracellular accumulation of transfected chicken Bves. Treatment with *bves* MO results in a loss of Bves at the cell periphery, as compared to untreated and control MO (C). TER measurements were performed on HCE and HCE-R (D). Parental HCE cells exhibited a TER value of approximately 390 Ω•cm2. Treatment with *bves* MO resulted in a 40% decrease (p<0.05) in TER (asterisk), while control MO did not significantly alter the TER. The TER of HCE-R cells doubled to 775 Ω•cm2 and, following *bves* MO (double asterisk), a drop of only 20% (p<0.05) in TER was observed. ZO-1 immunofluorescence performed on Transwell inserts following TER measurements are shown (E). Parental HCE cells and HCE-R cells were treated with control and *bves* MO. Bves and ZO-1 expression is disrupted in HCE cells treated with *bves* MO (top right panel). MO treatment does not alter the expression pattern of ZO-1 in HCE-R cells (lower right panel). Western blotting demonstrates that membrane ZO-1 and Bves increase with exogenous expression of Bves in HCE-R cells, but decreased following treatment with *bves* MO, as compared with the parental HCE line (F). Membrane integrity of junction proteins occludin, E-cadherin, and β-catenin is disrupted following *bves* MO treatment. Scale bars, 10 μm.

Discussion

The current study was initiated to investigate the putative involvement of Bves in cell adhesion and to test the hypothesis that Bves functions endogenously to regulate epithelial integrity. Given the broad distribution of Bves in a variety of epithelial types, the present data predict a conserved, significant and wide-ranging function of this novel gene family. In addition, the dynamic distribution pattern during contact formation correlates with a proposed involvement in cell-cell adhesion. Immunolocalization and physiological challenge studies suggest Bves resides at or near the TJ. Thus, Bves is spatio-temporally regulated in a manner necessary to fulfill requirements of a junction/adhesion protein. GST pull-down assays demonstrate a physical link to an established TJ component, ZO-1. Furthermore, functional MO knock-down and rescue experiments reveal that Bves is necessary for the stability of an epithelial monolayer in vitro. Taken together, our data establish that Bves exhibits properties in accordance with a role in the maintenance of epithelial integrity and are the first to show that Bves could be an important molecular element at the TJ.

Significant to this study is the placement of Bves into a definitive junction within the terminal bar of epithelial cells. While previous findings determined that Bves localizes to the cell membrane and confers adhesion to L-cells after transfection, the protein had not been assigned to a subcellular domain prior to these studies. Our current immunohistochemical, confocal, and immuno-EM analyses of cell lines and the gastric epithelium indicate that Bves colocalizes with occludin and ZO-1. Also, Bves responds like both TJ markers following TPA challenge, an assay used to identify components of the TJ, such as JEAP and MAGI/BAP1 (Ide et al., 1999; Nishimura et

al., 2002). Localization studies on confluent and mature epithelia support a function for Bves at the TJ with occludin and ZO-1.

Thus, while we establish that Bves is concentrated at the TJ, we find that Bves parallels the behavior of ZO-1 more closely that that of occludin. For example, the pattern that Bves localizes to primordial cell-cell adhesions mimics what is observed with ZO-1 during contact formation. ZO-1 can be detected at contact points with E-cadherin and catenin proteins, well before occludin, claudin, and other TJ components translocate to the cell periphery (Ando-Akatsuka et al., 1999). Moreover, in non-epithelial cells such as NRK fibroblast cells where TJ are not formed and occludin is not expressed, ZO-1 and Bves colocalize at the cell periphery. In these cells, ZO-1 is thought to function as a crosslinker between the cadherin complex and actin network through interaction with AJ protein α-catenin (Itoh et al., 1993) and the nectin-afadin complex (Yokoyama et al., 2001). Interestingly, persistent culture of EMCs in low calcium permitted the localization of ZO-1 and Bves at the cell periphery, even when E-cadherin and occludin failed to localize to the membrane. Bves.

Importantly, of the two TJ proteins ZO-1 and occludin, only a complex containing ZO-1 was shown to interact with Bves C-terminus. We postulate that we detect an interaction with ZO-1 and not occludin due to the relative strengths of interaction between Bves and these proteins. It is possible occludin could be a member of this complex in its native state, but due to the detergent-induced disruption, some of the protein-protein interactions could be disrupted. It is critical to point out that while ZO-1 binds both occludin and claudin proteins, the two proteins do not co-immunoprecipitate (Chen et al., 2002). While it is unclear whether the interaction is direct or indirect, the

fact that the C-terminus can pull down ZO-1 suggests that Bves is tightly associated with ZO-1. Alternatively, Bves and ZO-1 interact via other proteins not contained in the TJ. Although we believe that the ZO-1/Bves interaction occurs at the membrane, it is not out of question that the association we detected occurs elsewhere in the cell. Also, it is interesting to note that although ZO-1 and occludin both localize and interact at the TJ, ZO-1 clearly has roles outside of the TJ, and its behavior is often different than occludin (Fanning et al., 1998; Gottardi et al., 1996; Itoh et al., 1997; Schneeberger and Lynch, 2004). Likewise, while Bves clearly has a role at the TJ, a high possibility exists that Bves's functional niche encompasses more than just the TJ. For instance, Bves was occasionally detected outside of the predicted TJ domain in ultrastructural studies. The literature agrees that ZO-1 is a scaffolding protein that interacts with occludin, claudins, and ZO proteins at the TJ in epithelial cells but also possesses an increasing number of other binding partners (Balda and Matter, 2000b; Gonzalez-Mariscal et al., 2003; Matter and Balda, 2003). ZO-1 provides an example of a TJ-related protein that may have functions beyond those at the TJ in epithelia as well as in other cell types. An explanation for this dynamic distribution has not been proposed, although it is likely that the spatio-temporal recruitment of ZO-1 and Bves can be correlated with a common function. However, with these observations, it is now possible to more carefully probe the function of Bves.

To probe Bves function in epithelia, we established the SV40-t HCE cell model system where Bves function can be disabled following morpholino treatment and rescued with exogenous protein expression. We combined these MO experiments with TER analysis, a method used to confirm a functional role for proteins at the TJ

(Cereijido et al., 1978; Gonzalez-Mariscal et al., 2003; Gumbiner and Simons, 1986; Sonoda et al., 1999). Recently, proteins CLMP and MLCK have been established as TJ components using this assay (Clayburgh et al., 2004; Raschperger et al., 2004). Bves knock-down by MO in HCE cells results in the rapid loss of TER, epithelial polarization, and the disassembly of cell junctions. Similarly, RNAi suppression of JAM-1 or Par-3 disrupted TJ integrity, caused a mislocalization of related proteins, and caused a drop in TER values (Chen and Macara, 2005; Mandell et al., 2005). In addition, Chen *et al*. rescued the alterations by expression of human Par-3 (Chen and Macara, 2005). It is important to note that the transfection of chicken Bves into HCE cells not only rescues the MO knock-down effects as demonstrated by retention of ZO-1 at the membrane, but drastically increases the TER of both control and MO-treated HCE-R cells. This signifies that Bves could be required for integrity of the epithelial sheet by its influence at the TJ. We postulate that the overexpression of Bves strengthens the TJ seal, thus generating a significantly higher TER value, as has been shown with overexpression of other TJ proteins (Cohen et al., 2001; McCarthy et al., 1996; Raschperger et al., 2004). It is possible that through its interaction with the ZO-1 molecular complex, Bves can modulate the integrity of the TJ paracellular seal. Furthermore, this increase in TER and rescue demonstrates that the observed phenotype is Bves-dependent and suggests a conservation of Bves function between species. Taken together, the present study is the first to assign a function for Bves in epithelial maintenance and integrity and establishes that Bves may be an important molecular component of the TJ.

The mechanisms that control the sealing capabilities of the TJ are poorly understood. However, a measure of the transepithelial electrical resistance has become

the gold standard for assessing whether proteins influence TJ integrity. We show that depletion of Bves in human corneal cell lines results in a breach of barrier function, as indicated by a drop in the TER. Furthermore, we have shown previously that similar treatment of these cells shows a faster migration of cells in a wound-healing assay. This finding suggests that without Bves, cells are more motile due to a decrease in the adhesive nature of the cells, potentially due compromised cell junctions. Currently, occludin and the claudins are the only known membrane-spanning proteins forming the backbone of the TJ strands. The finding that occludin-null mice can form functional junctions further suggests that other molecules must contribute to TJ integrity (Saitou et al., 2000). Sufficient evidence is lacking as to the exact mechanism of Bves action at the TJ. However, this study is the first demonstration that Bves localizes to a specific junction and is a critical step in affixing a specific function to Bves during epithelial cell-cell adhesion.

In conclusion, we find Bves to be a functional component of the TJ complex. However, this finding is merely reveals the "tip of the iceberg" of how Bves could be involved in cell-cell interactions in epithelia. Clearly the investigation of Bves in epithelial cell types is not complete. However, this study has advanced our knowledge on Bves significantly. Prior to this analysis, the expression and distribution of Bves in epithelia was not defined, and a function in epithelia had only been a speculation. Now, both the localization and a function at a specific cell junction have been assigned. Future studies will be directed at determining the protein domain responsible for interaction with the ZO-1 protein complex in order to unravel how Bves is important for TJ function. Taken together, the functional insight provided by this study gives a new meaning to the

expression pattern observed during embryogenesis. At present, this work is in press

(Osler et al., 2005).

CHAPTER IV

XBVES IS NECESSARY FOR EPITHELIAL MORPHOGENESIS AND INTEGRITY DURING _XENOPUS LAEVIS_ DEVELOPMENT

Introduction

The work in this chapter focuses on elucidating how the _Xenopus_ homolog of Bves, Xbves, functions in epithelial development and morphogenesis in vivo. The ultimate goal is to connect an in vivo function with a role of Bves/Xbves at epithelial cell junctions, in accordance with our central hypothesis that Bves proteins are essential for epithelial cell-cell interactions and integrity. Upon the isolation of _Xbves_ from a cDNA library, a detailed study of mRNA and protein expression was initiated (Ripley et al., submitted). These early studies from our laboratory demonstrate the presence of _Xbves_ in epithelia during the earliest phases of frog development and the conservation of its adhesive function. To explore the function of Xbves, global disruption of Xbves using a MO-based knock-down approach was performed. Xbves depletion was found to arrest development at gastrulation, a stage of development where epithelial tissues are undergoing dynamic morphogenetic movements. This first demonstration of Xbves function underscored the essential nature of Xbves during epithelial morphogenesis and warranted a further and more comprehensive look at the fundamental role of Xbves in _X. laevis_ development. To expand this investigation, Xbves was knocked down using MO at various concentrations and developmental stages. Xbves depletion in a clonal population of cells inhibits proper movements and alters the ultimate fate of the affected progeny. Disruption of Xbves in one half of an embryo yields defects in epithelia of the

eye, epidermis, and other structures. While a thorough analysis of our findings from these two methods is far from complete, taken together, the outcomes of these experiments are consistent with a role for Xbves in epithelial morphogenesis and regulation of cell adhesiveness.

Movement and reshaping of epithelia are essential during embryogenesis. In vertebrate embryos, some of the first and most fundamental examples of epithelial morphogenesis are those that drive epiboly and gastrulation (Berditchevski, 2001; D'Urso et al., 1990; Heasman et al., 1994a; Keller, 1980; Nieuwkoop, 1973). In *X. laevis,* these movements are governed, in part, by rearrangements of cells within epithelia (Keller et al., 2003; Wallingford et al., 2002). Epiboly in *X. laevis* is regulated by the epithelial movements, which include the intercalation of deep cells and thinning of surface cells that affect spreading of the animal cap tissue towards the vegetal pole (Keller, 1991). These morphogenetic movements are governed by convergence and extension, which ultimately contribute to the closure of the yolk plug and neurulation. The activity of different signaling and adhesive systems permit repositioning and reorientation of cells within epithelial sheets that occur during gastrulation and epiboly (Keller, 1991; Wallingford et al., 2002). A dynamic expression of cell adhesion molecules is correlated with these embryonic movements. For example, EP/C and XB/U cadherins are expressed in cells at the marginal zone undergoing convergent extension (Kuhl et al., 1996; Lee and Gumbiner, 1995; Zhong et al., 1999). During involution, integrin and fibronectin interactions are required for the migration of cells along the blastocoel roof (Beddington and Smith, 1993; Winklbauer, 1990). While many critical players involved in these processes have been identified, there is still far from a

complete understanding of the cell biology and biochemical mechanisms/processes that balance plasticity with structural integrity in epithelial sheets during tissue elongation, and involution (Bradley et al., 1998; Brieher and Gumbiner, 1994; Broders and Thiery, 1995; Cordenonsi et al., 1997; Fagotto and Gumbiner, 1994; Heasman et al., 1994a; Heasman et al., 2000; Marsden and DeSimone, 2001; Wallingford et al., 2001b).

Following gastrulation, epithelial movements and rearrangements are also critically involved during later stages of development for proper formation of specific organs or tissues. Of specific interest are the epithelial interactions involved in epidermal and eye development. The ectoderm/primordial epidermis surrounds and protects the developing embryo throughout development. The embryonic skin differentiates to become a two-layered structure, with a columnar epithelial outer layer of tightly adherent cells over an inner, sensorial layer of less adherent epithelial cells (Deblandre et al., 1999). However, in addition to its protective function, it exhibits a high degree of proliferation and differentiation at specific sites where it contributes to the formation of other organs, for example, in the head region where the neural tube and eye will develop. The ectoderm exhibits a high expression of adhesive molecules, including desmosomes (Ohga et al., 2004) and a variety of cadherins. Specifically, the spatio-temporal expression of cadherin proteins suggests that cell adhesion/interactions are essential to the structure, integrity, and fate of the epidermis (Angres et al., 1991; Izutsu et al., 2000; Kintner, 1988). For example, the specific expression patterns of N-cadherin and E-cadherin help drive the process of neurulation. N-cadherin is expressed in the region of the ectoderm fated to become the neural tube, while E-cadherin is expressed in the flanking areas (Kintner, 1992). The N-cadherin-positive neural plate

epithelium undergoes dynamic cellular rearrangements and shape changes in order to fold and involute, forming the neural tube. This example underscores the requirement of cell adhesion molecules in organogenesis and illustrates how a molecule like Xbves may function to regulate epithelial interactions during embryogenesis.

The formation of the vertebrate eye is as an ideal model system for epithelial differentiation and cell adhesion processes (Jeffery, 2001; Pichaud and Desplan, 2002; Tripathi et al., 1991). The eye is derived from three embryonic sources: neuroectoderm of the diencephalon, head mesoderm (product of neural crest), and surface ectoderm. During neurulation the ectoderm invaginates and pinches off, forming the neuroectoderm/neural tube (Colas and Schoenwolf, 2001). Next the neuroectoderm of the diencephalon expands to form the optic vesicle, which will ultimately give rise to the retina. The optic vesicle comes into proximity with the surface ectoderm and induces it to form the lens placode (Baker and Bronner-Fraser, 2001; Faber et al., 2001). This epithelial placode quickly thickens, forms an invaginating cup and loses contact with the surface to form the lens vesicle. Meanwhile, the head mesoderm contributes to the iris and other supporting cells. Subsequently, the newly formed lens sends reciprocal signals back to the surface ectoderm and induces the formation of the cornea (Beebe and Coats, 2000). At this time, all the elements needed to form the eye are present and, thereafter, these embryonic structures will be modified and reshaped to form the adult eye. Following lens induction, retinal development begins as the epithelial optic vesicle invaginates to form a double-walled optic cup. This causes the apical surfaces of the inner and outer retinal progenitor layers to be apposed. The outer epithelial layer of the retina will become a simple, cuboidal retinal pigmented epithelium (RPE), while the

inner layer will differentiate into a multilayered and synaptically-competent neural retina (Cepko, 1993; Livesey and Cepko, 2001).

Cell adhesion plays a critical role in the induction, growth, movement, and stability of epithelia during formation of the eye (Erdmann et al., 2003; Xu et al., 2002; Zelenka, 2004). Interactions between these epithelial structures as well as adjacent mesenchymal cells are critical for eye development and disruption of any one component can lead to major defects in the entire structure. For example, cell adhesion is required for lens development, detachment of the lens, and the morphogenetic movement of cells in the lens vesicle. The cells of the lens express proteins of the tight, adherens and gap junction (Leong et al., 2000; Lo et al., 2000; Zampighi et al., 2000), and lens differentiation is blocked by antibodies to N-cadherin (Erdmann et al., 2003; Ferreira-Cornwell et al., 2000). Also, contact between the optic vesicle and lens via the ECM is thought to be important during early eye development to "cement" the two layers together (Webster et al., 1984). Regulation of cell adhesion plays an essential role in the production of the cornea (Meier, 1977; Xu et al., 2002). Adhesion of corneal epithelium is essential for formation and maintenance of barrier function and also to allow stem cells to migrate in as a sheet in the event of corneal wounding. TJs, desmosomes, AJs and gap junctions are present between these cells and contribute to the integrity of the corneal epithelium (Suzuki et al., 2003; Wang et al., 2004). Cell adhesion plays a critical role in the intimate association of the epithelial layers of the retina and is essential to both the establishment of the RPE as an effective blood-retinal barrier and the proper lamination of the mature neural retina (Bora et al., 1999; Erdmann et al., 2003; Jablonski and Ervin, 2000; Jensen and Westerfield, 2004; Kojima

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et al., 2002; Raymond and Jackson, 1995). Also, without N-CAM, retinal morphogenesis is impaired (Buskirk et al., 1980; Gundersen et al., 1993), and the loss of TJ function leads to deleterious effects in the development of the RPE (Jensen et al., 2001; Jensen and Westerfield, 2004). Consequently, the eye is a unique developmental system to study epithelial movement, morphogenesis and function. The formation of each layer relies on proper interaction within the epithelial layer itself, but also with the other layers of the eye and, thus, it serves as an excellent model to analyze epithelial cell adhesion.

Because epithelial movements are important to so many processes and stages of development, identification of new classes of molecules that regulate these movements is critical for a meaningful understanding of early development as well as epithelial morphogenesis. No studies have, to date, demonstrated a function for any *pop* family gene in epithelia in vivo, particularly during early development. Because early epithelial morphogenesis in the amphibian embryo is well characterized and yields predictable defects in epiboly and gastrulation with experimental intervention, *X. laevis* embryos provide an excellent model to test Bves function. Furthermore, epithelial movements involved in later stages of development such as those involved in eye maturation provide an opportunity to study the role that Bves in organ formation.

The *X. laevis* homolog of Bves was identified in 2003 by A. N. Ripley, who performed the initial characterization studies (Ripley et al., submitted). Sequence data obtained from cDNA clones predicted a 338 amino acid protein that was termed *X. laevis bves* (*Xbves*). The predicted sequence contained the canonical Bves/Pop protein family structure, including a short N-terminus with two invariant N-linked glycosylation sites, three hydrophobic domains separated by two intervening loops and a long C-

terminus (Knight et al., 2003). Xbves protein shared over 90% amino acid similarity with mouse and chick Bves, with significant variation in the N-terminus (2-28) and in the extreme C-terminus (316-338). Like chick *bves, Xbves* confers adhesiveness to fibroblast cells following transfection, as we observed with other Bves homologues, and traffics to cell contact points of A6 *X. laevis* kidney epithelial cells, demonstrating a conserved function across species (Ripley et al., submitted).

The distribution of *Xbves* mRNA and protein during early embryogenesis was determined (Ripley et al., submitted). Whole mount in situ hybridization clearly detected *Xbves* transcripts throughout the unfertilized egg (data not shown), but as cleavage initiates, the distribution of *Xbves* mRNA was restricted to the animal pole (Figure 21A,B). During gastrulation (stages 10-12), epithelial surface cells expressed *Xbves*, while yolk plug cells remained negative (Figure 21C). From stages 12.5 to 35, *Xbves* expression persisted in the dorsal regions of the embryo and *Xbves* became progressively restricted to specific structures, including the heart, somites, cement gland, and eyes (Figure 21D-E). Regions of immunoreactivity with an antibody to Xbves corresponded to those that expressing *Xbves* mRNA. Xbves protein is detected in all, or nearly all, epithelia in the gastrulating embryo and is predominantly localized to the interface between epithelial cells (Figure 21F,G). 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 (Osler and Bader, 2004; Ripley et al., 2004; Wada et al., 2001). This distribution along with its demonstrated adhesive properties predicts a broad role for Xbves in *X. laevis* gastrulation. Throughout neurulation and
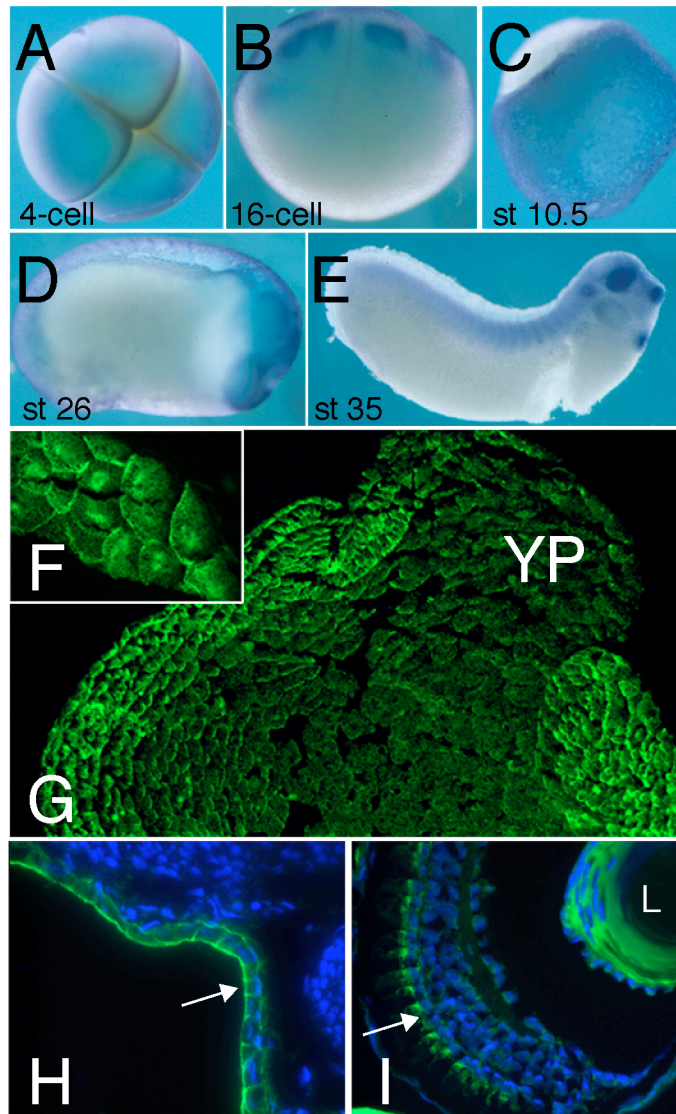
Figure 21. *Xbves* mRNA and protein expression patterns.
Whole mount in situ hybridization analysis shows *Xbves* expression throughout early development. *Xbves* message is restricted to the animal pole during early cleavages stages, as shown in a 4-cell embryo (A) and a 16 cell embryo (B) *Xbves* expression is observed in the surface epithelium in gastrulation stage embryos but is excluded from the yolk plug (C) and a more restrictive dorsal expression pattern upon neurulation (stage 25) (D). *Xbves* expression is observed in the heart, eye, cement gland, and somites of a stage 35 embryo (E). Control sense probe hybridization was negative. Immunohistochemical analysis demonstrates Xbves protein distribution in early embryos. At stage 11.5, α-Xbves (green) reacts with surface and involuted cells but is negative for endodermal cells of the yolk plug (YP) (F). A high power view of the epidermal region demonstrates that Xbves is confined to regions of cell/cell contact (G). α-Xbves (green) reacts with epithelial tissues such as the epidermis (H, arrow) and the lens (L) and photoreceptors (arrow) of the eye (I). DAPI nuclear stain is shown in blue.

tadpole stages, Xbves protein expression persisted in the dorsal ectoderm/epidermis, the underlying neural tube and optic vesicle. Other epithelial structures derived from mesoderm such as the notochord and somites were also positive for α-Xbves. Later, epithelial elements such as the epidermis (Figure 21H), velar plate, pronephros, somatically-derived striated muscle, epithelial layers of the eye (Figure 21I), and heart expressed Xbves protein. Clearly, this characterization of Xbves message and protein distribution demonstrates high expression in epithelia, suggesting a conserved role of Xbves during frog development. Thus, these findings support the use of *X. laevis* as an in vivo model system to study the function of Bves/Xbves during development and in epithelia.

In the absence of recognizable functional protein motifs, known interacting protein partners, or linkage to any established molecular pathway, a broad-based approach to ascertain the function of Bves proteins in development was necessitated. Global MO knock-down of Xbves during early development leads to a general arrest of gastrulation characterized by the failure of epiboly, yolk plug closure, involution and mesodermal patterning. When a 40 ng dose of *Xbves* or control MOs was injected into both cells of the two-cell embryo, *Xbves* MO-injected embryos appeared to undergo normal cleavage and are indistinguishable from control MO-injected and non-injected embryos until stage 9+ (Figure 22A,F). At stage 10, slight differences in the size of the yolk plug 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, movement of the epithelial layers was greatly inhibited (Figure 22G). *Xbves* MO-injected embryos had a large yolk plug, did not form a neural
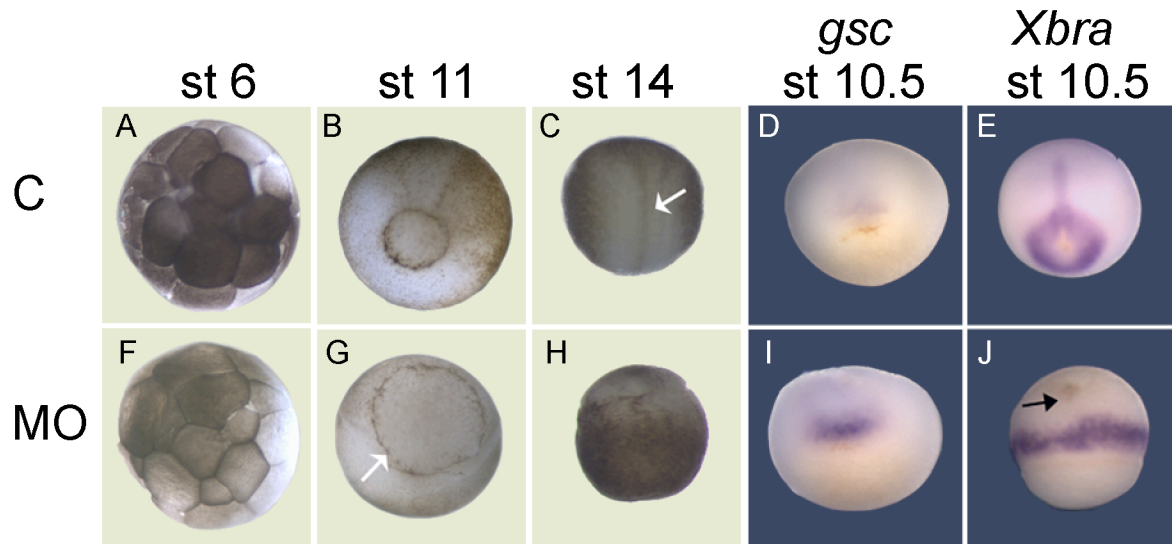
Figure 22. *Xbves* depletion inhibits gastrulation movements.
Uninjected embryos proceed through development normally and serve as a control (A-E). The neural plate is denoted by the white arrow at stage 14. Control sense MO-injected embryos complete gastrulation and undergo neurulation at the same rate compared with uninjected embryos (not shown). Injection of *Xbves* MO into a 2-cell *X. laevis* embryo blocks gastrulation and proper expression of mesodermal markers (F-J). At stage 6, Xbves MO-injected embryos develop in an apparently normal fashion (F). By stage 11, experimental embryos are delayed or inhibited in yolk plug closure (G, arrow denotes the edge of the yolk plug). By stage 14, most embryos lack closed yolk plugs and have become necrotic (H). In situ hybridization analysis demonstrates altered expression of mesodermal markers *goosecoid (gsc)* and X*bra*. Expression of *goosecoid* and X*bra* markers is unaffected in embryos injected with control MO at stage 10.5 (D and E). *Xbves* depletion leads to an abnormal accumulation of *gsc* staining immediately anterior to the dorsal lip of the blastopore (I) and the lack of X*bra* in midline mesendoderm (J, arrow).
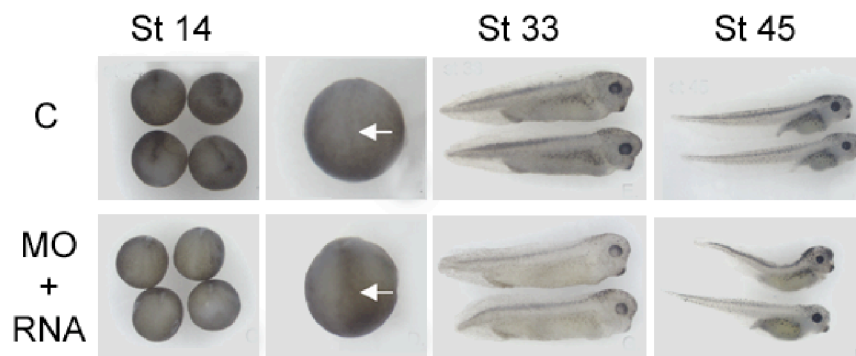
Figure 23. Xbves depletion by MO is rescued with co-injection of *Xbves* mRNA. Uninjected control embryos at stage 14, 33, and 45 (top row) demonstrate normal development. Co-injection of *Xbves* MO with a MO-resistant *Xbves* mRNA rescues the developmental defects caused by Xbves depletion. Embryos at stages 14, 33, and 45 appear grossly normal, with the exception of a slight tail curve observed at stage 45.

plate and became necrotic soon thereafter (Figure 22H). Control MO had no effect, except a slight delay in development, completing gastrulation and forming a neural plate along the dorsal axis (Figure 22B,C). Disruption of *Xbves* function also affected mesoderm formation and patterning, as the expression of two mesodermal markers, *goosecoid* and *Xbra,* (Cho et al., 1991; Smith et al., 1991), was altered in *Xbves* MO injected embryos (Figure 22I,J). These data demonstrated that mesoderm formation was not inhibited with loss of Xbves function, but that the proper movement and positioning of mesendoderm was severely disrupted. The specificity of the morphant phenotype was verified by co-injection of MO-resistant *Xbves* RNA with *Xbves* MO. In those embryos, the gastrulation defect was rescued (Figure 23). Importantly, the efficacy of MO in Xbves elimination is demonstrated by assessment of the loss of protein, and rescue experiments show the nearly complete reversal of the morphant phenotype with reinstatement of *Xbves* function. The Xbves message and protein expression profile in *X. laevis* embryonic epithelia coupled with the MO knockdown studies reveal an important role for this protein in epithelial morphogenesis.

All of these anomalies in Xbves morphant embryos 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 (Brieher and Gumbiner, 1994; Heasman et al., 1994a; Heasman et al., 1994b; Levine et al., 1994; Marsden and DeSimone, 2001). While these phenotypes vary, global inhibition of any of these molecules results in a cessation of epithelial movement and a failure of embryos to rearrange cell sheets during gastrulation. For example, inhibition of EP/C-cadherin decreases intercellular adhesion and disrupts blastocoel integrity (Heasman et al.,

1994b) while disruption of XB/U-, C-, or E-cadherin prevents embryos from completing gastrulation (Kuhl et al., 1996; Lee and Gumbiner, 1995; Levine et al., 1994). Furthermore, disruption of the integrin/fibronectin interaction with α-fibronectin antibodies inhibits gastrulation by affecting radial intercalation movements and cell polarity (Longo et al., 2004). Thus, it is reasonable that depletion of a component such as Xbves from epithelial adhesion complexes may result in a similar phenotype.

Initial studies from our laboratory have demonstrated the presence of *Xbves* in epithelia during the earliest phases of frog development and the conservation of its adhesive function. To further investigate Xbves function during embryo morphogenesis, we expand these studies by disrupting *Xbves* with MO using two distinct methodologies. First, Xbves knockdown in a select clone of cells was achieved by injecting one of 32 cells. Clonal inhibition of Xbves activity within a specific blastomere and its derivatives completely randomizes the movement of progeny within otherwise normally differentiating embryos. The resulting data demonstrate that Xbves plays a critical role in epithelial morphogenesis and specifically in the cell movements essential for the large-scale epithelial rearrangements that occur during early *X. laevis* development. In a second experiment, half of the embryo was depleted of Xbves by injecting one of two cells following the initial cleavage event. The major finding from this analysis is a significant disruption in epithelial cell types following neurulation. Initially, we chose to focus on analysis of the epidermis and the eye, both of which are severely affected by Xbves depletion. Importantly, Xbves is present during critical periods of eye and epidermal development and is in a position to mediate cell/cell interaction events. The present study of the "eyeless" embryos is a work in progress, with full analysis currently

underway. However, to this point, the findings clearly demonstrate the importance of Xbves in epithelial development in the early *X. laevis* embryo.

Materials and Methods

Animal and embryo care

Adult *X. laevis* were obtained from commercial sources (*Xenopus* Express). *X. laevis* eggs were harvested and fertilized by standard methods (Danilchick et al., 1991) and staged according to Nieuwkoop and Faber (1967).

mRNA constructs for injection

An *Xbves* cDNA that lacks 5'UTR and begins with the start ATG codon was constructed and used to test the rescue capacity of cloned *Xbves* mRNA with MO treatment. This transcript excludes the most of the MO target sequence found solely in the 5'UTR (i.e. the first 17 bp of the MO). This construct successfully rescued *Xbves*-MO induced developmental abnormalities. Nuclear lacZ mRNA was synthesized by mMESSAGE mMACHINE (Ambion) and co-injected into developing *X. laevis* embryos.

Morpholino treatment

A series of antisense MO had been synthesized previously (Gene Tools). The most efficacious morpholino in terms of eliminating protein synthesis in developing embryos was: ATCTTTCTTATACCTGGATGTGCAG that contains the reverse complement to the AT of the start codon and sequences immediately 5'. Control and

experimental MO was dissolved in water and diluted in water to appropriate concentrations based on the experimental design. The sense complement had no influence on development up to 80 ngs/blastomere.

In one 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 according to published protocols (Morasso et al., 1995). Staining of control and experimental groups were measured and the average dimensions determined using the Student's t-test. In a second set of experiments, control or *Xbves* MO was injected into one cell of the 2-cell embryo (20 ng/cell), with 1.5ng of lacZ tracer RNA in a 5 nl volume. *Xbves* MO or control MO (20 ng) was injected into 1 of 2 cells of a 2-cell embryo. Embryos were allowed to develop to 2 days (stg 35-38). This experiment was designed to eliminate Xbves in one *half* of the embryo. The uninjected half of the embryo develops normally and serves as an internal control (this experiment was initially designed by A.N. Ripley). Whole mount, lacZ-stained embryos were photographed and captured by Magnafire (NIH).

In situ and histological analyses

Processing of embryos was performed as described (Sive et al., 2000). Embryos for whole mount analysis were fixed in MEMFA for 20 minutes to overnight and subsequently washed in PBS and transferred to MeOH for long-term storage at -20°C. Embryos selected for sectioning were paraffin embedded and stained with hematoxylin

and eosin. LacZ injected embryos were processed for β-gal staining and refixed with MEMFA prior to storage in MeOH.

Whole mount *in situ* hybridization analysis was conducted according to published methods (Harland, 1991). *Xbves*, *Xbra* (Smith et al., 1991), *goosecoid* (Cho et al., 1991) probes were DIG labeled and hybridized with experimental or control embryos. Following substrate color reaction, embryos were post-fixed in Bouin's fixative, bleached for 1-2 hours, and stored in methanol.

For clonal analysis of lacZ-labeled A1 progeny, images of cross-sectioned stage 12 embryos were analyzed to determine the location of labeled nuclei relative to the embryo surface. Randomly selected blue nuclei (>250) were counted for each group and were scored as 0-20, 20-40, 40-60 and >60 μm from the surface. The statistical significance was determined by Chi square analysis.

Electron microscopic analysis

Embryos were fixed in 2.5% glutaradehyde in cacodylate buffer for 2 hours at room temperature, washed in PBS, and stored in cacodylate buffer. Further processing and generation of thick and thin sections were performed by standard methods in collaboration with the Vanderbilt Research EM resource laboratory. Statistical analysis of epidermal cell size was performed using Microsoft Excel and the Student's T-test.

BrdU analysis

Cell proliferation was assessed using a BrdU incorporation kit by (Roche). BrdU is incorporated into DNA and provides a measure of DNA synthesis, and thus cell

proliferation. Cells that have incorporated BrdU into dividing cells can be detected using an antibody to BrdU and a fluorochrome-conjugated secondary antibody. Briefly, embryos at 1 day post fertilization were immersed in BrdU containing media (40ul of 1000x BrdU diluted in 0.1x Steinberg's solution) for 24 hours. BrdU-exposed animals were collected at ~stage 35. Embryos were flash frozen in OCT and sectioned. Sections were incubated with α-BrdU antibody (1:10) and fluorescence of the fluorescein-coupled secondary antibody was evaluated.

Results

Progeny of the *Xbves*-depleted A1 cell display rogue cell movements

The initial MO-injection experiment, performed by A.N. Ripley, focused on a global depletion of Xbves and was accomplished by injecting both cells of a two-cell embryo after the first cleavage event (Ripley et al., submitted). This treatment led to a general inhibition or altered regulation of epithelial movement and an overall failure in development. After confirming the results of A.N. Ripley, I expanded these studies by determining whether Xbves knockdown within a 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. We selected the A1 blastomere (note: there are two A1 blastomeres at the 32-cell stage) as the target cell for this experiment because the progeny of A1, the dorsal animal clone, are thought to undergo significant morphogenetic movements of convergence, extension, and intercalation during gastrulation (Bauer et al., 1994),

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(Figure 24). Extensive fate mapping studies have been published that determine the developmental program of all cells at the 16- and 32-cell stages. These studies have defined that the descendants of the A1 cell ultimately reside in cranial regions and somites. Thus, the cell derivatives of the A1 blastomere exhibit dynamic movements and predictably assume a specified identity as organ structures begin to form (Figure 24), making this an appropriate target cell to assess the role of Xbves in cell movement and cell determination.

To examine the role of Xbves in the controlled movement of epithelial cells towards the blastopore, we used MO to disrupt the expression of *Xbves* in the A1 cell at the 32-cell stage. Successfully injected cells were identified by a β-gal expression. Gross morphology was indistinguishable in groups of *Xbves* and control MO-injected embryos over the period of development studied but striking differences were observed in the distribution of A1 progeny between control and experimental groups. In the controls (uninjected or control MO-injected), labeled A1 descendents formed a tightly compacted continuous strip of cells at stage 12 (Figure 25A,D, Figure 26A). This configuration of cells has been observed previously and results from convergent extension movements in the gastrulating embryo (Bauer et al., 1994). This strip was narrowest at the blastopore where cells had begun involution and broadest near its anterior margin. The tightly packed array of cells was observed in 73% (117/161) of control embryos (Figure 25I). The remaining 27% embryos display a pattern of cell movement that is more irregular. In contrast, progeny of *Xbves* MO-injected A1 blastomeres had a completely different distribution (Figures 25B,E, 26B). Most clones had a highly irregular shape and did not extend to the blastopore. Some clones were

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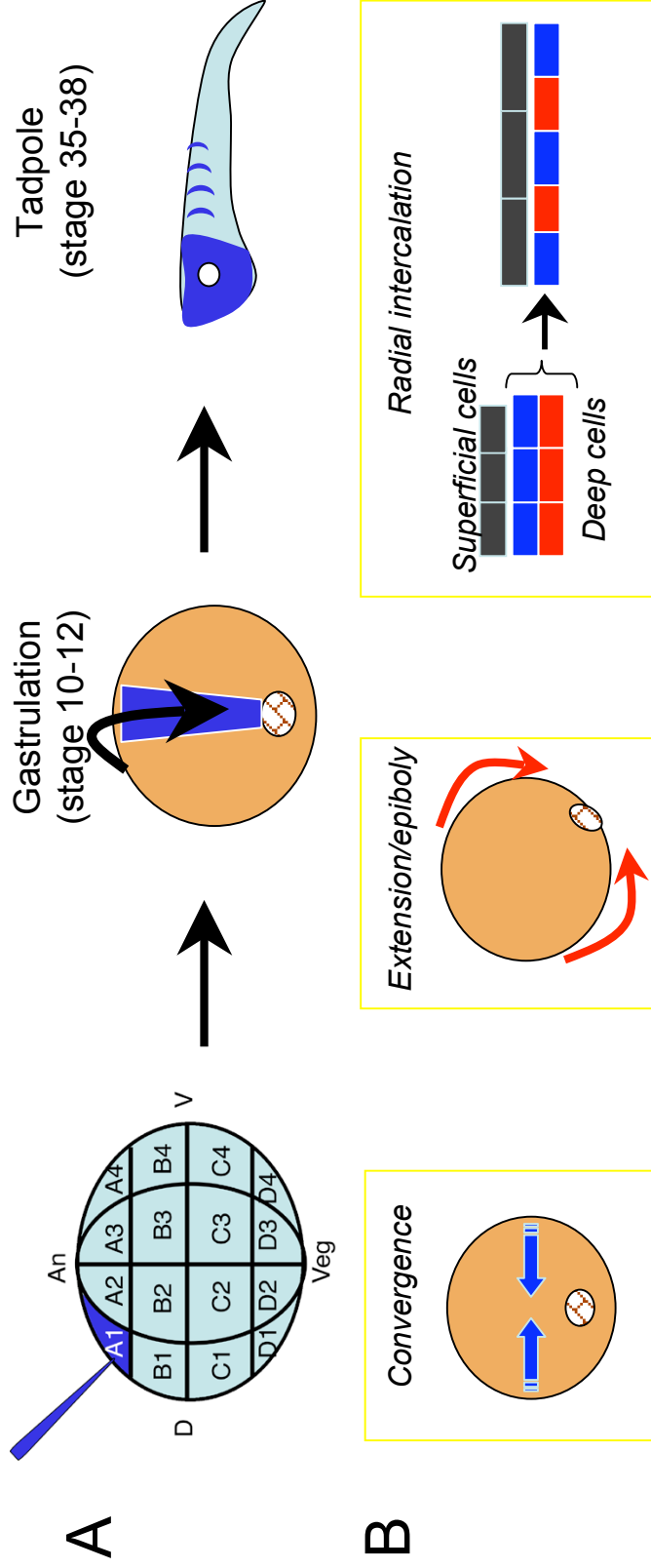
Figure 24. A1 blastomere movements and cellular rearrangements during embryogenesis.
The progeny of the A1 cell of the 32-cell embryo exhibit dynamic movements and rearrangements. The migration pattern of A1 cell descendants, as predicted by fate mapping studies, is depicted in blue (A). During gastrulation, A1 derivatives are found in a cone-shaped stripe directed toward the dorsal lip of the blastopore (stage 11-12). At stage 35, A1 progeny reside primarily in cranial structures, including the cement gland, olfactory and lens cells, and many neural cell types. Descendants are also found in the pharynx, foregut, and somites. Cells undergo several types of morphogenetic movements during gastrulation (B). Cells exhibit convergence as they move inward toward midline. Cells also extend around the embryo, moving toward the blastopore in both directions. Radial intercalation of deep cells and surface cells helps to drive the processes of convergence and extension. These cell movements, in addition to others, facilitate the initiation and completion of gastrulation and ultimately allow the formation of additional germ layers.
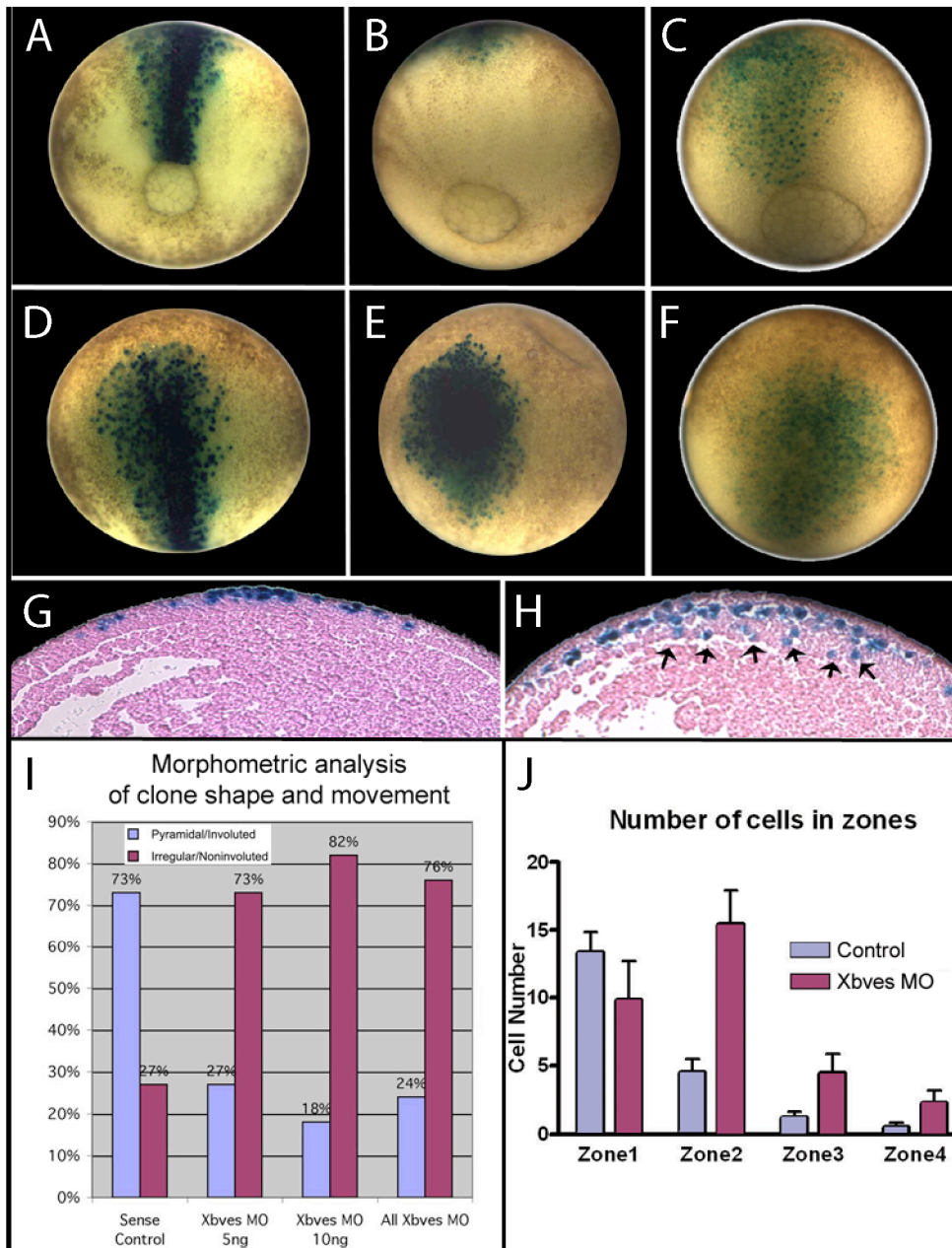
Figure 25. Xbves depletion in the A1 cell alters movements of progeny during gastrulation.
Three embryos were imaged to show the blastopore (A-C) and reimaged after rotation (D-F).
Injection of control MO with lacZ tracer into the A1 blastomere demonstrates the convergence of
A1 progeny toward the blastopore at stage 12 (A,D). Progeny of *Xbves* MO-injected A1 cells do
not follow normal paths of movement (B,E and C,F). Cross-sections of these embryos
demonstrate that progeny of the control MO-injected A1 blastomeres remain closely associated
with the embryo surface (G) while derivatives of *Xbves* MO-injected cells are often detected
below the surface (H, arrows). The shape and position of lacZ-labeled cells relative to the
blastopore was determined for control and experimental groups at stage 11.5 is graphically
represented (I). Within each group, clones with a pyramidal shape extending to the blastopore
are demarcated in blue. Red denotes those clones that do not have this configuration. The
derivatives of the A1 cell were counted in sections through control and *Xbves* MO-injected
embryos (J). The position of lacZ-labeled nuclei relative to the embryo surface was measured
and values were placed in groups (Zone1: 0-20, Zone2: 20-40, Zone3: 40-60 and Zone4: >60 m
from the surface). Data show a significant shift of *Xbves* MO-injected cells away from the surface.
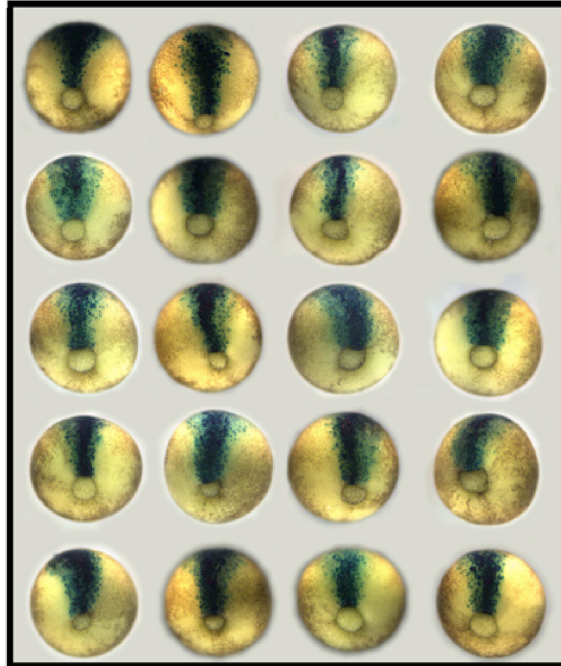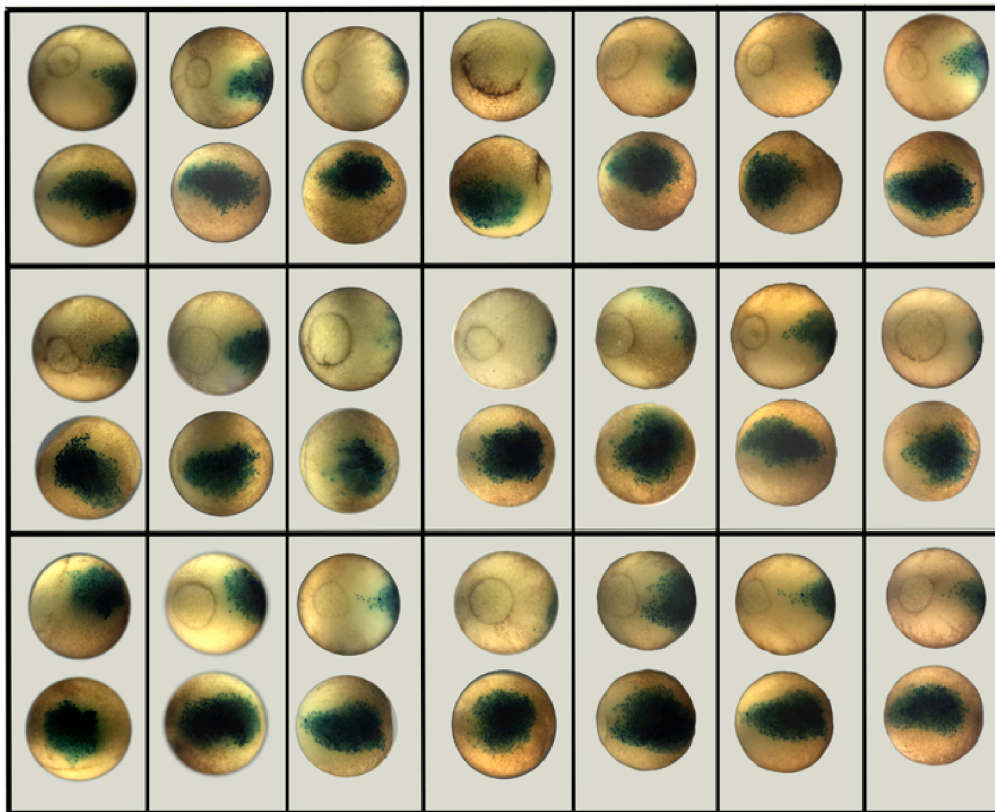
Figure 26. Control and *Xbves* MO injection of the A1 blastomere. Control MO injection demonstrates the normal pattern of movement of A1 progeny (A). Injection of *Xbves* MO results in wide variation of clone movement (B). Each box in B shows two views of the same embryo. Labeled clones of cells from *Xbves* MO-injected embryos did not demonstrate a directed movement toward the blastopore as observed in control embryos.

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located on the pole opposite the blastopore and did not possess a discernable narrowed leading edge orientated toward the blastopore. Other *Xbves* MO-injected clones had a diffuse appearance with many interspersed non-labeled cells (Figure 25C,F). A small subset exhibited a narrowed leading edge but this did not reach the blastopore lip. Morphometric analysis of these clones determined that 73% (55/72 embryos with 5 ng of *Xbves* MO) and 82% (29/35 embryos with 10 ng of *Xbves* MO) had an irregular shape that did not extend to the blastopore (Figure 25I). The remaining 27% (5 ng MO) and 18% (10 ng MO) of embryos show a pattern similar to controls, demonstrating a slight dose-dependency of the MO-induced defect. Also, the average width of the labeled patch of cells from 34 randomly selected *Xbves* MO-injected clones was 855 μm, compared to 585 μm in control embryos (p<0.0001; Student's t-test).

To quantify the extent of dysregulation, the stage 12 embryos were fixed and sectioned. Histological analysis of sections through control and *Xbves* MO-injected embryos revealed that lacZ-labeled cells had differing distributions relative to the embryo surface. Sections were divided into four equivalent zones with the uppermost epithelial cell layer classified as zone 1. Zones 2, 3, and 4 represent the layer of cells penetrated by the random migration of β-gal stained cells. As seen in Figure 25G, labeled cells of the control group 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 (Figure 25H, arrows). To quantify the result, the location of labeled nuclei relative to the surface was measured in 20 μm increments. The number of cells within each increment (0-20 μm, 20-40 μm, 40-60 μm, 60+ μm) of control embryos was compared to that of *Xbves* MO-injected embryos. As seen in

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graphical form (Figure 25J), the distribution of cells from *Xbves* MO-injected blastomeres shifted away from the embryo surface indicating a repositioning within those epithelial structures. Embryos injected with *Xbves* MO demonstrated a significant number of cells in zones 2, 3, and 4 as compared to embryos injected with control MO (p<0.001, by ANOVA with Bonferroni test for multiple comparions).

Fate maps have established that A1 progeny contribute to anteriorly located tissues in the head region including the skin, connective tissue, and neural components, as well as to the head and trunk somites (Moody, 1987). To determine whether cell fate and location was altered by Xbves depletion, control and experimental embryos were allowed to develop to stage 37 (two days post fertilization). During this timeframe, the gross morphology and behavior of control and experimental embryos were indistinguishable. As seen in Figure 27A, control injected cells faithfully differentiated into predicted structures (as diagrammed in Figure 24) with little "scatter" of labeled cells. Note that β-gal staining in the head totally obscured visualization of the eye and that somites were labeled (Figure 27A). Section analysis demonstrated the extensive incorporation of lacZ-labeled cells in the eye, skin, brain, connective tissue of the head (Figure 27D) and anterior somites (data not shown). β-gal-positive cells within the deepest portions of the embryos indicated the lacZ staining could penetrate throughout the embryo.

In contrast, the progeny of *Xbves* MO-injected A1 cells were found to have a wide and inconsistent range of distributions throughout the embryo. In most cases, β-gal-positive cells were dispersed throughout the embryo without concentration in any particular structure and, notably, with no enrichment in the head or somites (Figure

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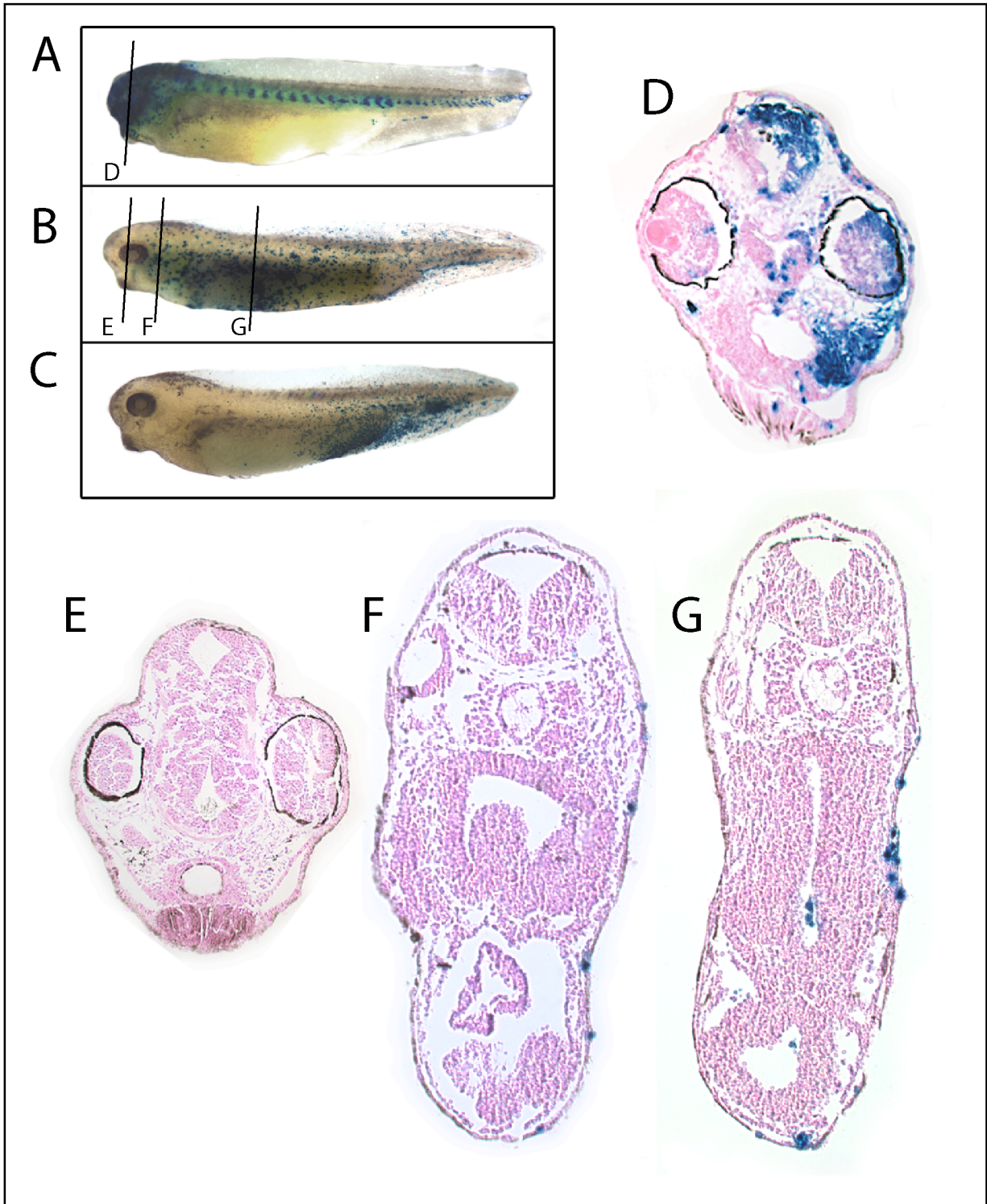
Figure 27. *Xbves* MO injection randomized movement of A1 progeny.
At stage 37, A1 derivatives are observed in head structures and in somites of control MO-injected animals. (A). A1 progeny are randomly distributed throughout the embryo after *Xbves* MO injection (B,C). Note the absence of labeled cells from the head region with *Xbves* MO injection. A cross section through the head region of a control embryo (A) reveals blue cells in various cranial structures and tissue types (D). This distribution correlates with predicted fate mapping studies. In sections through an *Xbves* MO-injected embryo (B), few labeled, if any, cells are detected in the head region (E). Trunk cross-sections show that most blue cells reside in the outer epidermis of the embryo (F,G).

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27B,C). Additionally, a subset of embryos had labeled cells located exclusively in the caudal region, a position normally devoid of A1 progeny (compare to Figure 27A). 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 (Figure 27E-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. Furthermore, we postulate that this outcome results from a dysregulation of cell adhesion and/or cell-cell interaction, which supports a function for Xbves in epithelial junction integrity.

Depletion of Xbves results in epithelial defects

The *X. laevis* embryo is distinct in that, following cleavage into two cells, each cell gives rise to either the left of right half of the embryo (Hirose and Jacobson, 1979). This property provides a unique opportunity to challenge the developmental potential of one side of the embryo, while the other side serves as an internal control. In the previous "global" knockdown study, a high dose of *Xbves* MO (40 ng) injected into both cells of a two-cell embryo results in a block in early epithelial movements. To explore how developing embryos respond to selective inhibition of Xbves function in one half of the embryo, one of two cells of a two-cell embryo was injected with 20 ng of *Xbves* MO. This is a lower dose than used for previous studies. Following the titration of MO injection into one of two cells, 20 ng was sufficient to generate the "eyeless" defect and

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was used for the remainder of our studies. Control embryos are either uninjected or treated with 20 ng of control MO in one of two cells. *Xbves* MO-injected embryos are able to undergo gastrulation and demonstrate no overt developmental inhibitions up to tadpole stages (Figure 28A-C). Occasionally, MO-injected embryos are slightly delayed in development as compared with controls. However, as the embryos continue to grow, the resultant phenotype is striking (Figure 28D,E). While the embryos are able to generate head, trunk, and tail structures, their development is significantly disturbed. MO-injected embryos are much shorter and exhibit a severe body curvature at stage 42 (Figure 28D). Often the embryos curve inward on the affected side. Uninjected or control MO-injected embryos are longer and develop a straight tail structure with dorsal fin that the experimental embryos appear to lack. By five days of development, a distinction is easily made between the MO-injected tadpoles and the control embryos (Figure 28E). The animals are severely deformed and are even shorter and more curled than even those at two days of development. Furthermore, the state of the body curvature causes the animals to swim in circles. Pigmentation also appears to be increased in *Xbves* MO-injected embryos. Clearly, the depletion of Xbves in one of two cells after the first cleavage event results in severe defects in epithelial tissues which appear restricted to one side of the embryo. This finding again highlights the importance of Xbves for epithelial development and suggests an important function in regulation and maintenance of this cell type. Co-injection with MO-resistant *Xbves* mRNA rescues these embryos and allows development to proceed normally, as observed in Figure 23.
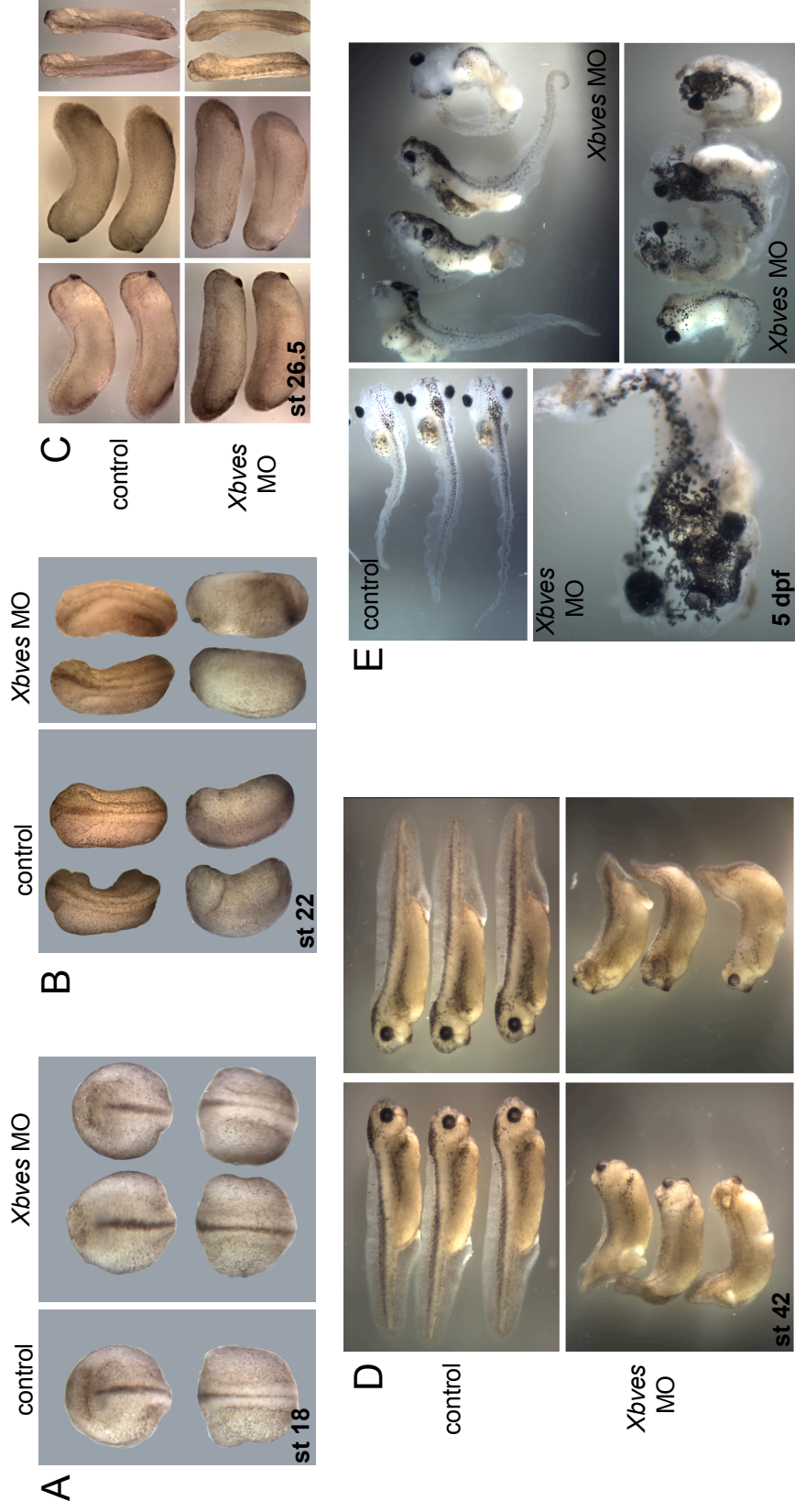
Figure 28. Comparison of uninjected and *Xbves* MO-injected embryos.
Development proceeds past gastrulation, but arise at 2 days following low-dose *Xbves* MO injection. Embryos injected with 20 ng of *Xbves* MO in 1 of 2 cells after one cleavage successfully undergo gastrulation and do not appear strikingly different, externally, than control embryos at stages 18 (A), 22 (B), or 26.5 (C). Embryos subject to *Xbves* MO injection show significant external signs of developmental anomalies after 48 hours of development. Stage 41 MO-injected embryos are shorter and exhibit a sideways curvature compared to controls (D). Occasionally, MO-treated embryos can live to 4 and 5 days post-fertilization (E). The bodies of these embryos are severely deformed and curled. External pigmentation appears to be increased.

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Eye development is inhibited following *Xbves* MO injection

Depletion of Xbves protein in one of two cells at the two-cell stage, and its progeny, resulted in severe malformations on one side of the embryo. A detailed close-up external examination of *Xbves* MO-injected embryos at stages 34-37 reveals that embryos are unsuccessful in the formation of an eye on one side (Figure 29A4, arrow, A5, asterisk), as compared to controls (Figure 29A1-3). This is most readily apparent by the lack of the dark round eye structure on one side of the head, which is given its pigmented color from the RPE (Figure 29A4,6, arrowheads). Histological analysis of transverse sections through *Xbves* MO-injected embryos reveals striking internal differences (Figure 29B). While the formation of ectodermal, mesodermal, and endodermal layers appears to proceed without obstruction on both sides of the embryo, the disruption of epithelial structures, including severe eye defects, was a hallmark of this phenotype. One side of the embryo exhibits a developing eye, in which a retina and lens structure can be clearly distinguished (Figure 29B1, box2). Higher power images allow the visualization of the RPE, the dark, pigmented cell layer around the retina (Figure 29B2,4, arrowheads). In addition, the epidermis covering the inner structures is thin and smooth (Figure 29B4, arrow). The other half of the embryo, presumably the *Xbves*-MO injected side, appears highly disorganized. Eye structures can be resolved, although highly undeveloped, and there is an overall loss of tissue integrity throughout the entire side (Figure 29B1). A close examination of the eye region reveals that the RPE surrounding the defective eye is lacking (Figure 29B3). As seen in Figure 29B5, the lens is not clearly separated from either the retina or overlying epithelium. Normally, the covering epidermal layer over the lens is fated to form the cornea. On the disrupted

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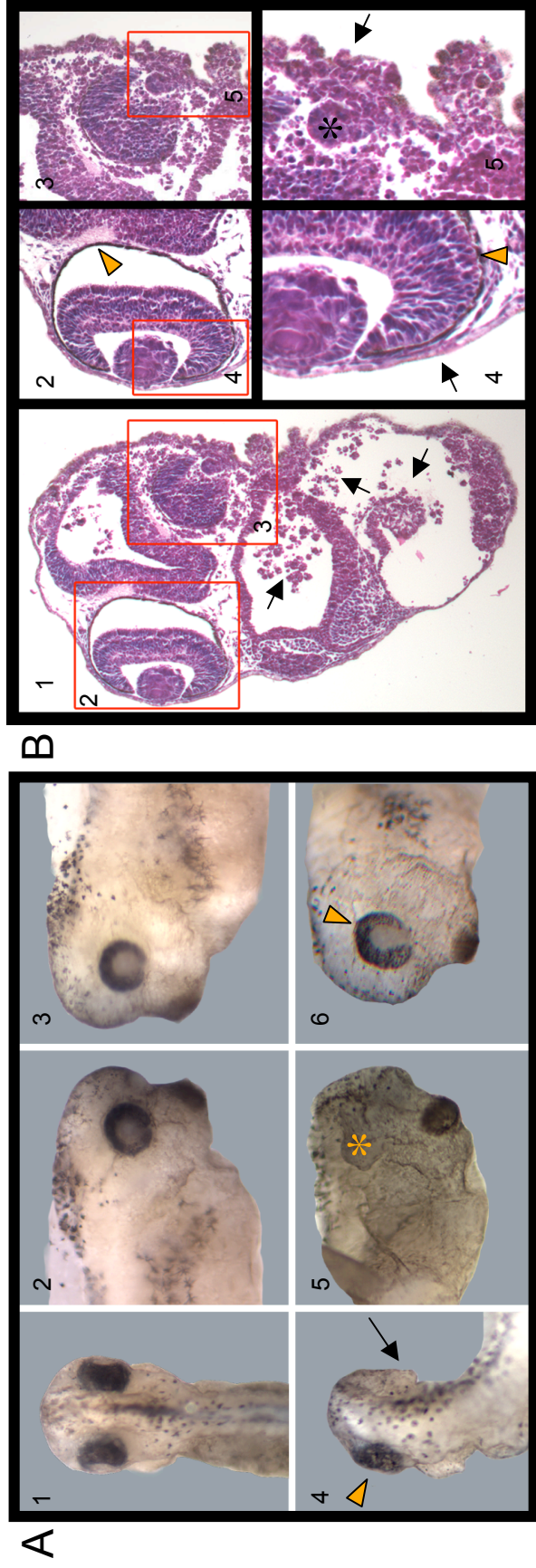
Figure 29. Xbves MO-injected embryos fail to develop a fully-formed eye structure.
Control embryos clearly have two eyes, as seen by the dark pigmented eye structures on either side of the head (A1-3). The bodies of control embryos are straight and symmetrical (A1). Close examination of 2 day (stage 35 embryos) MO-injected embryos reveal that Xbves depletion can block eye development (A4-6). Embryos lack eye pigment on one side (A5, asterisk) and exhibit a trunk curvature on the same side (A4,arrow). The uninjected side of the embryo forms an eye similar to controls (A4,6, arrowheads). H&E stained sections through MO-injected embryos show a severe disruption of many internal structures, including the eye (B1). The uninjected side of the embryo forms a fully developed eye (B2). An ordered retinal pigmented epithelium surrounds the retinal layers, which encompass the lens (B4). The RPE on the control side is indicated by arrowheads (B2,4). The MO-injected half of the embryo lacks an RPE, has an unorganized retina, and lacks a fully formed lens (B3,5, asterisk). High power images show that the control eye has a smooth corneal epithelium covering the lens and retina, whereas the MO-injected eye develops a bubbled, disorganized epithelial structure over the lens (B4,5, arrows). Loss of cellular integrity is observed in the internal cavities (B1, arrows).

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half of the embryo, this layer is much thicker, and ruffled, as compared with the smooth, ordered epithelium on the control side (Figure 29B5, arrow). In addition to clear defects in eye formation, the entire outer epidermis appears to be affected, and the detached clumps of cells within the body (neural, pharangeal and cardiac) cavities suggest that internal tissue integrity is disrupted (Figure 29B1, arrows). Also, note that both whole embryos and transverse sections show a body curvature, an outcome characteristic of this *Xbves*-MO injection experimental protocol. Internal structures such as the heart and liver diverticulum and lateral mesoderm tissue can also be distinguished and do not appear to be as developmentally delayed or inhibited as the eye.

Coinjection with lacZ tracer confirms specificity of MO-induced defects

To establish the specificity of Xbves MO-treatment and to confirm that the "eyeless" side correlates with the half of the embryo receiving the MO, co-injection of lacZ mRNA and *Xbves* MO into one cell of a two-cell embryo was performed (Figure 30A). In early tadpole stage whole embryos, β-gal staining can be detected in nearly all cells on the injected half of embryos throughout varying stages of development, ranging from stage 18 to stage 27-28 (Figure 30B-E). Also demonstrated in Figure 28A-C, no overt external defects due to MO-injection are observed at these stages. Although some crossover of cells from one half to the other does occur infrequently (see Figure 30D, arrow), β-gal expressing cells are primarily restricted to one side of the embryo. This system is ideal since the MO-injected cells, marked in blue, provide a high contrast from the uninjected side, which serves as an internal control. As with other injection
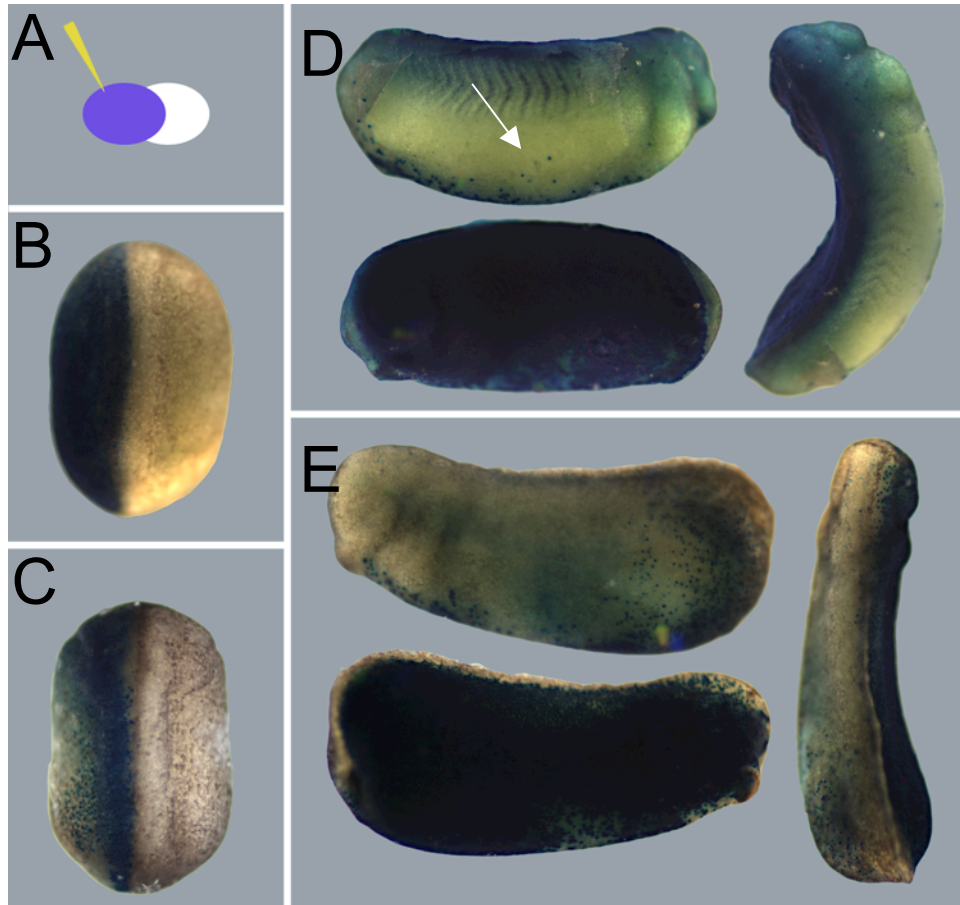
Figure 30. LacZ mRNA injection analysis.
LacZ tracer mRNA injection into 1 of 2 cells labels half of a developing *Xenopus* embryo. Injected cells can be detected by labeling for β-galactosidase. Embryos of various stages received lacZ in 1 of 2 cells following the first cleavage event (A). Half of a stage 18 (B), stage 22 (C), stage 25 (D) and stage 28 (E) embryo has been labeled blue after fixation and processing for β-gal.

protocols, morphological aberrations were not observed in uninjected control embryos, control MO-injected embryos, or embryos co-injected with rescue RNA.

Embryos lacking Xbves consistently demonstrate one-sided epithelial defects

To probe further into the defects observed with Xbves depletion in one half of an embryo, a detailed histological examination of stage 35 MO/lacZ-injected embryos was performed following paraffin embedding and eosin staining (Figure 31). Co-injection with lacZ provides a confirmation of which cells are Xbves-depleted and allow a confident assessment of cellular defects that is not possible with unlabeled embryos (Figures 30,31). Three whole mount embryos were examined to demonstrate the consistency of the developmental aberrations. Transverse sections through these embryos reveal that one half of the embryo contains β-gal expressing cells, while the other side is nearly free of any blue cells. The labeled side of the embryo exhibits external and internal defects, indicating that MO-induced Xbves depletion is responsible for the alterations. Specifically, as shown in Figure 31D-F, β-gal positive cells were located on the half of the embryo lacking a developed eye and several disrupted cell layers and tissues. On the β-gal expressing MO-injected side, the outer epithelial layer is highly irregular, much thicker, and often has a ruffled appearance (Figure 31E4). Contrarily, the epidermis found on the unlabeled, control side is a thin, smooth, cell layer, as observed in Figure 29. Note the sharp contrast in cell morphology from the uninjected, unlabeled side to the β-gal expressing/MO-injected half of the embryos (Figure 31F1, arrow). In several cases, the labeled epidermis has lost integrity and has separated from internal cell
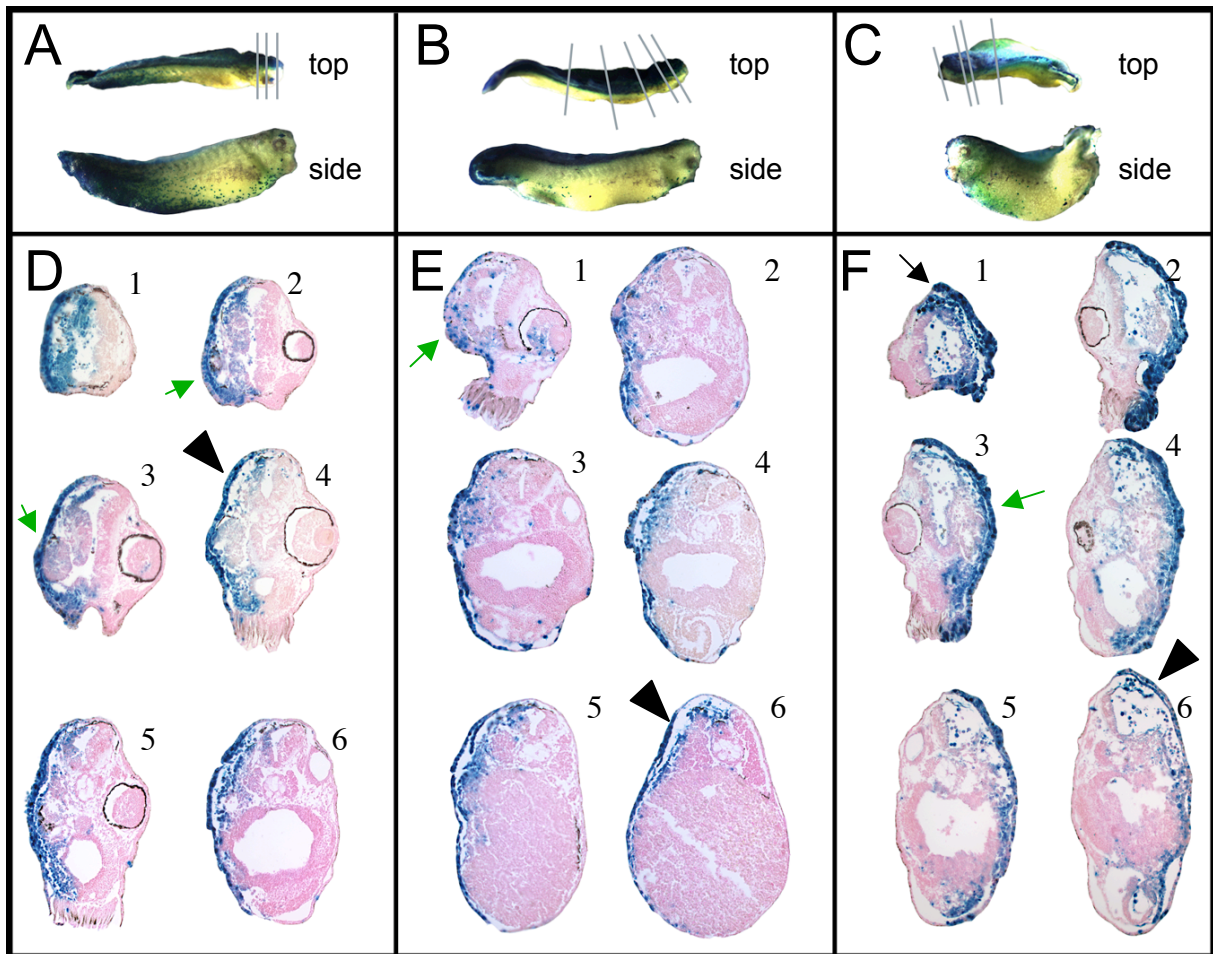
Figure 31. *Xbves* MO induces global epithelial defects on injected side of embryo. β-gal positive cells signify MO-injected progeny in three stage 35 whole mount embryos (A-C) and sections labeled with eosin (D-F). Gray lines indicate approximate location of cross sections (A-C). The uninjected side of embryos exhibits a fully developed eye (D2-5, E1, F2-4), with a single layer retinal pigmented epithelium around the retina, a circular lens structure and a smooth corneal epithelial layer covering the lens (D4, E1,F3). All three embryos lack a fully developed eye on the side co-injected with LacZ mRNA and Xbves MO, as indicated by the absence of the RPE on the side with blue cells (D-F, green arrows). The epithelial layer on the 'blue' side is also thicker, often bubbled, and occasionally even separated from the internal layers of the embryo (D-F, arrowheads). Note the abrupt change of cell morphology of the outer epithelium from the uninjected to injected side, most apparent in F1 (arrow).

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layers (Figure 31D4,E6,F6, arrowheads). However, despite these defects, the overall structure of the embryo is maintained and intact.

As a gross morphological assessment of whole embryos and preliminary sectioning had predicted (Figure 29), this inspection revealed that the arrest of eye development occurs to some degree in all of the three stage 35 embryos examined. Retinal development is initiated but the stage of optic cup maturation is highly variant when compared to the contralateral control. At present, it is unclear whether retinal cell types are completely blocked in their differentiation or if they have emerged but are incapable of proper alignment and significant differentiation. In some embryos, the pigmented retina does appear to form, but does not have significant accumulation of granules (Figure 31D3,5). Further analysis is necessary to determine the degree to which RPE differentiation has taken place. Furthermore, formation of the lens and the cornea is affected also. While a rudimentary invagination of surface ectoderm is observed, lens development appears to be inhibited beyond that point. Delamination of the vesicle from the surface is variable but formation of a hollow vesicle and subsequent alignment of lens fibers are never detected. Finally, surface ectoderm that would presumably differentiate into cornea is highly unpatterned. This epithelium is very disorganized and appears ruffled and unpolarized (see Figure 29B5). Strikingly, this epithelium is significantly thicker than the contralateral control side (see Figure 29B4). This result is also observed within the epithelia of the lens and retina. Formation of the otic vesicle on the MO-injected/labeled half is also not complete (Figure 31D6, E3, F5). The defects observed in the epidermis and eye correlate with a high expression of Xbves message and protein. Thus, we have determined that inactivation of Xbves

activity leads to an inhibition of retinal differentiation, lens delamination, corneal formation, and otic vesicle formation. Taken together, inhibition of Xbves function produced a phenotype consistent with our central hypothesis that this protein plays an essential role in the morphogenesis of epithelia, specifically in eye development. However, the exact mechanism underlying this phenotype is unknown. Other structures expressing Xbves such as the heart and somites appear to be present in all embryos examined, however a detailed investigation to determine the nature of defects in these tissues has yet to be performed.

Ultrastructural analysis underscores severity of epidermal defects

In order to closely examine the nature of the MO-induced cellular defects at the ultrastructural level, we performed an EM study of treated and control embryos. Histology of fixed embryos in thick sections reflects what has been observed with paraffin-sectioned embryos (see Figures 29, 31). At the light microscopic level, a developed eye and a smooth, thin epidermis on the uninjected, control side provide a striking contrast with the Xbves-depleted half of the embryo, which contains large, bubbled epidermal cells covering an underdeveloped eye structure (Figure 32A). For this preliminary ultrastructural study we have focused on the outer epidermal epithelium. The striking defects caused by depletion of Xbves are demonstrated by the extreme differences in cellular arrangement/structure. Electron microscopic images of the control, uninjected half of the embryo (Figure 32B) reveal an ordered two-layer structure. The epidermis at this stage is composed of an outer layer and an inner sensorial layer (Deblandre et al., 1999). Cells on the MO-injected half of the embryo are
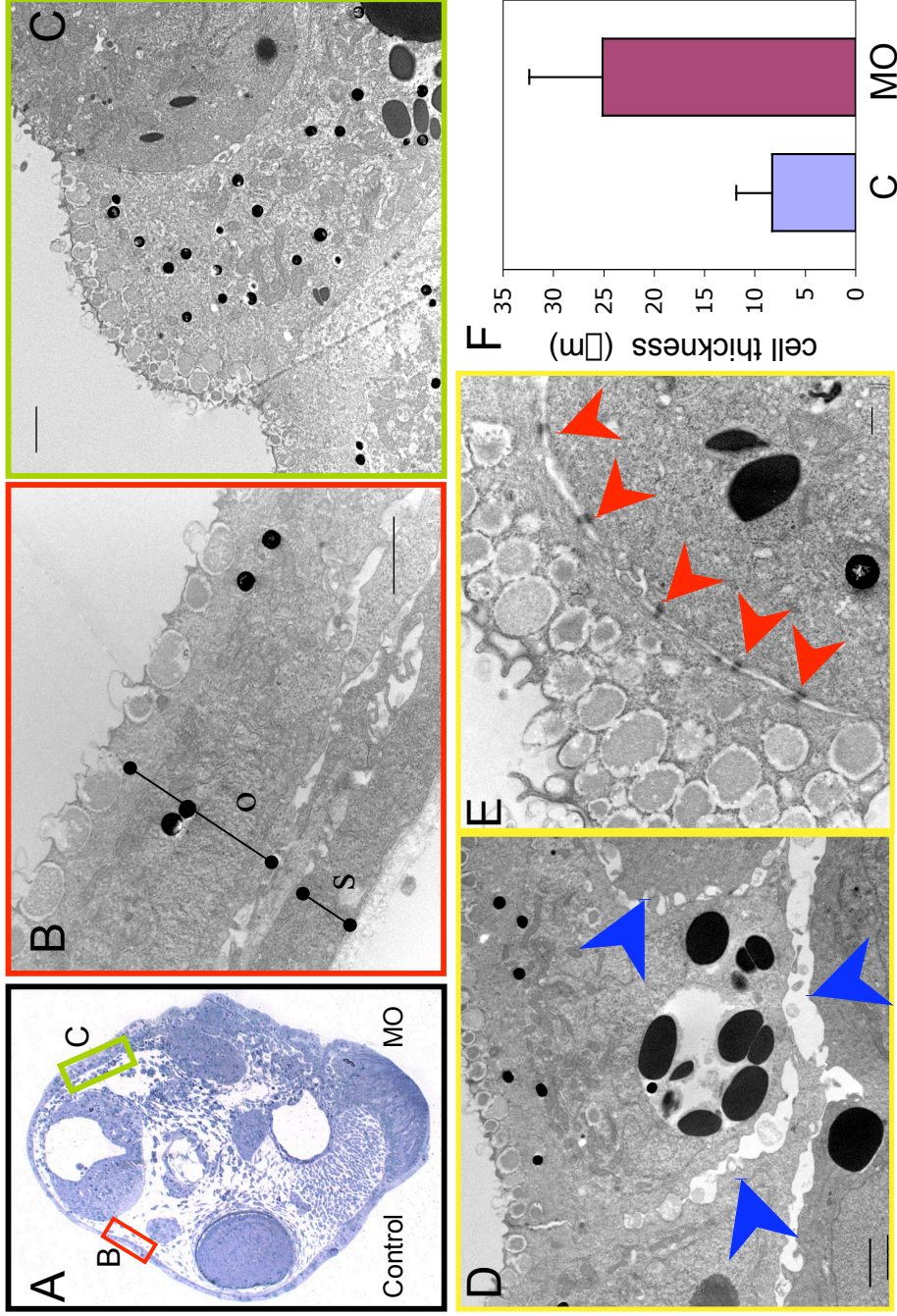
Figure 32. Epithelial layers exhibit marked ultrastructural defects in *Xbves* MO embryos.
Electron microscopic analysis of *Xbves* MO-injected embryos shows that epithelial cells of the affected epidermis (MO) are highly irregular as compared to the uninjected half (control) of the embryo (A). Epidermal cells on the uninjected side are ordered in two layers, the outer (o) layer and sensorial (s) layer (B). The epithelium on the MO-injected side is highly disorganized, much thicker and contains more spaces between cells (C-E). The average thickness of the epidermis on each side are compared (F). The average thickness of the *Xbves* MO-injected epidermis is 25.1 μm, which is 300% larger on average than cells on the control side (8.3 μm average thickness). Scale bars, 2 μm.

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larger, more bubbled, and contain intercellular spaces (Figure 32C-D, arrowheads), and also lack the ordered structure possessed by control cells in Figure 32B. MO-injected cells appear to have more and larger pigment granules and, in some cases, show more cell junctions (Figure 32E, arrows). However, it is unclear how the loss of Xbves leads to these changes in cellular structure. A comparison of average cell size between epidermal cells of control and MO-treated halves demonstrates also a significant difference. Cells composing the epidermal layer on the *Xbves* MO-treated side are, on average, over 300% larger than epidermal cells on the control side (Figure 32F). This finding supports our hypothesis that Xbves is essential for proper epithelial maintenance and integrity during development and suggests that Xbves is necessary for the generation of an ordered two-layer epidermal structure.

Xbves depletion results in excess cell proliferation

Our histological and ultrastructural analyses have demonstrated a dysregulation of the ordered epithelial epidermis and eye development, among additional unexplored defects. In many cases, the alterations in the epithelia following Xbves knockdown were defined by thicker, bubbling cell layers, which could have been generated through an increase in cell proliferation. To investigate whether an overproliferation of cells occurred, incorporation of BrdU (5-Bromo-2'-deoxy-uridine) was determined. Sections through the head region of stage 35 control and MO-treated embryos bathed in BrdU for 24 hours were closely examined. Control embryos showed a uniform basal level of incorporation throughout (Figure 33A). Analysis of sections through embryos injected with *Xbves*-MO on one side revealed an increase in BrdU labeling was detected in the
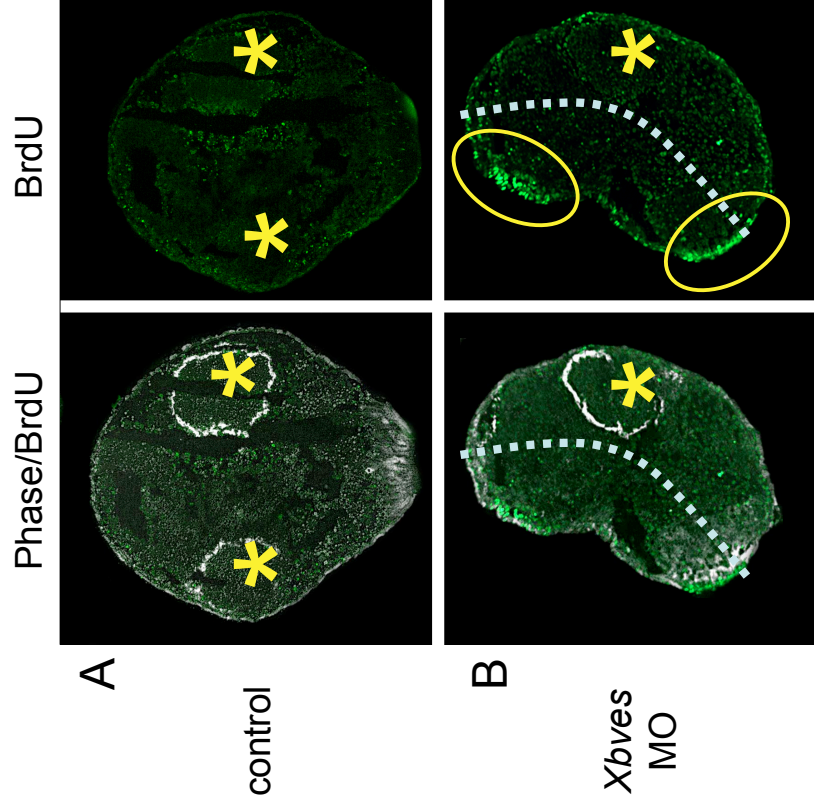
Figure 33. *Xbves* MO-injection induces an increase in cell proliferation.
Embryos injected with *Xbves* MO in 1 cell at stage 2 were grown in BrdU to assess incorporation into dividing cells. Control embryos had uniform incorporation throughout (A). *Xbves* MO-injected embryos exhibited an increase in BrdU uptake on the injected side of the embryo (B). A high BrdU incorporation is observed at the epidermis (B, circles). Location of the eyes is denoted by asterisks. Note the lack of RPE on the injected side of the embryo.

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"eyeless" half (Figure 33B). Note that punctate spots indicating high BrdU incorporation are clustered at the epidermis (Figure 33B, circles). For reference, inverse phase contrast of the pigmented epithelium has been used to determine the location of the eye (Figure 33, asterisks). This finding allows the speculation that Xbves could be regulating the proliferative nature of epithelia during epidermis and eye development.

Epidermal defects can be detected post-neurulation

Clearly, a MO-induced decrease of Xbves expression in one half of a *X. laevis* embryo yields a striking phenotype. Stage 35 embryos exhibit a severe defect in eye formation and a highly irregular epidermis on the injected side. However, eye development begins much earlier with the branching of the diencephalon, the epithelial structure that gives rise to the optic cup and the brain. To determine how early the developmental defects can be detected, one cell of 2-cell-stage embryos were co-injected with MO/lacZ and processed for β-gal labeling at a range of timepoints after neurulation is complete (Figure 34A). At stage 20, the diencephalon outgrowth has begun and appears to be unaffected (data not shown). Sections through an embryo later in development, at approximately stage 24-25, do not suggest that eye development is impeded at the phases of epithelial outgrowth but clearly, further examination is needed (Figure 34B). However, it is obvious, even at this early stage that labeled blue cells in the outer epithelial epidermis do appear to be thicker (Figure 34D,E, arrows) and separated from the inner layers (Figure 34F,G, arrows), in contrast with the intact epithelium on the control uninjected side of the embryo. These results show that developmental defects due to Xbves depletion are already detectable by this
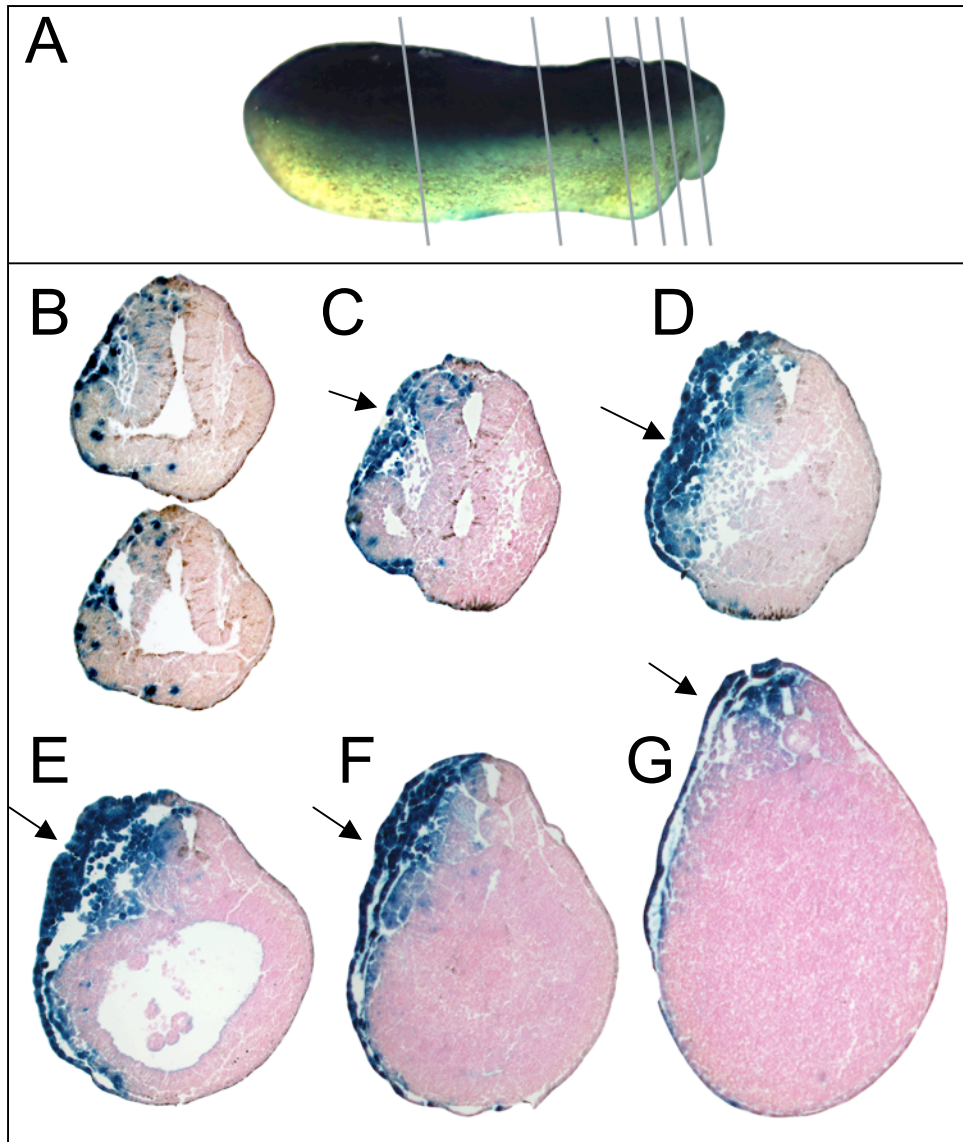
Figure 34. Epithelial defects can be detected during day 1 of embryo development. At stage 25, an embryo co-injected with lacZ mRNA and *Xbves* MO appears developmentally unaffected externally (A). Sections through the embryo show that the epidermis on the injected side, marked by blue cells, is thicker, ruffled, and separated from the internal tissues, most noticeably in the trunk region (B-G, arrows).

point of embryo formation. Furthermore, loss of Xbves results in a separation of cells from their neighboring cells and tissues, even at early stages. This investigation is obviously at preliminary stages and analysis of additional embryos and varying timepoints is necessary to determine the timeframe in which defects are detected. Still, this finding clearly demonstrates that the effects of Xbves depletion arise well before the obstruction in eye formation is apparent. Thus, even at early stages, the loss of Xbves results in a recurring phenotype that can be characterized by loosely associating cells and epithelial layers that fail to arrange and/or move properly.

Discussion

This chapter investigates the function of Bves/Xbves in an in vivo system of development, the *X. laevis* embryo. *X. laevis* proved to be an excellent model system to study the role of the putative adhesion/junction protein Bves/Xbves because the earliest morphogenetic movements of the embryo involve epithelial tissue layers. Furthermore, the simplicity and the bilateral symmetric cell lineages of the *X. laevis* embryo were ideal for probing the development of epithelial structures such as the epidermis and the eye at later stages. The studies described here follow our initial MO knock-down experiment, which demonstrated that global inhibition of *Xbves* function is incompatible with successful gastrulation. This chapter describes how disruption of Xbves within a small population of cells affects the movement and/or fate of those cells specifically, as well as embryo development as a whole. Overall, growth of the animal was unaffected using this method but the clonal disruption of Xbves randomized cell positioning and/or movement of the injected cell's derivatives. Taken together, these two studies that

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probe Xbves function during the early stages of development reveal that Xbves is essential for gastrulation and suggest that Bves proteins represent a new class of molecules essential for regulation of epithelial movements. Using a different method that takes advantage of the bilateral development of *X. laevis*, Xbves was disrupted on only one half of the embryo, such that the contralateral side could serve as an internal control. *Xbves* MO-injected embryos exhibited prominent defects that became most obvious at two days post-fertilization. The affected half of the embryo lacks a fully developed eye, the covering epidermis is highly disorganized, and in many cases, the integrity of internal tissues appears to be lost. Findings from this experimental method certainly correlate with the global and clonal MO knockdown studies in that Xbves plays a role in epithelial cell homeostasis. In addition, all three protocols demonstrate an epithelial function for Xbves, which supports the central hypothesis that Bves proteins maintain the integrity of epithelial tissues during dynamic developmental processes.

Through extensive cell rearrangements, gastrulation transforms a sphere of epithelial and yolk cells into an elongated tadpole with well-defined differentiated structures. Epithelial movement in *X. laevis* gastrulation is driven by convergence and extension (Keller, 1991; Keller and Danilchik, 1988; Wallingford et al., 2001a; Wallingford et al., 2002). 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 (Keller, 1991; Keller and Danilchik, 1988; Marsden and DeSimone, 2001; Wallingford et al., 2002). Cell adhesion is a crucial contributor to many events in this process, such as the convergence of cells toward midline, the

epibolic movements forming the blastopore, and involution of cells to form mesoderm. 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 (Figures 25,26). 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 convergence and 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. Our findings are reminiscent of those observed by Broders and Thiery (1995), who examined the adhesive nature of cadherins using a similar method. Clonal overexpression of dominant-negative E- or EP-cadherin in one of 32 cells caused a dispersion of injected cells at early blastula and gastrula stages, a perturbation in convergent-extension movements, and a lack in coherent directional migration. Furthermore, the distribution of proteins is often correlated with their specific function during gastrulation. In other words, defects in cell movements are easily explained when a protein is "in the right place at the right time" to exhibit a role. For example, Disheveled is found at the membrane in cells specifically undergoing convergence and extension, but is cytoplasmic in cells not actively participating in these dynamic movements (Wallingford et al., 2000). Similarly, Xbves protein is expressed on the cell surface between epithelial cells of the cleaving and gastrulating embryo. Thus Xbves is spatio-temporally regulated such that it can function in epithelial cell associations during these processes. Taken together, these data suggest that Xbves is essential for the appropriate interaction and movement of neighboring epithelial cells during *X. laevis* gastrulation.

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Clonal disruption of Xbves function leads to randomization of cell movement. These results suggest that this in turn may lead to alterations in cell differentiation. The production of mesendoderm in the frog is dependent on the involution of epithelial cells from the embryo surface (Keller, 1991; Kwan and Kirschner, 2003). 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 is due to the disruption of coordinated cell movement within epithelial sheets leaving the cells unable to reach deeper positions in the embryo. We propose that Xbves depletion alters the adhesive properties of cells and inhibits the ordered assembly and/or necessary cell interactions that otherwise would occur with the non-perturbed cells. While global inactivation of Xbves function is not incompatible with mesoderm production (Figure 22), we cannot exclude the regulative capacity of morphant cells within a normally differentiating embryo. Therefore 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. In either case, surviving cells appear to favor epidermal over neural fates within derivatives of the ectoderm. Thus, while 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.

When administered at a high dose into both of two cells, *Xbves* MO inhibits gastrulation. Injecting a lower MO concentration into only one cell permits a progression to neurulation and continued development. However, defects arise and are externally apparent at two days post-fertilization. The most obvious defects are found in the

epidermis and in the eye, two epithelial structures that exhibit a high degree of Xbves expression. In the tadpole stage embryo, Xbves protein is observed at the borders between epidermal cells (Figure 21F,H) and in the epithelial components of the retina, lens and cornea (Figure 21I). Work discussed in Chapters II and III define Bves as transmembrane protein that is highly expressed in a variety of epithelial cell types and functions at the TJ complex through interaction with the ZO-1 protein complex. It is not surprising that depletion of a molecule regulating cell junctions/cell adhesion could give rise to such developmental defects in epithelia as observed in the present in vivo study.

Examination of cell-cell adhesion/association in the X. laevis has been ongoing since the beginning of the last decade, with a concentration on cadherin proteins. Several cadherins are expressed in the developing embryo. XB/U-cadherin and EP/C-cadherin are maternally-expressed adhesion proteins while expression of E-cadherin and N-cadherin begins at midblastula transition just before gastrulation initiates. Disruption of cadherins during the early phases of development results in decrease of intercellular adhesion, ectodermal lesions, and a failure to undergo gastrulation (Detrick et al., 1990; Heasman et al., 1994a; Levine et al., 1994). However, several studies explore the role of cadherins at later stages of development, and while the methods are varied, both find defects in eye development (Dufour et al., 1994; Kuhl et al., 1996). Specifically, a study by Kuhl et al. directed DN expression of XB/U-cadherin to post-MBT stages avoiding disruption of early cleavage stages (1996). In one experimental protocol, injection of a DN XB/U-cadherin construct lacking the "adhesion" domain in the dorsal two blastomeres of a four-cell embryo produces embryos with reduced eye anlagen or complete lack of eye formation, among other posterior defects. Interestingly,

this group noticed a dose-dependency with their method and found that injection of a higher dose yields developmental defects in gastrulation. Injection of high doses into two cells out of four, gastrulation movements were severely distorted and mesoderm involution was hindered. The injection of the DN construct at lower doses resulted in the "eyeless" embryos. XB/U-cadherin is ubiquitously expressed at cell membranes up to gastrula stage, expressed in all three germ layers during gastrulation, declines during neurulation, and is restricted to specific epithelia (Dufour et al., 1994) and is thought to mediate interblastomere adhesion. Thus, not surprisingly, its function could be important to the movements of the neuroepithelium that gives rise to the layers of the eye. Importantly, we also find that a global depletion of Xbves with a high dose of MO yields a gastrulation block, but lower doses of *Xbves* MO permit gastrulation to occur with epithelial defects emerging at later stages. Thus, the studies involving XB/U cadherin manipulation provide a parallel example of a molecule with adhesive function that contributes to proper development (Dufour et al., 1994; Kuhl et al., 1996).

The level of *Xbves* MO that induces the "eyeless" embryos is much lower than the concentration of MO that blocks gastrulation. Interestingly, the interference of XB/U cadherin with varying doses of a dominant negative construct results in a gastrulation block at high dose, but causes a disorganization of neural structures at lower doses. This parallels what we observed in our Xbves studies and we interpret our results in a similar manner. As described in Kuhl et al, a large decrease of cellular adhesion during gastrulation would inhibit or severely hinder the involution of cells and, thus, the formation of mesoderm (1996). Upon injection of a high dose of *Xbves* MO, we observed a gastrulation block. Contrarily, a slight decrease in cell adhesion would

impair cellular intercalations and epibolic movements to a lesser extent. This may allow more cells to involute, potentially shifting the major defects to later in development when the embryo is undergoing elongation. In our experiments, a lower dose of *Xbves* MO allows development to proceed past gastrulation, but defects are apparent soon thereafter.

The mechanism by which Xbves promotes cell movements is currently undefined. A homophilic binding property has been shown for chick Bves, and the amino acids responsible for this interaction have been determined (Wada et al., 2001). With the finding that Bves physically interacts with the ZO-1 protein complex (Chapter II), a peripheral TJ protein that functions as a molecular scaffold, it remains highly possible that junctional integrity is disrupted upon Xbves depletion. During epithelial morphogenesis, whether it is gastrulation or eye formation, cell-cell interactions within the epithelial layers rely on intact cell junctions to maintain integrity and allow movements to occur. The distribution analysis of early *X. laevis* (current chapter) and chick embryos (Chapter II) highlighted Bves expression in epithelia during embryonic stages. High expression of *Xbves* in regions undergoing active morphogenetic movements (marginal zone of gastrulae, epithelial structures like the eye and epidermis) suggests that the protein is involved in dynamic adhesion events, such as gastrulation and germ layer development. Our findings are supported by reports where other cell junction/cell adhesion proteins expressed at these stages are important for gastrulation movements and epithelial morphogenesis (Bradley et al., 1998; Brieher and Gumbiner, 1994; Broders and Thiery, 1995; Cordenonsi et al., 1997; Fagotto and Gumbiner, 1994; Heasman et al., 1994a; Heasman et al., 2000; Marsden and DeSimone, 2001;

Wallingford et al., 2001b). While the cellular basis of Xbves function is unknown, inactivation of Xbves leads to severe disruption of gastrulation as well as the epithelial primordia of the eye. We postulate that depletion of Xbves results in the inability of cells to interact with one another or form functional cell junctions. Future studies will focus on unraveling the cellular nature of Xbves in epithelial morphogenesis.

Current analysis

We have demonstrated a function for Xbves in the developing *X. laevis* embryo using several protocols to deplete Xbves at various stages. We have defined that epithelial movements during gastrulation and the integrity of epithelial tissues, including the epidermis and the eye, are disrupted following Xbves knock-down. However, we are continuing to perform experiments to elucidate Xbves function. While we are confident that Xbves is essential for epithelial development, we do not currently understand the nature of its functional role and thus, it is critical to continue our examination of Xbves during *X. laevis* development. At present, our investigation of Xbves role in gastrulation includes several key experiments: animal cap extension assays, which specifically examine the movements of convergence and extension, assessment of junction protein distribution, and an EM analysis of junctional integrity. Also, considerably more analysis is required to clarify how Xbves depletion results in an "eyeless" morphant embryo. We plan to determine not only when earliest defects can be observed, but also if other tissues are altered, and whether the patterning of the embryo is affected. In the following discussion, I outline ongoing and future experiments to further examine the role of Xbves during development.

Clearly, gastrulation movements of Xbves-depleted cells are disrupted. This is demonstrated by both the global elimination of Xbves (injection of MO into two of two cells) as well as clonal knock-down of Xbves (injection of MO into one of 32 cells). However, the mechanism or pathway in which Xbves functions to regulate these movements is unclear. In light of findings that Bves/Xbves proteins exhibit adhesive properties, we are continuing this investigation using several techniques. First, immunochemical analysis of cell adhesion and cell junction proteins in gastrulation-stage embryos is underway to determine whether expression or distribution changes could provide an explanation for the MO-induced epithelial disruption. If Xbves affects the adhesive properties of epithelial cells during convergent extension and epibolic movements, a disruption in adhesion/junction markers within epithelial layers involved is highly probable. To this point, the autofluorescent properties of the yolky endoderm has posed some problems, but we hope that in collaboration with experts in this field, including Dr. Doug DeSimone, we can circumvent that issue. Another method is currently being employed to assess epidermal integrity, which will be used in concert with EM studies. Ruthenium Red (RuR) is an inorganic dye that is excluded by plasma membranes with intact TJs (Luft, 1971). Xbves-depleted embryos (one of 32 cells or two of two cells), co-injected with the lineage tracer horseradish peroxidase (HRP), will be fixed for ultrastructural study in the presence of RuR. Should the TJ integrity be breached, the dye will permeate between the lateral membranes of HRP-marked epidermal cells, signifying that Xbves functions to maintain the TJ integrity in the epithelia of developing embryos. Finally, animal cap assays are a technique used to confirm the role of a protein in convergent extension movements. The blastula animal

cap is composed of pluripotent epithelial cells that can be induced to form endoderm, mesoderm and ectoderm cell types in response to exogenous signals. In this assay, the animal cap tissue is dissected from a stage 8.5-9 embryo and incubated in the presence of the mesoderm inducer, activin-A. Activin-A triggers gastrulation movements of convergence and extension. Thus, if the cap is competent to undergo convergent extension movements and form mesoderm, the cap cells will literally grow and extend over the course of 12 hours, converting the ball of cap cells to a rod. From work described here, we would expect to observe a lack of convergence and extension movements and therefore, no extension of the mass of cells dissected from embryos where Xbves has been globally depleted. However, preliminary animal cap studies where Xbves has been knocked down by MO-injection into two cells of a two-cell embryo show that, not only does extension occur, it may be even more pronounced. While this experiment must be repeated and the data quantified, if this preliminary finding holds true, additional examination will be required to clarify the role that Xbves plays during gastrulation movements. Potentially Xbves depletion results in a gastrulation block by an alternative mechanism, rather than interfering with convergent extension movements.

The analysis of *Xbves* MO embryos with the "eyeless" developmental defect is in the early stages and far from complete. As shown with lacZ co-injection and rescue experiments, Xbves depletion is responsible for the observed alterations in epithelial and other cell types. To this point, we have chosen to more closely examine the malformations in the epidermis and eye, as they are the most apparent and the disrupted layers are epithelial in origin. Still, the nature of the developmental

malformations in epithelial structures is not understood. An assessment of cell adhesion molecule distribution in eye and epidermis of Xbves-depleted embryos could provide important clues about the state of junction integrity. While it is readily apparent that certain stages of epithelial morphogenesis in the eye, for example, are inhibited, we do not know whether inactivation of *Xbves* alters the expression and/or distribution of adhesion molecules such as cadherin, β-catenin and ZO-1. Furthermore, potential changes in the cytoskeleton are likely to be observed given the interaction of these structures and cell junctions. Any changes in polarity of epidermal cells could be addressed by labeling with tubulin. Also, the epidermis exhibits a bubbled appearance, suggesting a dysregulation in the adhesive capacity of this epithelial layer.

Concurrently, ongoing experiments are focused on identifying the nature of the "eyeless" defect. The failure of Xbves-depleted embryos to form a fully developed eye is not only a striking malformation, but provides an example of impaired epithelial function. The eye provides a complex, but ideal model of development in which the integrity of the epithelial layers themselves is as important as the signaling between the layers that will become the retina, the lens, and the cornea. Interestingly, the retina, lens, and cornea are derived from embryonic epithelia that undergo dynamic changes in shape and differentiation during development. Interactions between these epithelial structures as well as adjacent mesenchymal cells are critical for eye development and disruption of any one component can lead to major defects in the entire structure. Importantly, cell adhesion plays a critical role in the induction, growth, movement, and stability of epithelia during formation of the eye. Preliminary analysis to determine how early the "eyeless" phenotype is detectable has begun, as demonstrated in Figure 34, but

examination of additional embryos between stages 20-28 is necessary to determine when eye development is disrupted. Also, closer examination of the components of the eye is essential to unravel the function of Xbves in the developing embryo. For example, a disruption of the adhesion system may underlie the changes in retinal differentiation. From the preliminary studies, we observed that the neural retina does not undergo lamination to become a mature ten-layered structure and has a "disorganized" appearance. It is possible that certain cell types do not form, leading to failure in retinal development. Immunolabeling of sections through "eyeless" embryos at later stages (two to five days post fertilization) using antibodies to the distinct neural and glial cells that reside within this highly organized structure may indicate whether these cell types are just disorganized or misaligned, or are completely absent. Likewise, while initiation of lens differentiation occurs after *Xbves* inactivation, detachment of the lens vesicle, alignment of lens fibers and maturation of the substructures of the lens appear to be inhibited. Also, as corneal differentiation is dependent on lens development (Henry et al., 2002), it is not surprising that defects in this epithelium are seen. Our preliminary analyses show very little patterning in the stratification of presumptive cornea/surface ectoderm and a clear increase in the number of cells in this epithelium (Figure 29, 31). Histological, immunochemical and ultrastructural analyses are underway to determine how *Xbves* inactivation changes the formation of these structures. Again, we postulate that changes in localization of adherence proteins will be evident and result in abnormal morphogenetic movements of lens epithelium.

Furthermore, specification of the eye anlagen is an early event that occurs shortly after neurulation is complete (Jeffery, 2001; Tripathi et al., 1991). Following the

outgrowth of the optic vesicle from the diencephalon at early tailbud stages (stage 23), additional eye-specific proteins including crystalline are formed. Whole mount in situ analysis of neural and presumptive eye specification markers may also suggest which processes of eye development are affected by Xbves depletion. Preliminary data show that the expression of Pax 6 is not altered (Pichaud and Desplan, 2002), suggesting that eye specification may be unaffected. The potential relationship between Xbves, generation of cell junctions and regulation of cell division may be analyzed in this setting. Using immunochemistry and TEM, we are particularly interested to determine whether junctional complexes form properly in eye epithelial layers. BrdU staining and cell counts will determine whether alteration of Xbves function results in increased DNA synthesis and mitosis in epithelial progenitors. Taken together, these analyses should determine which developmental events proceed normally in the eye or are inhibited in the absence of Xbves.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Summary

Bves was independently discovered by our laboratory and by Thomas Brand's group in 1997. Both groups found that Bves was expressed in cardiac and skeletal muscle. However, our initial studies using Bves-specific antibodies demonstrated expression of the protein not only in the myocardium, but in the epicardial epithelium surrounding the heart and other cell progenitors found in the coronary vascular lineage. Nevertheless, while Brand and colleagues stressed that expression was restricted to heart and skeletal muscle, their published Northern blot and in situ data detected mRNA in brain, stomach, kidney, lung, and spleen. After performing an extensive protein expression and distribution analysis in three organisms, it became clear that Bves was not a muscle-specific molecule, but was widely expressed in developing and mature epithelia across species. We also determined that Bves was a transmembrane protein that could confer adhesion to fibroblast cells and, therefore, we postulated that Bves functions to regulate epithelial cell adhesion. The results from my dissertation present major and critical findings that will impact the direction of future studies on Bves. The first finding (discussed in Chapters II and III) is that Bves is a functional component of epithelia and interacts with the protein complex containing ZO-1 at the TJ in epithelial

cells. Chapter II outlines Bves expression the developing chick embryo. This work highlights the expression and localization of Bves in epithelial layers throughout early stages of embryo morphogenesis that is, importantly, a timeframe before heart and muscle development has been initiated. Chapter III subsequently addresses the central hypothesis that Bves plays a role in epithelial cell-cell interaction and integrity. Here, Bves is assigned to the tight junction complex through localization, interaction, and functional assays. The second major contribution of my work demonstrates that depletion of Bves from developing embryos disrupts epithelial integrity, suggesting that Bves provides an essential function in vivo. Chapter IV describes in vivo experiments showing that Bves is important for epithelial morphogenesis during *X. laevis* development. While a mechanism for Bves proteins at the TJ has not been determined, a correlation exists between the results from in vitro studies in cell lines (Chapter III) and the in vivo investigation in the frog (Chapter IV). Future studies will be designed to elucidate a connection between the function of Bves at the TJ in vitro and in the regulation of epithelial morphogenesis during embryonic development. The following discussion addresses several points and presents ideas about potential regulatory mechanisms in which Bves could participate in the control of epithelial integrity.

Bves function at the AJC

Initially, we focused our investigation of Bves on elucidating the structure of the protein, hoping that this effort would direct future studies. Bves has three hydrophobic regions, thus spanning the membrane three times, two N-linked glycosylation sites at the N terminus, and an intracellular C-terminal tail (Knight et al., 2003). Identification of

an interaction domain in the investigation of an uncharacterized molecule such as Bves, can provide a starting point for functional studies (M. Kawaguchi, in preparation). As discussed in the Introduction, many TJ proteins have interaction domains, including PDZ, SH3, and MAGUK protein motifs. Outside the putative transmembrane regions, the primary sequence of Bves lacks these and other known protein-protein interaction domains, functional motifs, or consensus regions. Although the sequence and overall structure of Bves are highly conserved between species, computer modeling and database searches revealed no structural homology with other proteins. Therefore, predictions of Bves function from proposed protein structure were purely speculative and failed to provide a significant direction to our investigation.

Due to the lack of any identifiable domains, we used our initial antibody localization studies to generate a hypothesis about Bves function and to guide the direction of our future experiments. We observed a dynamic change in protein distribution of Bves in cells undergoing EMT during heart development. Cells in the epicardial epithelium localized Bves to cell borders whereas delaminated, single cells show an intracellular localization of the protein. Also, L-cell assays ("hanging-drop" and DiO-DiI segregation assays (Kubota et al., 1999; Thoreson et al., 2000)) demonstrated that Bves exhibits adhesive properties. Although we have not resolved the exact mechanism by which adhesiveness is conferred to L-cells, several in vitro studies demonstrate a Bves-Bves interaction through the intracellular tail of the protein. Using both GST pull-down assays and a SPOTS blot assay (Sigma-Genosys), we have identified a homophilic interaction domain within the internal C-terminus (Knight et al., 2003; Wada et al., 2001). Our results suggests that this interaction is mediated by two

lysine residues found within this domain (M. Kawaguchi, in preparation). We also predict

that this domain is responsible for the oligomerization of Bves (Knight et al., 2003).

However, these early efforts failed to identify a function or suggest a mechanism for

Bves. Comprehensive studies of other proteins in the Popeye gene family are lacking,

but existing expression data provides evidence that other Bves/Pop proteins have

similar functions. Furthermore, global inactivation of *bves/pop1a* in mice lacks an

epithelial phenotype, suggesting a redundant function of family members. Still, based

on protein localization and topology, we postulated that Bves was a member of a new

class of junctional/adhesion molecules involved in EMT and/or epithelial regulation.

Significant to the work discussed in this dissertation is the placement of Bves into

a definitive junction within the AJC. Our localization and functional studies in cell lines

demonstrate that Bves is a functional component of the TJ and that Bves interacts with

a ZO-1-containing protein complex. This supports our initial postulation that Bves may

play a role in adhesion/epithelial regulation. It is now possible to more carefully probe

the function of Bves, since early structural analysis failed to suggest a role for Bves.

With the finding that Bves is a functional TJ component, we can propose ideas for how

Bves may facilitate this intercellular interaction seen in L-cells. This cell-cell adhesion

could occur in several ways. Bves may generate/establish a multiprotein junctional

network that facilitates intercellular adhesion through ZO-1 or another protein. Currently,

we have not explored how this may occur. This phenomenon, however, is of interest

since L-cells lack cadherins and many other epithelial adhesion proteins with which

Bves could interact, if indeed Bves solely responsible for providing the intercellular

adhesion. Also, Bves may cluster internally via homophilic interaction and create an

adhesive extracellular "sugar tree" via N-linked glycosylation. Interestingly, a similar type of cell adhesion occurs during the extravasation of leukocytes through cell-surface proteins called selectins (Ebnet and Vestweber, 1999). Selectins are found on the apical surface of endothelial cells and, with only three members, comprise the smallest family of cell adhesion molecules, known as the sialomucin adhesion family (Cambi and Figdor, 2003; Lasky, 1994). Selectins mediate cell contact through mucin-like carbohydrate ligand structures at the amino terminus and predominantly function as cell adhesion receptors that direct the homing leukocytes across the endothelial linings of blood vessels as part of the inflammatory response (Lasky, 1995). Of particular interest, the binding interactions mediated by the selectins appear to be of lower avidity than those of the integrin-immunogobulin superfamily (Lasky, 1995), which also participate in leukocyte-endothelial interactions (Zen and Parkos, 2003). If Bves mediates adhesion through a similar method, it might explain the spatio-temporal regulation of Bves in epithelia undergoing dynamic movements during early embryogenesis. While cadherin-mediated adhesion is necessary during these events as well, it is possible that Bves can contribute to transient and less robust interactions to achieve the proper level of cell adhesion to permit the cell rearrangements that must occur. This idea is supported by data showing that Bves-expressing L-cells aggregate into clumps, but not as robustly as E-cadherin-expressing L-cells (A. Wada, unpublished data).

Taken together, we have shown that Bves mediates cell adhesion and influences the TJ, but the physiological roles of this family of proteins are unresolved. How could a depletion of Bves affect TJ integrity? To review, the two primary functions of the TJ are to maintain the transepithelial barrier and to establish apical-basal polarity of the

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epithelial sheet. It is unclear whether Bves contributes to TJ integrity by regulating the paracellular seal via cell adhesion or has a role in polarity. While the mechanisms that control the sealing capabilities of the TJ are poorly understood, a measure of the transepithelial electrical resistance has become the gold standard for assessing whether proteins influence tight junction integrity. We show that depletion of Bves in human corneal cell lines results in a breach of barrier function, as indicated by a drop in the TER and a mislocalization of junction markers, but did not investigate whether the polarity was altered. Previously, we have shown that similar treatment of these cells shows a faster migration of cells in a wound-healing assay. This finding suggests that without Bves, cells are more motile potentially due to compromised cell junctions (Ripley et al., 2004). Furthermore, these studies provide another example supporting the hypothesis that Bves plays a role in regulating epithelial interactions in some way. With the current studies, we can say only that Bves **does** influence the TJ in some way, and that the interaction with a ZO-1 containing complex supports that finding. Clearly, future studies are necessary to unravel the precise role of Bves and family members at junctional complexes.

Bves and ZO-1 at the Tight Junction

Bves localization at the TJ and the interaction with the ZO-1 complex provides a significant advancement for the Bves/Pop field. Unfortunately, the finding fails to sufficiently narrow the potential function of the protein family. Along with Bves, over 40 proteins have been localized to the mature TJ complex and these proteins exhibit a wide range of roles in paracellular barrier and polarity establishment, signal

transduction, and structural support (Gonzalez-Mariscal et al., 2003). For example, PAR proteins (partitioning-defective proteins), which interact with PKC and JAM, primarily function to establish polarity (Itoh et al., 2001). The PAR protein complex also influences TJ assembly and interacts with several Rho GTPase family members, and thus has additional functions at the TJ (Gao et al., 2002; Johansson et al., 2000). Another TJ protein, cingulin interacts with ZO proteins, afadin, JAM, F-actin and myosin, and is thought to function as a cross-linker between TJ proteins and the actomyosin cytoskeletal network (Bazzoni et al., 2000; Cordenonsi et al., 1999; D'Atri and Citi, 2001). Also, cases exist where membrane-spanning proteins that co-localize with TJ in epithelia have unique rolls in specific tissues (Gonzalez-Mariscal et al., 2003). For example, a peripheral membrane protein PMP22/gas-3 is observed at TJ but may play a role in compaction of myelin sheaths by heterophilic adhesion with a myelin protein, Protein 0 (Berditchevski, 2001; D'Urso et al., 1990). This suggests that proteins found at the TJ in epithelia may have undergone an evolution of function in a tissue-specific manner. As described, proteins that have been defined as TJ components exhibit a vast array of different functions and, based on our limited data thus far, it is possible that Bves could play a role in many TJ-associated functions.

The question of how Bves functions at the TJ could be more easily addressed if we understood the relationship between Bves and ZO-1. Analysis of Bves localization, compared with TJ proteins, particularly ZO-1, reveals intriguing similarities throughout stages of epithelial cell-cell adhesion. For example, the pattern that Bves localizes to cell-cell contacts mimics what is observed with ZO-1 during contact formation. ZO-1 can be detected at contact points with E-cadherin and catenin proteins, well before occludin,

claudins, and other TJ components translocate to the cell periphery (Ando-Akatsuka et al., 1999). Likewise, Bves mimics ZO-1 and localizes to forming junctions with E-cadherin and catenins. Also, in non-epithelial cells that lack TJ, ZO-1 is recruited to cadherin-based AJ via its interaction with α-catenin (Itoh et al., 1997; Itoh et al., 1993). Bves colocalizes at these junctions with ZO-1 in non-epithelial cells, such as NRK cells. These localization findings have led us to postulate that the parallels observed between Bves and ZO-1 localization could be important. Then, we discovered that the Bves C-terminal tail co-precipitates a protein complex containing ZO-1. Currently, ZO-1 is considered a scaffolding protein that binds occludin and claudins at the TJ in epithelial cells. It is possible that Bves localization at the cell membrane is modulated by ZO-1, or rather ZO-1 is tethered at the membrane by Bves. With the exact function of ZO-1 being unknown, further studies will be necessary to explore the function of Bves at the TJ and the nature of the Bves/ZO-1 interaction.

It is possible that ZO-1 coordinates recruitment of other junction molecules, including Bves, to form fully-functional TJs. The ZO proteins are members of the MAGUK family, which has been implicated in the formation of membrane microdomains by serving as a scaffold/cross-linking protein for cytoplasmic domains of integral membrane proteins. In neurons, PSD-95, a MAGUK family member, cross-links the NMDA receptor, K channel and neuroligin, and organizes the postsynaptic membrane domain in neurons (Irie et al., 1997; Kim et al., 1995; Kornau et al., 1995; Niethammer et al., 1996). Thus, ZO-1 could aid in establishing and/or sorting proteins into respective junction, either AJ or TJ, within the lateral compartment (Ando-Akatsuka et al., 1999). An explanation for the dynamic distribution of Bves has not been proposed, although it

is likely that the spatio-temporal recruitment of ZO-1 and Bves can be correlated with a function. It is possible that Bves is a component of the membrane microdomain organized by ZO-1 at early adhesive contacts, and is subsequently sorted into the TJ. Furthermore, this dynamic localization could suggest an early role during the formation of adhesive contacts, and then a later role in established polarized epithelia. Also, the finding that occludin-null mice still form tight junctions suggests that other proteins can contribute to TJ integrity (Saitou et al., 2000). Our studies show that Bves appears to be an additional transmembrane constituent of the TJ. This provides an example where Bves could fill a gap in the understanding of TJ biogenesis and maintenance.

To reiterate, the localization/interaction data does not provide many clues to Bves function within the molecular context of the TJ. However, we can speculate as to its role at the AJC based on our findings. Bves depletion in cultured epithelial cells causes a decrease in transepithelial resistance (Chapter IV). This suggests that Bves influences the barrier function of the TJ. However, as with PAR proteins, it is possible that Bves has a primary and/or additional function in cell signaling or structural support, and this result is purely a secondary effect. It is important to note that in addition to epithelial TJs, Bves proteins are highly expressed in cardiac and skeletal muscle (Andree et al., 2002a; Andree et al., 2000; DiAngelo et al., 2001; Reese et al., 1999; Vasavada et al., 2004). Like epithelia, cell adhesion is also an important property of striated muscle (Forbes and Sperelakis, 1985). Bves localizes to the intercalated disc, an adhesive structure in muscle. At this time, it is unclear whether Bves is a functional component of this structure and/or what other known adhesive proteins with which Bves interacts in this setting. Thus, since both cell types require intimate cell associations for

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function, it is possible that Bves participates in the generation of adhesion in both cell types. In any case, this study is the first demonstration that Bves localizes to a specific adhesive junction and is a critical step in affixing a specific function to Bves during epithelial cell-cell adhesion.

Bves and epithelial integrity of embryos

Based on our early findings, we postulated that Xbves expression/localization during the early embryogenesis correlates with a function during *X. laevis* gastrulation. A global depletion of Xbves resulted in a gastrulation block and demonstrated that our hypothesis was correct. Clonal depletion studies again highlight the importance of Xbves in epithelial integrity, since epithelial movements of Xbves-depleted progeny are impaired. Likewise, the distribution pattern observed during neurulation and organogenesis suggest a tissue-specific function at later stages. Thus, it is not surprising that the elimination of Xbves disrupts the development of epithelia including the epidermis and the eye, where Xbves is highly expressed. Together, we show that a knock-down of Xbves results in epithelial perturbations in both injection protocols described in these studies, strongly suggesting that Xbves is essential for epithelial integrity during morphogenesis.

Tight control of junctional integrity is critical as the cell layers undergo significant cell movements and shape changes during gastrulation and epiboly, and organogenesis at later stages (Keller et al., 2003; Marsden and DeSimone, 2001; Marsden and DeSimone, 2003). In the literature, manipulation of other cell adhesion and/or junctional proteins demonstrates a dysregulation of epithelial integrity. Molecules that generate

adhesion structures like cadherins and integrins allow embryos undergo fundamental tissue rearrangements that may require cells to vary adhesiveness within an epithelium (Jamora and Fuchs, 2002). With the findings from Chapter III that Bves exhibits a function at the TJ, it remains possible that the defects we observed in epithelial development can be correlated with Bves's function at the apical junctional complex.

The strength of cell adhesion also regulates the migratory ability of epithelial sheets (Brieher and Gumbiner, 1994; Winklbauer, 1998). For example, several different cadherin proteins are important in *X.laevis* development. Ectopic expression of N-cadherin causes a thickening and fusion of cell layers (Detrick et al., 1990; Fujimori et al., 1990). Depletion of maternal EP/C cadherin disrupts inter-blastomere adhesion and knockdown of zygotically-expressed E-cadherin causes ectodermal lesions and a gastrulation block (Broders and Thiery, 1995; Heasman et al., 1994b; Levine et al., 1994).   For optimal cell movements, the strength of cell-cell adhesions must be at an intermediate level (Huttenlocher et al., 1995; Palecek and Ubbels, 1997). In hanging-drop adhesion assays, transfection of Bves into L-cells causes the cells to cluster into adhesive clumps (Ripley et al., submitted; Wada et al., 2001). Parallel experiments using E-cadherin-transfected L-cells yield large aggregates of cells, signifying robust cell adhesion (Chen et al., 1997). Because fewer Bves-transfected cells comprise each aggregate than clumps formed by E-cadherin-transfected cells, we can speculate that cell adhesion generated by Bves may be weaker than E-cadherin. At this time, no quantitative experiments to determine strength of cell-cell adhesion mediated by Bves have been performed. Still, a potential role for proteins like Bves may be to modulate the TJ and/or regulate adhesion between cells to hold the sheet together when other

proteins like cadherins may not have the same capability at a given developmental timepoint. Alternatively, Bves could function in concert with other adhesion forces to contribute to cell sorting efforts during early development (Kim et al., 2000).

Investigation of junction proteins other than cadherins in the frog is not abundant, but does exist. Overexpression of *X. laevis claudin* (*Xcla*) causes cells to disperse as adherent clumps in gastrulating embryos, demonstrating that Xcla plays a role in cell adhesion of the epithelial layers during this dynamic process (Brizuela et al., 2001). Overexpression of *Xcla* at the four-cell stage results in bilateral *XNR-1* expression and left-right randomization of organs at stage 45. GJ have also been implicated in the control LR asymmetry (Levin and Mercola, 1998). Interestingly, very little is known about ZO-1 in *X. laevis* beyond the expression pattern in the cleaving embryo (Fesenko et al., 2000; Merzdorf and Goodenough, 1997). Thus the work on Xbves in *X. laevis* contributes not only to the field of Bves/pop proteins, but to the *X. laevis* cell adhesion field as well. The developmental defects that we observed in our studies on *X. laevis* parallel others in which adhesion proteins have been manipulated (Broders and Thiery, 1995; Dufour et al., 1994; Kuhl et al., 1996). Our interpretation of a role for Xbves in epithelial integrity in the embryo correlates with our in vitro findings in which Bves exhibits a function at the TJ. Thus, the potential involvement of Xbves in epithelial morphogenesis could be due to an influence on cell-cell adhesion, potentially through an interaction with ZO-1.

Bves and Rho signaling pathway

Currently, results are being analyzed from a yeast two-hybrid interaction screen designed to detect interactions between Bves and proteins from an embryonic mouse heart library (screen performed by T. Smith). Although the analysis is far from complete, we identified the RhoGTPase exchange factor GEFT as a Bves-interacting partner (T. Smith, unpublished data). This is intriguing since, as previously discussed in Chapter I, the Rho family of proteins function as molecular switches involved in the regulation of junctions that participate in cell proliferation and differentiation, cytoskeletal regulation, and signal transduction in response to external cellular stimuli (Carlier et al., 1999; Matter and Balda, 2003; Rossman et al., 2005). Guanine nucleotide-exchange factors (GEFs) activate Rho-family GTPases, specifically by controlling the rate at which GDP dissociates from the Rho GTPase (Erickson and Cerione, 2004). GEFT is a Rac/Cdc42-specific GEF protein that regulates cell morphology, proliferation, and migration (Guo et al., 2003). GEFT is highly expressed in excitable tissues such as the brain, heart, and muscle, as is Bves, suggesting that they may function together in these tissues. GEFT expression has also been observed in the small intestine, liver, lung and placenta by Northern blot, but the specific regions of these organs have not been determined so the possibility of an epithelial localization for this protein remains. Overexpression of GEFT leads to alterations in cytoskeletal rearrangements and cell morphology, and cell proliferation (Guo et al., 2003). Although no reports of GEFT action have been reported in epithelial cells, Bves may interact with another epithelial-specific GEF protein and support a similar function as the GEFT/Bves interaction during heart morphogenesis.

When epithelial cells reach confluence, Rho proteins are downregulated, which leads to an inhibition of the signaling pathways that stimulate proliferation (Braga, 2002; Fukata et al., 2001; Noren et al., 2001). Not surprisingly, examples of interactions between epithelial junction proteins and GEFs exist in the literature. For example, RhoA regulation is mediated by an interaction between a TJ protein, cingulin, and a GEF protein, GEF-H1/Lfc (Aijaz et al., 2005). GEF-H1/Lfc associates with the TJ and regulates the permeability of the TJ (Benais-Pont et al., 2003). A direct interaction has been demonstrated between GEF-H1/Lfc and cingulin, which also binds ZO-1 at the TJ as well as actin (D'Atri and Citi, 2001; D'Atri and Citi, 2002). When cells become confluent, an increase in cingulin expression results in an interaction with GEF-H1/Lfc, which in turn, leads to an inhibition of RhoA function. If a connection can be made between the Bves/GEFT and Bves/ZO-1 interactions, it is possible that Bves may function to regulate epithelial integrity through the Rho family at the TJ.

Evidence also exists that Rho family proteins are important regulators in *X. laevis* development. The *X. laevis* homolog of Rho GTPase Cdc42 (XCdc42) is expressed in tissues undergoing extensive morphogenetic changes, such as the deep layers of involuting mesoderm and posterior neuroectoderm during gastrulation, and somitic mesoderm at neurula stages. Overexpression of either wild-type or dominant-negative XCdc42 interferes with convergent extension movements and alters the adhesive properties of cells. Interestingly, these effects occur without affecting mesodermal specification, as we see following Xbves depletion (Choi and Han, 2002). It is possible that Xbves participates in the Rho pathway during *X. laevis* development, especially in light of the Bves/GEFT interaction.

Finally, an interaction with the Rho pathway could provide an explanation for several results that we have obtained in different experimental systems. The Brand group reported a delay in muscle regeneration in *pop1a*-null mice following cardiotoxin injection. In *X. laevis*, an increase in BrdU incorporation on the *Xbves*-MO injected side of the embryos suggests a dysregulation of cell proliferation. An involvement of Bves with the Rho signaling pathway via a GEF would provide a parallel connecting molecular and cellular events of muscle regeneration and the proliferatory nature of *X. laevis* epithelial cells. Perhaps Bves controls or maintains the proliferative activity via its interaction with a GEF protein. It is interesting to postulate that this complex could be tethered at the TJ via ZO-1, and thus can quickly respond to the state of the epithelial cell layer.

Future Directions

Importantly, this dissertation presents findings that have shown Bves to be a necessary component of epithelial tissues and provides an important demonstration of functional significance for Bves in epithelia. Our earliest studies of Bves investigated its potential role in coronary vasculogenesis, since we originally discovered Bves as a transcript enriched in the heart, in agreement with the Brand group. Thus, the question of how the dynamic distribution of Bves at the cell membrane of the epicardium impacts development of the coronary vessels is one of interest. The current work clearly demonstrates a role for Bves in the regulation of cell adhesion and/or epithelial integrity through its localization at the TJ. However, how Bves is involved in heart development is unresolved. Is Bves permitting dynamic movement via transient adhesions generated

through the TJ as the epicardium moves over the heart? How can Bves be an important factor when other adhesion molecules like cadherins are also spatio-temporally regulated to play a role in this process? How might Bves regulate EMT? In light of the data connecting Bves to the TJ, it is now possible to speculate that Bves exhibits a direct or modulatory role in this and other processes through its interactions with ZO-1 at cell junctions. Although this work investigates Bves function in epithelia, Bves is clearly expressed in cardiac and skeletal muscle types that lack a TJ, although adhesive structures do exist. Do Bves family members have a similar role to regulate the integrity of cell-cell adhesive complexes in muscle cell types? Currently, a basis for speculation on this topic is lacking and a parallel examination of Bves in heart and muscle tissue will hopefully address the mechanism of Bves activity.

Our discovery that Bves/Xbves family members exhibit a functional role at the TJ and are essential for integrity in X. laevis embryos supports our original central hypothesis stating that Bves plays an integral role at cell junctions and in the fundamental processes of epithelial cell-cell interaction. Findings from the work discussed in this document have elevated what we know about Bves and laid groundwork for future studies. Several simple but key questions, alluded to in the discussions above, must be addressed in these studies. First, what is the nature of the Bves/ZO-1 protein interaction and how does it influence the TJ? Second, if this interaction exists in the developing embryo, how is embryogenesis affected by Xbves MO treatment in order to produce the defects we observed at both high and low doses, and in the clonal depletion studies? Finally, how does Bves function in epithelial cells correlate with its function in muscle types?

Clearly, we have much to learn about how Bves interacts with and influences the TJ. The nature of the Bves/ZO-1 interaction is left to be resolved. From our studies thus far, we can only say that an association with Bves and a protein complex containing ZO-1 exists. We are uncertain whether this interaction is direct or indirect. We can resolve this issue by performing a yeast two-hybrid screen or by using purified proteins to identify a direct interaction. Also, the domain of Bves that interacts with the ZO-1 protein complex is unknown. Pull-down experiments using Bves truncation mutants can be performed to elucidate the critical regions of Bves responsible for protein-protein binding with junction components. Identifying the exact region that Bves binds to ZO-1 and/or GEFT is essential to the forward progress of research in epithelial regulation. Currently, results from a split-ubiquitin screen for Bves-interacting proteins are under analysis. Characterization of binding partners and the nature of these interactions is necessary to understand and make further speculations about how Bves could be influencing cell-cell adhesion and interaction in both epithelial sheets and well as striated muscle during embryogenesis and maintenance of adult tissues.

To understand how our molecular findings may be important in vivo, we used *X. laevis* to study how Bves influences epithelial movements, a critical component of early embryonic development. We depleted Xbves with MO to block translation of the endogenous protein. Analysis of the embryological defects from these experiments is highly intriguing but far from complete. In one method, we examined the behavior of Xbves depleted progeny of one blastomere of a 32-cell embryo. With the recent finding that Bves modulates TJ integrity through a physical interaction with the ZO-1 protein complex warrants an assessment of cell junctions in the disturbed tissue layers. Thus,

an assessment of the cell junctions in embryos where Bves levels are significantly reduced in a clone of cells may reveal why they do not migrate properly during gastrulation, and why they fail to become anything but ectoderm. To explore this, an electron microscopic analysis using RuR is underway. Also, immunostaining must be performed to explore whether the distribution of junction proteins has changed between two affected cells. These experiments are currently underway and were discussed in Chapter IV.

The "eyeless" embryos also present an interesting developmental defect that requires further examination. Determining when and how eye formation is blocked and/or severely delayed is critical and distribution of eye and head markers will be assessed as described in Chapter IV. Also, future studies will focus on how inactivation of Xbves within individual cells affects their ability to incorporate into the epithelia of the eye. This will address the autonomous nature of Xbves function and also investigate how cell junctions respond to Xbves depletion. This will be accomplished by targeting the eye (Moody, 1987) with a single blastomere injection of the 32-cell embryo and following the fate of its progeny. Specific targeting of head structures has been performed previously (Dufour et al., 1994). Our prediction is that inactivation of Xbves within a single cell will result in its inability to interact with normal cells or form junctions. Changes in the expression and distribution of adhesive proteins such as the catenins, cadherins, and ZO-1 will be monitored and indicate that disruption of adhesion/interaction at specified stages of development. Potential alterations in the cytoskeleton are likely to be observed given the interaction of these structures and cell junctions. Here the alignment of cells in the developing retina, cornea and lens will be

monitored along with these immunochemical reagents. In addition, the GFP-Xdsh tracer will be used in conjunction with antibodies against the cytoskeleton to determine whether changes in cell shape are concurrent with changes in cytoskeleton (Wallingford et al., 2000). We predict that the cytoskeleton and overall cell shape will be greatly altered in this model. Finally, addressing how these defects are related to the Bves/ZO-1 relationship at the TJ may provide a link between the in vivo and in vitro studies. Also, it is unclear whether Bves/Xbves is a component of a well-defined signaling pathway. Thus, directing future efforts to elucidate a molecular signaling cascade in which Bves participates would present new avenues to investigate Bves/Xbves function.

Thus far, we have identified a homophilic interaction domain within the Bves C-terminal tail, but no other domains, including those that mediate ZO-1 or GEFT interactions, have been identified. Clearly, this is also a critical component of future study on the Bves family of proteins. We plan to use the *X. laevis* system we have established to identify functional domains within Xbves. Endogenous Xbves function will be inhibited in one half of an embryo with MO to generate the "eyeless" embryos described in Chapter IV. Modified *Xbves* mRNAs with deleted or modified sequences will be used to "rescue" eye development. Embryos with mutated Xbves will be analyzed to determine whether rescue has been inhibited. If eye development is not rescued, it would signify that important sequences have been lost or modified. Using eye development as a "read-out", we will identify essential elements of molecular function and pinpoint specific aspects of eye development that are regulated by Xbves. Another important factor in choosing the frog model for these analyses is that Xbves appears to be the only major isoform expressed at this stage of development (EST

database, Ripley et al., submitted). Thus, problems with redundancy of protein function observed with the mouse model are eliminated. Third, mutation of rescue mRNAs is a much faster and economical way of analyzing a multitude of protein modifications when compared to mouse knockout and transgenic studies. Scores of embryos can be produced in a single day. Thus, multiple mutations of the protein can be rapidly analyzed.

Finally, after we determine the nature of Bves protein-protein interactions, the functional domains of the protein, and how these findings play into embryonic development, we can hopefully approach the third question of how Bves function in epithelial cells correlate with its function in muscle types. The expression of Bves/Pop1a proteins in cardiac and skeletal muscle is indeed very robust, suggesting a critical function. Although redundancy of Pop family members has complicated strategies to generate knockout mice, the Brand group continues their efforts to identify roles for these proteins in myocytes. Currently, contributions from both laboratories have begun to piece the molecular puzzle of this novel protein family together, but clearly, a complete understanding of the regulation of these proteins is far from complete.

In conclusion, this dissertation provides an important contribution to the body of literature on the Bves/Pop family of proteins. First, my work demonstrates that Bves is a component of epithelial cell types and exhibits a protein distribution in both cells and tissues that would suggest a function in cell junction regulation and cell interaction. My studies also establish that Bves plays an important role in TJ integrity, supported by functional knock-down experiments and an interaction with a molecular complex containing ZO-1. These findings provide the groundwork for further examination of how

Bves participates in cell adhesion and cell interaction, as our earliest studies had implied. Finally, I present evidence that Bves/Xbves plays a critical role in embryo morphogenesis, which establishes an excellent model system to perform future studies. In sum, my work demonstrates the importance of Bves at the TJ in epithelia and during embryo development, and defines the essential nature of Bves/Xbves in epithelial integrity.

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