ANALYSIS OF BVES FUNCTION IN VESICULAR TRANSPORT AND CELL MORPHOLOGY

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

This chapter will briefly introduce three topics: GEFT, VAMP3, and *Xenopus laevis (X. laevis)* gastrulation. An in depth discussion of Bves protein, the focus of this work, is given in Chapter II. The aforementioned three topics are critical for the understanding of this dissertation for the following reasons: Bves interacts with GEFT and VAMP3 and influences the signal cascades underlying cell adhesion and migration that are regulated by these two proteins. *X. laevis* gastrulation is a developmental stage characterized by cell movement and differential adhesion and thus can be exploited to examine the cohesive function of Bves, GEFT, and VAMP3. As detailed in Chapters III, IV, and VI, the *X. laevis* model system is utilized to investigate how this triad of proteins influences cell migration and adhesion.

GEFT

Rho GTPases are small molecular switches that regulate diverse functions such as cell polarity, adhesion, movement, and vesicular transport through downstream effector proteins (Etienne-Manneville & Hall, 2002). Specifically, Rac1 and Cdc42 have been shown to induce actin-rich stress fibers leading to process extension and subsequent cell movement. Three different subsets of proteins regulate the vast array of cellular processes in which Rho GTPase

functions: GTPase-activating proteins (GAPS), guanine nucleotide dissociation inhibitors (GDIs), and guanine nucleotide exchange factors (GEFs) (Larsen et al, 2003; Ridley, 2001a) (Figure 1.1). Of these three groups of regulatory proteins, GEFs are the signal cascade activators, catalyzing the molecular switch of GDP for GTP through their Dbl Homology (DH) domain (Ridley, 2001a). Rac1 and Cdc42 are Rho GTPases that are, in part, regulated by GEFT, which is highly expressed in brain, skeletal muscle, and heart (Guo et al, 2003). GEFT has been shown to promote neurite outgrowth and spine formation in neural tissue and promote skeletal muscle regeneration and *de novo* myogenesis (Bryan et al, 2004; Bryan et al, 2006; Bryan et al, 2005). Furthermore, overexpression of GEFT results in increased cell movement and proliferation through activation of Rac1 and Cdc42 (Guo et al, 2003).

The importance of understanding GEFT activity in these diverse cellular functions is that Bves directly interacts with GEFT and that disruption of Bves function leads to inhibition of GEFT effectors Rac1 and Cdc42.

VAMP3

Vesicular transport is a general eukaryotic cell process in which individual cells communicate with the environment through translocation of specific proteins to plasma membranes and extracellular spaces (Alberts, 2002). Membrane trafficking is tightly regulated and underlies several fundamental cellular processes including migration, epithelial biogenesis, and cell signaling. SNARE proteins regulate the final step in vesicular transport: fusion of a vesicle to a

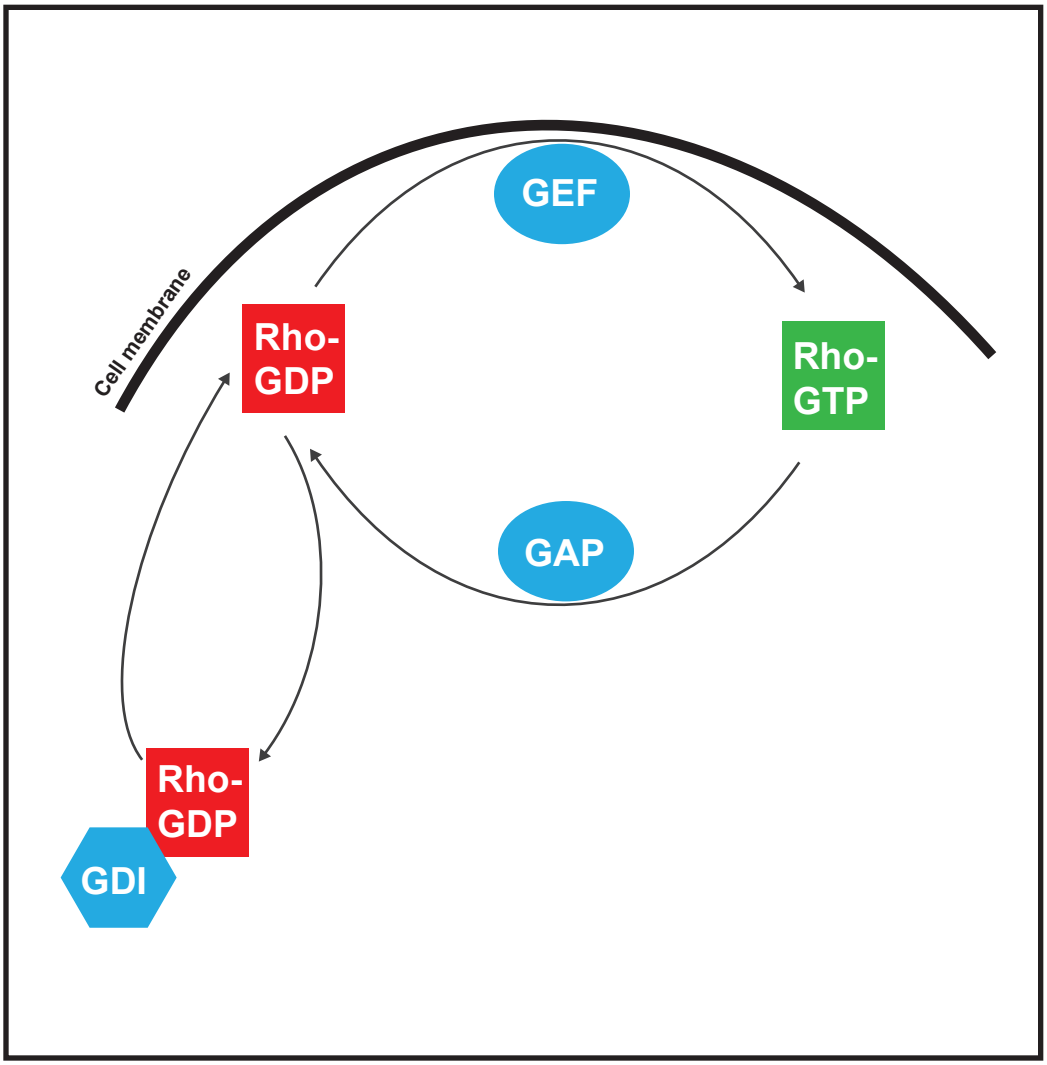


Figure 1.1 Regulation of Rho GTPases. GEFs stimulate Rho GTPase activity by catalyzing the exchange of GDP for GTP; GAPs perform the converse reaction by exchanging GTP for GDP to inactivate the Rho GTPase. GDIs prevent the release of GDP from a Rho GTPase and prevent activation. This figure was adapted from Larsen et al., 2003.

target membrane (Brunger, 2005). Although first described in the release of neurotransmitters, SNAREs are now known to function in a wide variety of tissues. There are three families of SNARE proteins that are essential for vesicular transport: synaptobrevins, SNAPs, and syntaxins (Jahn & Scheller, 2006; Leabu, 2006). These proteins interact via coiled-coiled domains, bringing two apposing membranes in close proximity so that membrane fusion can occur (Figure 1.2) (Alberts, 2002). Several accessory proteins assist the SNARE protein function by sorting specific vesicles to the membrane, or through facilitating docking of the correct cargo once in the vicinity of the SNARE motif (Jahn & Scheller, 2006). Rho GTPase activity has also been suggested to have a role in SNARE docking and fusion events, although the specific mechanism remains to be determined (Rodriguez-Boulan et al, 2005).

Vesicle-associate membrane protein 3 (VAMP3, cellubrevin, or synaptobrevin3) is a Tetanus Neurotoxin (TeNT) sensitive SNARE protein that is ubiquitously expressed in all non-neuronal tissue (McMahon et al, 1993). As a member of the recycling endosome, VAMP3 is involved in taking specific receptors to and from the plasma membrane (Borisovska et al, 2005). VAMP3 has an established role in transport of the transferrin receptor, and recently, several reports have demonstrated VAMP3 transports integrins to and from the membrane during cell migration (Galli et al, 1994; Luftman et al, 2009; Proux-Gillardeaux et al, 2005a; Skalski & Coppolino, 2005; Tayeb et al, 2005). Disruption of VAMP3 results in a reduced rate of migration in wounded epithelial cells, as β-1 integrin recycling is impaired (Proux-Gillardeaux et al, 2005a).

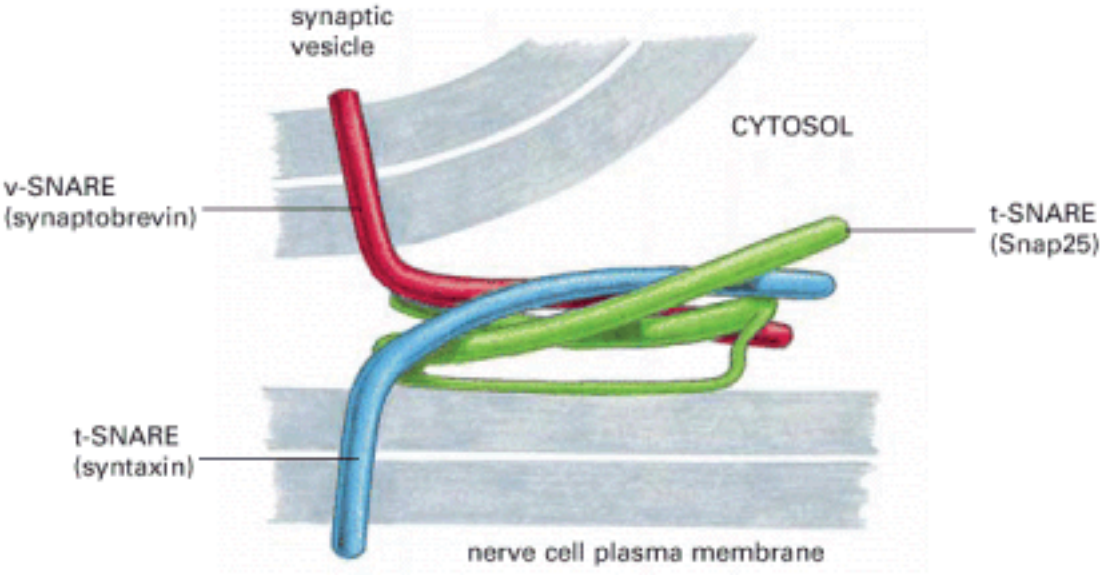


Figure 1.2 SNARE proteins. v-SNAREs (red, synaptobrevin or VAMP) bind t-SNAREs (green, SNAP; blue, syntaxin) to bring two membranes in close proximity so that fusion can occur. This image is from Alberts et. al., 2002.

Additionally, VAMP3 is required for specific sorting to the basolateral surface in polarized epithelia as VAMP3 cleavage results in the mis-sorting of both transferrin and the low density lipoprotein receptor (Fields et al, 2007). Interestingly, VAMP3 null mice do not exhibit a phenotype; this is attributed to potential redundancy in function of other VAMP family members (Yang et al, 2001). Overall, VAMP3 is necessary for efficient transport of specific vesicular cargos to the membrane, which subsequently act to mediate distinct cellular functions.

As mentioned above, VAMP3 and two other VAMP homologues, VAMP1 and VAMP2, are specifically cleaved by TeNT (Yamasaki et al, 1994). This property can be exploited as a means to effectively disrupt VAMP3 function in epithelial cells. Proux-Gillardeaux et al. (Proux-Gillardeaux et al, 2005a) utilized this property by co-expressing an inactive form of TeNT (only the enzymatic portion of the toxin that is missing the heavy chain that enables entry into the cell) and VAMP3-GFP. When VAMP3 is cleaved, GFP labeling is seen diffusely throughout the cytoplasm. However, in cells expressing mutated TeNT (a one amino acid mutation which renders the toxin enzymatically dead, thus preventing cleavage) VAMP3-GFP has normal distribution that is observed at the cell membrane and within the cell in vesicles. This method allows for visual selection of cells expressing mutated VAMP3 and can be used to elucidate the function of VAMP3 in cultured cells.

The relevance of understanding VAMP3 activity is based on the interaction of Bves with this protein. Many cellular functions dependent on

VAMP3 activity are disrupted with inhibition of Bves function (Chapter IV). The convergence of Bves, GEFT, and VAMP3 interactions on basic cell properties is discussed in depth in Chapter VII.

Xenopus laevis gastrulation

Gastrulation is a highly coordinated, dramatic reorganization of the embryo that is fueled by cellular movement (Slack, 2006). All triploblastic embryos undergo gastrulation, which functions to form and position the three germ layers: ectoderm, mesoderm, and endoderm (Gilbert et al, 2006). Specifically, gastrulating *X. laevis* embryos undergo extensive epithelial sheet rearrangement driven by differential cell adhesion and migration (Keller, 2002). These events have been studied for years and are well defined by various fate-mapping and explant studies (Keller, 2005; Keller, 1975; Wilson & Keller, 1991; Winklbauer & Nagel, 1991). Prior to gastrulation, the *X. laevis* embryo undergoes rapid cell divisions to produce a circular ball of cells surrounding a fluid-filled cavity called the blastocoel (Figure 1.3). During gastrulation, the embryo undergoes four defined, practically simultaneous movements: invagination, involution, convergent extension, and epiboly (Slack, 2006). The onset of gastrulation is characterized by pigment formation on the vegetal portion of the embryo, marking the dorsal lip of the newly forming blastopore (the future anus in deuterostomes) (Jones & Smith, 2008). This pigment soon encircles the blastopore as elongated cells, called bottle cells, begin to move inward, or *invaginate* to form the archenteron cavity (invagination is a minor cell movement

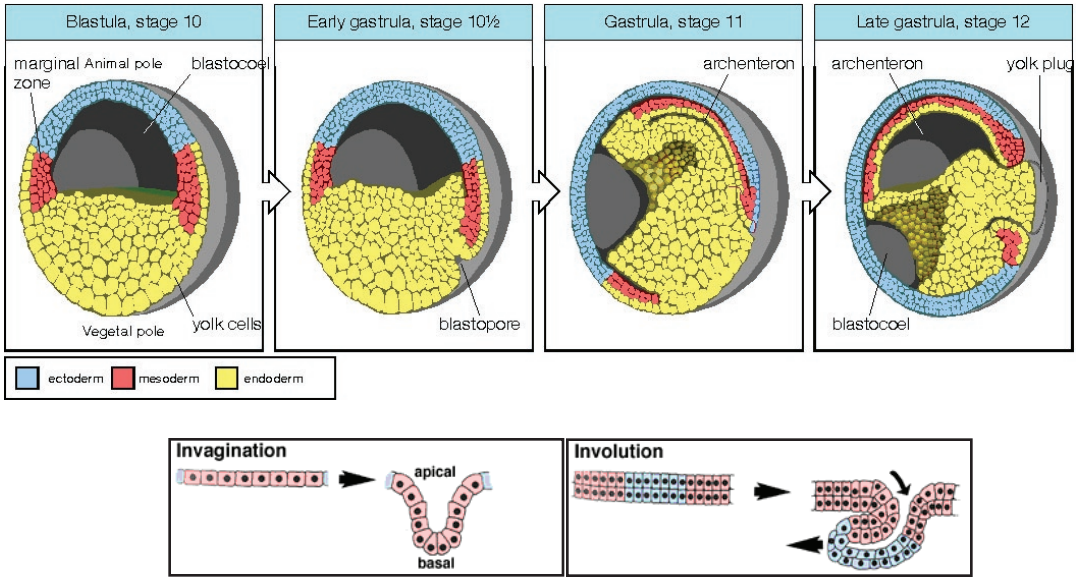


Figure 1.3 Gastrulation in *Xenopus laevis*. Prior to gastrulation, the blastula is defined by its hollow fluid filled cavity, the blastocoel. As gastrulation begins, cells undergo invagination and involution movements to form the archenteron and displace the blastocoel. Gastrulation defines and positions the three germ layers: ectoderm, mesoderm, and endoderm. This figure was adapted from Gilbert et al. (top panel) and Hardin et al. (bottom panel).

in *X. laevis* gastrulation, whereas involution is the major cell movement that drives archenteron formation and is described below; the difference between these cell movements is seen in Figure 1.3) (Keller, 1981; Keller, 1986). Simultaneously, the embryo elongates relative to its anterior-posterior (AP) axis; this initial extension is driven by the deep cells of the blastocoel roof (BCR) intermingling with the more superficial cells in a process called radial intercalation (Keller et al, 1992; Keller, 1975). Radial intercalation causes the thinning of the BCR, reducing this ectodermal layer to a thickness of two or three cell layers (Longo et al, 2004). Next, superficial cells of the BCR begin to converge towards the midline and intercalate, further driving extension along the AP axis (Keller et al, 1992). This movement is called mediolateral intercalation and pushes the tail region away from the future head region (Montero & Heisenberg, 2004). Together, radial and mediolateral intercalation comprise the characteristic gastrulation movement of *convergent extension* (Keller et al, 2008). Convergent extension fuels the previously outlined process of invagination and also drives the migration of mesodermal cells (cells that populate the marginal zone) during *involution* (Keller, 1981). In these cell movements, internal mesodermal cells migrate anteriorly along the BCR as invagination occurs, maintaining cell-substrate contact with the overlying ectoderm, but not intermixing with this germ layer (DeSimone & Johnson, 1991; Montero & Heisenberg, 2004). This set of movements displaces the blastocoel as the newly formed archenteron will develop into the future digestive organs of the animal (Gilbert et al, 2006). Involution and subsequent migration of head and trunk mesodermal cells is

required for future development of all anterior features (Ren et al, 2006a). Finally, the embryo also undergoes *epiboly* (Slack, 2006). Here, the most superficial, pigmented animal cap cells migrate as an epithelial sheet towards the dorsal and ventral blastopore lips to eventually enclose this region and envelop the entire embryo (Keller, 1980). As we will see later, these generalized cell movements and rearrangements require the interplay between cell-cell and cell-substrate adhesion molecules that have been studied in depth (DeSimone & Johnson, 1991).

As mentioned above, explants have been a powerful tool used to understand the complex movements that occur during gastrulation. *X. laevis* cells are particularly well suited for these studies because their high yolk content allows individual cells to survive outside the embryo in simple buffers for a relatively long period of time (Gilbert et al, 2006). Remarkably, not only will *X. laevis* cells survive *in vitro*, but gastrulation movements that occur *in vivo* also occur in culture (Wilson & Keller, 1991). This allows specific movements of interior cells to be studied in real-time, which would be impossible in the opaque embryo (and also demonstrates the level of cell fate commitment of gastrulation-staged cells). A variety of explants have been developed that allow one to study individual cell movements by disassociating a specific population of cells or keeping a tissue layer intact to study movement of cell sheets (Ren et al, 2006a; Tahinci & Symes, 2003; Wilson & Keller, 1991; Winklbauer & Nagel, 1991). Study of gastrulation in the frog has not only advanced the field of developmental biology, but has also greatly benefited biologists who study the regulation of cell

movement (DeSimone et al, 2005; DeSimone et al, 2007; DeSimone & Johnson, 1991). Thus, this is an ideal system in which to examine proteins that regulate cell migration and adhesion during development and/or during adult normal or diseased biological states.

As detailed in this thesis, Bves is a transmembrane protein that interacts with GEFT and VAMP3, and influences the downstream signaling cascades governed by these two proteins. Specifically, Bves is important for the vesicular transport and Rho GTPase activity underlying cell migration and adhesion. As tight regulation of cell motility and differential adhesion is critical for *X. laevis* gastrulation, this is an excellent system in which to study Bves function. Furthermore, Bves protein can be effectively depleted and restored in the developing frog embryo (Ripley et al, 2006), thus allowing for highly controlled experimentation. Finally, no other Bves homologue is present during early embryonic stages (EST databases), thus minimizing the effect of functional redundancy and simplifying phenotypic analysis. Taken together, *X. laevis* gastrulation provides a superb model system to examine the role Bves plays in regulating cell adhesion and migration.

CHAPTER II

BVES: TEN YEARS AFTER

This chapter was published under this title in *Histology & Histopathology*, June, 24th, 2009 (Hager & Bader, 2009).

Abstract

Bves was discovered in 1999 by two independent laboratories using screens to identify novel genes that were highly expressed in the developing heart (Andree et al, 2000; Reese et al, 1999). As an evolutionarily conserved transmembrane protein, Bves is postulated to play a role in cell adhesion and cell motility. In studies of Bves protein disruption, there have been multiple phenotypes, but few molecular mechanisms have been advanced to explain the underlying cause of these phenotypes. As the molecular function of Bves protein begins to be uncovered, it is now time to review the literature to examine the significance of this work and future directions of study. This review summarizes the literature on this unique protein and explores new and exciting data that support emerging themes on its molecular function.

Popdc Gene Family

The Popdc family, of which Bves is the founding member, is comprised of three highly conserved, completely novel genes (Andree et al, 2000; Reese et al,

1999). The products of these genes share no significant structural homology with any other established protein and thus it has been difficult to study protein function. Bves (Popdc1) is the most studied member of the *Pop*eye *d*omain *c*ontaining (Popdc) family. Popdc2 and Popdc3 comprise the rest of the family, however little is known about these homologs (Andree et al, 2000; Breher et al, 2004; Froese & Brand, 2008; Parnes et al, 2007). Recently, the expression pattern of Popdc2 in chick and mouse was reported, but no studies have been conducted to test the function of this protein. Popdc3 function remains completely unstudied (Froese & Brand, 2008; Parnes et al, 2007). Since its discovery, Bves transcripts have been identified in a wide array of eukaryotes ranging from honey bees to humans, whereas Popdc2 and Popdc3 are only found in higher vertebrates (NCBI Database). No known copies of Popdc genes are found in either plants or single celled organisms, suggesting these genes are important for complex cell-cell interactions that only occur within multicellular organisms in the animal kingdom. Within a single species, Popdc2 and Popdc3 are 50% conserved with each other, while Bves is only 25% homologous with either Popdc2 or Popdc3, suggesting Bves may be the outlier of this gene family (Figure 2.1) (Brand, 2005; Osler et al, 2006). It is interesting that Bves is present in both chordates and arthropods, while Popdc2 and Popdc3 are present only in evolutionarily younger chordates (NCBI Databases). Thus, Popdc2 and Popdc3 may have evolved in higher vertebrates to serve a function independent of Bves. Examination of these novel Popdc family members is essential as it will elucidate

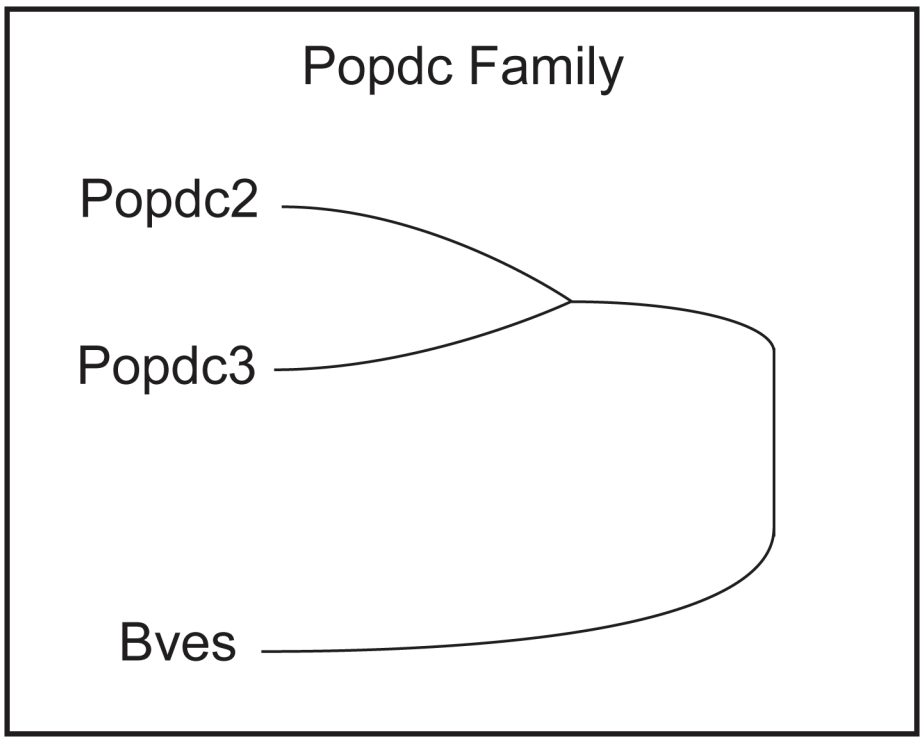


Figure 2.1 The Popdc family. Bves is only 25% conserved with either Popdc2 or Popdc3, suggesting it is the outlier of this gene family. The function of Bves is only now being uncovered, whereas the function of Popdc2 and Popdc3 are completely unknown.

the function of these structurally unique genes and underscore their overall biological significance.

Bves Structure

As mentioned previously, Bves protein structure is unique and displays no structural homology with any other protein. As structure most often predicts function, we postulate that Bves has a novel role in cell biology, and is likely to be linked to established pathways through mechanisms that cannot be predicted a priori. It is known that Bves (~360 amino acids, ~50kDa) is a three-pass transmembrane protein that has an intracellular C-terminus and an extracellular N-terminus (Figure 2.2) (Knight et al, 2003). Due to its position in the cell membrane, it can be postulated that Bves may act to recruit or dock intracellular proteins to membranes, or may play a role in cellular interactions with the environment or with other cells, as is typical for transmembrane proteins. The extracellular N-terminus (aa 1-42) of Bves has two invariant N-glycosylation sites, which may potentially protect Bves protein from proteolysis or may help to localize Bves to the membrane (Kukuruzinska & Lennon, 1998). However, the N-terminus may be dispensable (aside from N-glycosylation sites) or its structure less critical in regard to function as it is small and highly heterogeneous between species. Within the intracellular C-terminus (aa 113-360), there exists the novel Popeye domain, which was named for its homology throughout the Popdc family (Brand, 2005). Despite this conservation, no definitive homologous motifs are found within this domain, or,

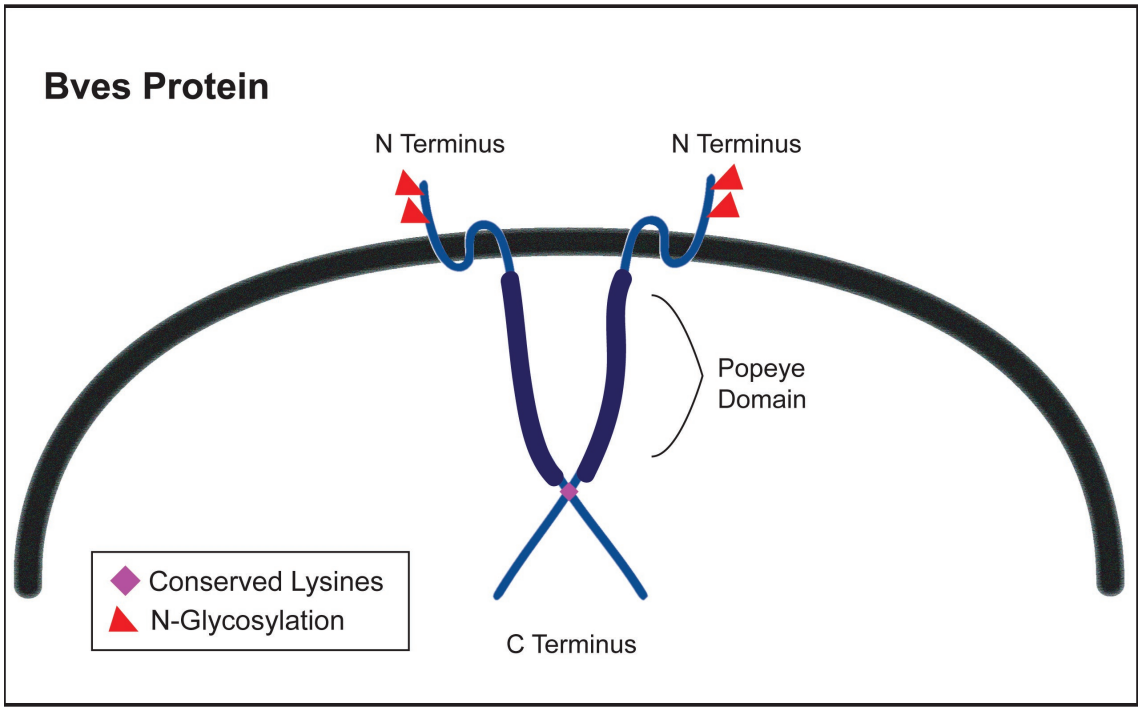


Figure 2.2 Bves protein. Bves is a transmembrane protein that exists in the plasma membrane as a multimer. There is a short, extracellular N-terminus with two N-glycosylation sites and an intracellular, self-associating C-terminus. Located within the C-terminus is the Popeye domain, named for its high conservation across species. To date, no function has been specifically linked to this motif.

for that matter, within Bves protein as a whole. Sequence alignment websites do predict a cyclic nucleotide binding domain fully contained within the Popeye domain (Finn et al., 2008). However, this alignment is not complete and biochemical function confirmation is required before this motif can be considered significant. Thus, no indication of Bves function can be deduced from its protein structure.

Although the function of the Bves Popeye domain is unknown, it is highly conserved throughout different vertebrates (~80%) (Brand, 2005; Osler et al, 2006). Evolutionary conservation of this protein suggests its function in cell biology is important and understanding the role of the Popeye domain is the key to understanding Bves biological function and significance. In this regard, Kawaguchi et al. recently reported that Bves exists as a dimer or multimer, self-associating in the cell within the Popeye domain (Kawaguchi et al, 2008). Additionally, they found that lysines 272 and 273 were essential for this function. Finally, this Bves-Bves interaction is necessary for maintenance of epithelial integrity and junctional stability (discussed below), further supporting the importance of the conserved Popeye domain to the overall function of Bves protein. Although the transmembrane domain and the C-terminus have been shown to have specific characteristics, many questions still remain concerning the biological role of Bves protein. For example, nothing is known about protein biogenesis, protein folding, or the kinetics of protein turnover. Exploration of these basic properties is critical to provide information about the spatial and temporal regulation of Bves in relation to cellular processes that are possibly

regulated by this gene product. Additionally, post-translational modifications of Bves, aside from N-glycosylation, are entirely uncharacterized. Identifying potential phosphorylation states, folding conformations, and enzymatic activity may elucidate Bves function and mechanism of interaction with other molecules, and would provide a molecular understanding of the phenotypes observed after disruption or elimination of the protein.

Expression Pattern

In order to predict biological significance and function, it is important to know the tissue distribution of the protein and where it is localized within the cell. Understanding the expression pattern of Bves protein, both within the organism and within the cell, has assisted in the initial steps to resolve gene function.

Bves is observed at high levels in the heart, thus initial focus was drawn to uncovering expression in this organ (Andree et al, 2000; Reese et al, 1999). Since its initial isolation, Bves expression has been identified in heart, smooth and skeletal muscle, brain, and various epithelia (Figure 2.3) (Andree et al, 2000; McCarthy, 2006; Osler & Bader, 2004; Ripley et al, 2004; Smith & Bader, 2006; Torlopp et al, 2006; Vasavada et al, 2004). As predicted by Expression Sequence Tag (EST) databases, Bves is present in a wide range of organs including spinal ganglia, thymus, and testis (NCBI). Although once thought to be present only in muscle, it is now clear that Bves protein is found in other tissues and, thus, when analyzing its role in the cell, broader biological functions must be considered (DiAngelo et al, 2001; Osler & Bader, 2004; Ripley et al, 2004; Smith

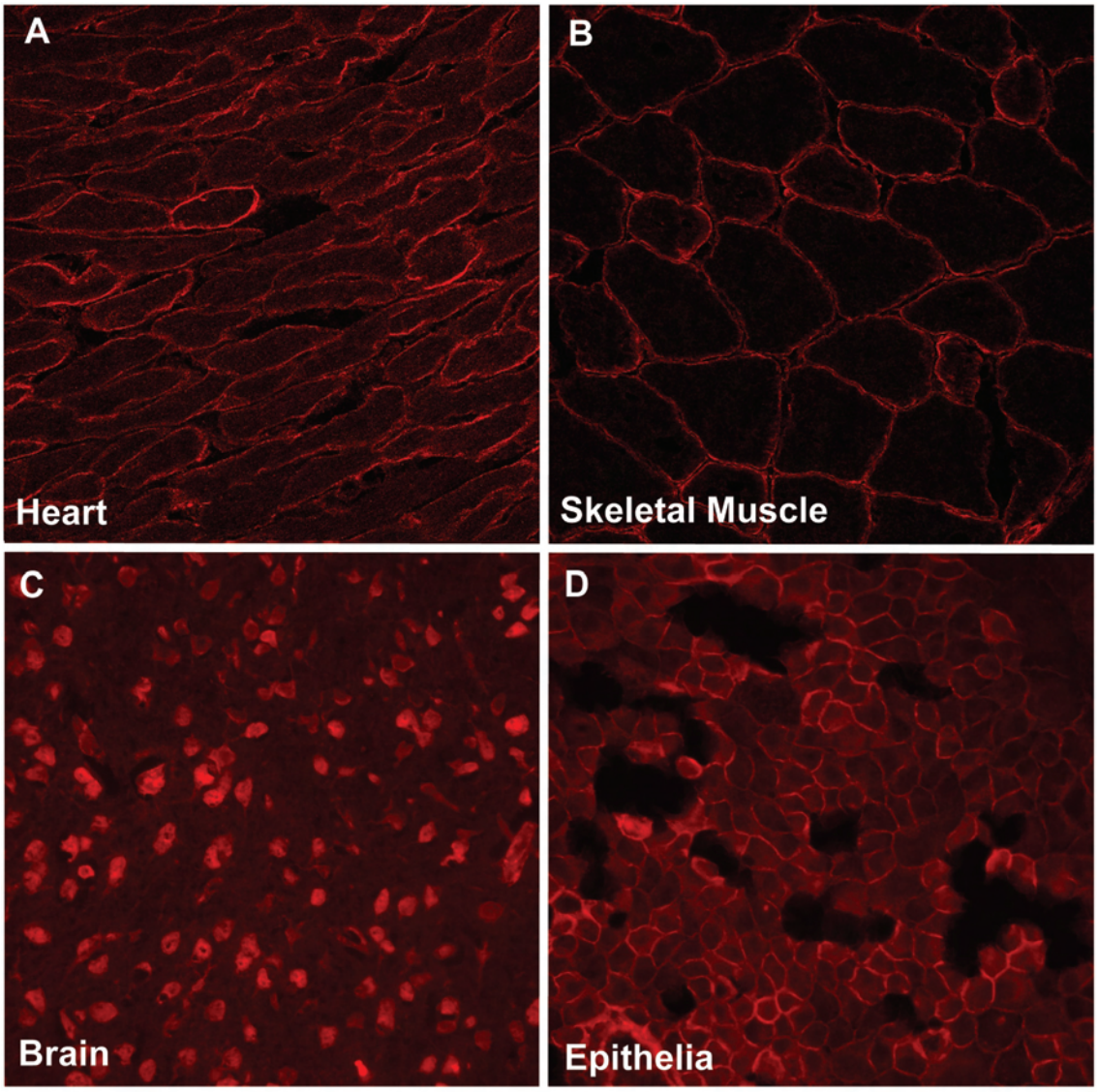


Figure 2.3 Bves expression. Bves is expressed in cells that associate or couple: Heart (A), Skeletal Muscle (B), Brain (C) and Epithelia (D).

& Bader, 2006). Interestingly, in the adult organism, most, if not all, of Bves-expressing cells share one common phenotype or function: they are adherent or are at least highly interactive in nature.

Bves expression in embryogenesis has been studied in several different organisms. In the chick, Bves is found in the epithelia of all three germ layers (Osler and Bader, 2004). In early development, Bves message is detected at Henson's node at HH stage 4 (Torlopp et al, 2006). Later during organogenesis, Bves expression is most prevalent in the heart, epidermis, and developing eye (Osler & Bader, 2004; Ripley et al, 2004). In the developing mouse, Bves expression is seen in heart, skeletal, and smooth muscle (Andree et al, 2000; Smith & Bader, 2006). Additionally, expression is detected in epithelial tissue such as the epidermis and gut throughout development, although analysis of the earliest stages of mouse development is incomplete (Smith & Bader, 2006). It was originally reported in 2002 that expression of the *X. laevis* homologue of Bves, *Xbves*, was confined to the heart, and expression in other organs, such as skin or skeletal muscle, was not observed (Hitz et al, 2002). Most recently, maternal transcripts were detected by *in situ* hybridization in blastula stage embryos and are localized to the animal pole; during gastrulation all animal pole cells express Bves (Ripley et al, 2006). Discrepancies in detection are most likely due to variation in protocols used to visualize expression of RNA. EST analysis of *X. laevis* eggs and early embryos clearly demonstrate the presence of this transcript as a maternal and early zygotic message. By stage 35 in *X. laevis*, expression is restricted to the heart, somites, cement gland, and eye. Protein

localization studies in *X. laevis* demonstrate Bves localizes to points of cell-cell contact, similar to the distribution seen in cell culture as described below (Ripley et al, 2006). Recently, the *D. melanogastor* homologue of Bves, DmBves, was characterized during oogenesis. DmBves is expressed in nurse cells and some epithelial follicle cells of the egg chamber (Lin et al, 2007). Anterior-dorsal and posterior follicle cells do not show DmBves expression. To summarize, Bves is found in a multitude of tissue types derived from all three germ layers both in the embryo and in the adult. Revealing the spatial and temporal expression pattern of Bves has brought about deeper insight of embryonic and adult function.

In order to better understand the function of Bves protein, it is clearly necessary to examine its subcellular localization during various events or changes in cell behavior. Because of its unique structure, it is difficult to predict a cellular function for Bves, thus, a broad spectrum of cell behaviors must be examined.

Generally speaking, Bves expression is strongest in cells that associate or couple, such as epithelia or cardiac muscle, and is less prevalent in non-associating cells, such as fibroblasts. This expression pattern indicates a role for Bves in cell communication or cell-cell adhesion. Consistent with *in vivo* expression in the developing embryo, Bves is detected in immortalized skeletal myoblasts and epithelial cells, and in isolated primary cardiac myocytes. (Osler et al, 2005; Smith & Bader, 2006). Interestingly, Bves exhibits a dynamic subcellular distribution pattern prior to cell-cell junction formation. When cells are not polarized or in contact with each other, Bves protein is observed within the cell

and not at the cell surface. As cells begin to adhere, Bves is one of the first proteins transported to the membrane, preceding membrane localization of classical junctional markers such as E-cadherin and ZO-1 (Osler et al, 2005; Wada et al, 2001). When epithelial cells form polarized sheets, Bves localizes primarily to points of cell-cell contact, and confocal Z-stack analysis reveals a lateral distribution (Osler et al, 2005; Smith & Bader, 2006). Another consistent yet unexplained result is that long-term confluent cultures of epithelial cells have nearly complete localization of Bves at the cell surface with no intracellular staining (Hager and Bader, unpublished results). As expected, Bves co-localizes at the tight junction with junctional proteins such as ZO-1 and Occludin (Osler et al, 2005). These data, published by Osler et al. in 2005, used a polyclonal antibody that specifically recognizes Bves at the tight junction. In 2006, Smith et al. generated new monoclonal immunoreagents that display a greater distribution along the lateral portion of the membrane (Smith & Bader, 2006). In addition to co-localizing with tight junction markers ZO-1 and Occludin, Bves monoclonal antibodies also co-localize with the adherens junction protein, E-cadherin. It is important to note that both the monoclonal and polyclonal antibodies were raised against the same epitope, and the reason for the discrepancy in distribution is unknown at this point. Nonetheless, in isolated cardiac myocytes, Bves displays this same dynamic subcellular re-localization. Initially, Bves is present within the cell, and then as the cardiomyocytes interact, Bves localizes to points of cell-cell contact (Smith & Bader, 2006).

Dynamic re-localization of Bves is not strictly confined to epithelial biogenesis. When Epithelial Mesenchymal Cells (EMCs) are in a confluent sheet, Bves is found at the membrane, but when induced to undergo EMT by stimulation with specific growth factors or high serum, Bves is seen in the interior of the cell and lost at the cell surface (Wada et al, 2001). This dynamic distribution of Bves protein may suggest that Bves function is regulated by its subcellular location. As seen with membrane receptors such as Glut4, receptor distribution is indicative of spatial regulatory states (Zaid et al, 2008). For example, when Glut4 is sequestered within the cell, it is unable to transport glucose into muscle and fat cells. Upon stimulation with insulin, Glut4 is translocated to the membrane and glucose enters the cell. Therefore, when Bves is localized within the cell, it may be spatially regulated by some unknown mechanism. Although the function of Bves is not entirely understood, it is known that it plays a role in maintaining epithelial junctions and this dynamic localization pattern supports a role for Bves in this process.

At this point, the expression pattern of Bves protein, both within the organism and at the subcellular level has been largely resolved. Bves is present in both vertebrates and invertebrates and displays a dynamic subcellular distribution pattern dependent upon the environment or context of the cell. From this expression pattern, a putative function can be postulated and tested. Thus, the next generation of experiments should focus on elucidating the molecular utility of Bves. The previous review from our group written by Osler et al. focused on gene expression and distribution patterns of Bves protein (Osler et al, 2006).

As a novel gene, it was necessary to outline discrepancies and consistencies in the literature. The current review will now focus on the molecular function of Bves, as it is important in explaining the mechanisms that underlie the developmental defects seen when Bves protein is disrupted.

Bves in Embryogenesis

Bves depletion or inhibition results in disrupted embryonic morphogenesis, however, studies that detail the underlying molecular mechanism of these developmental phenotypes are lacking. It is crucial to study Bves function *in vivo*, yet it is now not enough to simply describe these phenotypes. Previous reports have provided excellent detailed accounts of phenotypic variation upon inhibition of Bves (Andree et al, 2002; Lin et al, 2007; Ripley et al, 2006). Now, future experiments must provide evidence of Bves protein function in order to understand the significance of this novel protein in embryogenesis and homeostasis.

As mentioned previously, Bves is widely expressed during embryogenesis before expression is later restricted to specific regions (Osler et al, 2006). This suggests Bves plays a role in early development that may be reconstituted or maintained in specific adult tissues. Investigating Bves function in embryogenesis is important in understanding how Bves functions in basic cell processes *in vivo*. Bves has only one known gene copy in *D. melanogastor*, and Bves is the only Popdc member detected in the early *X. laevis* embryo, making them ideal systems in which to study Bves function (NCBI databases). Disruption of Bves in

both of these model systems leads to severe developmental defects, deeming them promising *in vivo* systems in which to study Bves function (Lin et al, 2007; Ripley et al, 2006).

In vitro studies of Bves with epithelial cell lines suggest a function in regulating cell adhesion, epithelial integrity, and cell motility (discussed in detail below), three cellular functions that are essential in embryonic gastrulation. Thus, gastrulation is a valuable developmental paradigm in which to probe the function of Bves. During gastrulation, the frog embryo undergoes a dramatic reorganization of cell layers that is fueled by sheet movement and differential cell adhesion (Wilson & Keller, 1991). Specifically, gastrulating *X. laevis* embryos undergo extensive epithelial sheet rearrangement driven by interdependent intercalation and convergent-extension events that drive blastopore closure and the involution of mesoderm (Keller, 1980; Nieuwkoop & Faber, 1994). The individual cell and subsequent progeny movements that occur during these gastrulation events are well defined by fate-mapping studies (Moody, 1987). As mentioned above, *Xbves* is widely expressed during early frog development, especially in these epithelial sheets, and is later restricted to specific regions (heart, eye, somites, and cement gland) in a two-day old embryo. Ripley et al. found that Bves-depleted embryos have disrupted gastrulation and aberrant individual cell movements. Specifically, blastopore closure is delayed and animal cap extension is impaired, suggesting epithelial sheets may not intercalate or converge and extend properly towards the blastopore (Ripley et al, 2006). This phenotype could be a result of impaired cell movements, cell adhesion, or

epithelial integrity, as all of these functions are necessary for cell rearrangement during gastrulation. As Bves protein is localized to the membrane in the developing *X. laevis* embryo, it is possible that Bves may play a role in all three of these cell processes, as they are interconnected. Therefore, additional experiments are needed to determine whether one or all of these cell processes account for the observed phenotype and link these phenotypes to disruption of specific molecular pathways.

In an accompanying set of frog experiments, individual blastomeres were depleted of Bves activity and shown to exhibit rogue cellular movements within the developing embryo, suggesting unregulated motility (Ripley et al, 2006). Still, while Bves is required for early epithelial cell movements, the exact mechanism underlying this phenotype is not specifically understood. As will be described in greater detail below, Bves interacts with GEFT to modulate process extension and cell motility through Rho GTPases, Rac1 and Cdc42. When mutant Bves is expressed in clonal cell lines, cells have decreased motility and become more rounded (Smith et al, 2008). Interestingly, in *X. laevis*, expression of mutant Rac1 causes decreased cellular adhesion and process extension, resulting in impaired gastrulation movements (Hens et al, 2002; Tahinci & Symes, 2003). Thus, it is possible that disruption of Bves protein in the developing *X. laevis* embryo results in unregulated Rho GTPase activity through inhibition of the Bves-GEFT interaction. While this hypothesis remains to be tested, this could provide an important link between an observed *in vivo* phenotype and an established molecular mechanism of Bves function.

Similar to *X. laevis* gastrulation, *D. melanogastor* gastrulation consists of dramatic rearrangement and movement of epithelial sheets (Gilbert et al, 2006). Lin et al. isolated DmBves, the *D. melanogastor* homologue of Bves, and characterized the role this single copy gene plays in embryogenesis of the fly (Lin et al, 2007). Antisense DmBves expression resulted in a failure of pole cells to adhere and migrate anteriorly, failure of posterior midgut invagination and germband elongation, and significant embryonic lethality. These defects suggest that Bves is required for proper *D. melanogastor* gastrulation movements, as was seen in *X. laevis*. However, these phenotypes were only seen in a small percentage of embryos (10-20%), suggesting the role of Bves is not strictly essential in these invertebrates. Alternatively, this could be due to difference in genetic penetrance or redundancy related to non-family member proteins that might compensate for Bves function.

Although the exact mechanisms underlying the observed phenotypes are unexplained, common themes are beginning to emerge. Bves disruption *in vivo* results in disrupted cellular movement during gastrulation, thus understanding how Bves functions in these processes will reveal the significance of Bves function during development.

Given the severe phenotypes seen in both *X. laevis* and *D. melanogastor* embryos when Bves is depleted, it was predicted that Bves-null mice would exhibit obvious developmental defects and would not live a normal lifespan (Andree et al, 2002). This, however, was not the case. Bves-null mice displayed no overt morphological defects. As the Popeye domain is highly conserved

throughout all Popdc family members, and all three members have similar tissue expression, it is possible that Bves, Popdc2, and Popdc3 have redundant functions in development (Parnes et al, 2007). This has yet to be studied, as functions of the latter proteins have not been tested and are entirely unknown (Andree et al, 2002; Andree et al, 2000; Breher et al, 2004; Froese & Brand, 2008; Smith & Bader, 2006). The possibility of overlap in function of Popdc family members warrants the creation of either a double or triple knockout mouse, or the creation of a transgenic mouse expressing mutated Bves. Thus, embryologic characterization of Bves function in mice may prove more challenging and different genetic strategies must be employed to characterize the function of Bves in this model system.

Despite the lack of an overt phenotype in development, skeletal muscle regeneration was impaired in Bves-null mice (Andree et al, 2002). Skeletal muscle regeneration is orchestrated by activated satellite cells; these cells migrate to the area of injury from healthy tissue and eventually fuse and mature into muscle fibers (Carlson & Faulkner, 1983). In Bves-null mice, skeletal muscle regeneration was initially delayed and disorganized when compared to controls. However, 20 days after injury, there was no apparent difference in tissue architecture between controls and Bves-null mice. This suggests satellite cells in Bves-null mice may have been delayed or impaired in their ability to migrate, interact, and subsequently heal the wound (Andree et al, 2002). This is also consistent with previous reports in development where disruption of Bves results in aberrant cell movement. Further studies are needed to elucidate the exact role

Bves plays in skeletal muscle regeneration and how this phenotype relates to promising areas of molecular function.

Taken together, these studies demonstrate that Bves plays an important role in development and regeneration. To fully understand the function of Bves, it is important to examine how disruption of this protein affects these different model organisms and how these phenotypes are linked to previously established *in vitro* mechanisms. Using these approaches in conjunction will provide a global perspective of Bves function.

Regulation of Bves expression

At the time of this writing, very little is known about the signaling events and transcriptional control regulating Bves expression. Again, we mention that Bves expression is not confined to a single cell type and thus, we predict that modulation of Bves transcription will be complex and not strictly mediated through a tissue specific regulatory pathway.

Although gene regulation of Bves has not been studied in detail, Barber et al. has reported Bves to be a putative target gene of PAX3 (Barber et al, 2002). PAX3 is a transcription factor important for neural, heart, and skeletal muscle development; PAX3 null mice die *in utero* and have defective myogenesis and impaired skeletal muscle formation. In these null mice, Bves RNA is downregulated in comparison to controls, further supporting Bves induction by PAX3 .

Additionally, Lin et al. reported that Bves is downstream of Gurken (Grk)/EGFR signaling. Grk, the invertebrate TGFα homolog, is important for dorsoventral patterning of the embryo (Lin et al, 2007). Grk is expressed in anterior-dorsal region of the oocyte, regulating downstream effectors in this area (Gilbert et al, 2006). In *Drosophila*, Bves is expressed in all follicle cells surrounding the ooctye, except in anterior-dorsal or posterior follicle cells where Grk signaling is active. In Grk mutants, where Grk signaling is depleted, Bves expression is present in all anterior-dorsal or posterior follicle cells, suggesting Bves expression is negatively regulated by Grk. In *fs(1) K10* grk mutants, where Grk expression is no longer restricted to the anterior-dorsal region, Bves expression is decreased in anterior-ventral follicle cells, further supporting a role for Bves regulation through Grk (Lin et al, 2007).

Prior to this report, the regulatory system of Bves protein expression was completely unexamined. Thus, Lin et al. has provided the first report detailing how Bves protein levels are controlled and has linked Bves to an established signaling pathway in development. While this clearly shows Bves linkage to this pathway, this most likely is not the only regulatory system directing Bves protein expression and it is still unknown how Bves is regulated in the adult. As new data emerges, it may become clear that Bves plays a role in adult disease states (described below). Therefore, it is plausible that tight regulation of Bves protein in the adult may be necessary to maintain tissue homeostasis.

Molecular Function

There are definitive phenotypes associated with Bves protein disruption during development, and the mechanism of these underlying phenotypes are beginning to emerge. Given its unique structure and the possible redundancy of function between Popdc family members, investigation into molecular function using *in vitro* techniques is essential to resolve how this gene exerts its influence at the cellular level. Bves was first identified as a putative cell adhesion molecule in 2001 (Wada et al, 2001). Since its initial characterization, Bves has been reported to play a role in maintaining epithelial integrity and regulating cell movement. Still, the global impact it has on the developing organism is only now being uncovered. Summarized below are the current data on the molecular function of Bves and speculation of how this data can account for the observed phenotypes.

Cell Adhesion

Bves has a definitive role in cell-cell adhesion although it is unknown how Bves confers this intercellular adhesion. Two reports show that previously non-adherent L-cells form adhesive clumps when transfected with wildtype Bves (Kawaguchi et al, 2008; Wada et al, 2001). These data suggest Bves confers an adhesive property to non-adherent cells, either directly through intercellular Bves-Bves homophilic interaction or indirectly through vesicular transport pathways or signaling cascades that would recruit or "assist" conventional adhesive molecules. The possibility that Bves induces cell-cell adherence through an

intercellular Bves-Bves interaction, as a junctional protein would, seems unlikely because the extracellular N-terminus is very short (~40 aa). In comparison, Occludin and E-cadherin, both of which self-associate intercellularly, have extracellular termini well over 200 amino acids. Additionally, Bves N-terminus is not homologous throughout species, suggesting conservation of this extracellular sequence is not essential for function. Most likely, Bves is enacting adhesion as an accessory protein by facilitating the transport or docking of bona fide cell adhesion molecules to the membrane, as Bves is one of the first junctional proteins to localize to points of cell-cell contact and is thought to be an early marker of cell adhesion (Osler et al, 2005; Wada et al, 2001). As the C-terminus of Bves is both highly conserved and unique, it is possible that Bves acts as a novel docking or recruiting protein for junctional proteins, allowing them to localize to the membrane to create cellular junctions. In order to test this possibility, other interacting proteins must be identified to link Bves to established biological processes leading to adhesion. Finally, Bves may be a critical component of a signal cascade that results in cell adhesion. As is discussed below, Bves regulates GEFT activity, which in turn modulates downstream Rho GTPases. Rho GTPases are known to be important in epithelial junction biogenesis, suggesting Bves may act through this pathway to induce cell adhesion (Braga & Yap, 2005). Exploring these avenues of Bves function in cell adhesion is vital to understanding the role Bves plays in the embryo and adult.

As Bves is similarly localized to the membrane in multiple epithelial cell lines, Osler et al. investigated the specific role of Bves at the tight junction (Osler

et al, 2005). In epithelia, the tight junction forms an impermeable barrier so that diffusion of molecules and intermixing of proteins between apical and basolateral domains does not occur, resulting in a polarized epithelium (Cereijido et al, 2008). It was reported that Bves co-localizes with components of the tight junction, particularly ZO1 and Occludin, in clonal epithelial cell lines and in adult mouse small intestinal epithelium using the polyclonal antibody described above. Furthermore, Bves forms a complex with tight junction component ZO1, although this interaction is not thought to be direct. When Bves protein is disrupted, junctional proteins such as E-cadherin are not localized properly to points of cell-cell contact. Additionally, the trans-epithelial resistance (TER), a measure of tight junction integrity, is decreased (Osler et al, 2005). Taken together, these data suggest Bves is integral in establishing and maintaining the tight junction and is critical for a properly polarized monolayer of epithelial cells. But, the exact mechanism by which Bves functions at the tight junction is not entirely understood. It is possible that Bves may function to maintain epithelial integrity by allowing junctional proteins to dock at, be transported to, or retained at the membrane. The size and conservation of structure in the C-terminus of Popdc family members might also suggest a scaffolding function at the membrane where interaction with many proteins may occur. In this way Bves could function to either organize or sustain adhesion, or maintain adhesion proteins at the membrane through a mechanism that has yet to be explored.

Recently, it has been established that Bves exists as a dimer or multimer, and this self-association is essential for Bves function in conferring cell adhesion

and maintaining polarity (Kawaguchi et al, 2008; Knight et al, 2003). Kawaguchi et al. identified the intracellular KK motif (aa 272, 273), located within the highly conserved Popeye domain, as a site that is necessary for Bves homodimerization. L-cells transfected with Bves mutated in the KK region (KK-mut Bves) do not form aggregates as wild type Bves transfected cells do. In a stable KK-mut Bves epithelial cell line, contiguous epithelial sheets are not maintained, junctional proteins such as E-cadherin are mis-localized or downregulated, and the TER is greatly reduced. Additionally, these cells display properties consistent with cells that have undergone epithelial to mesenchymal transitions (EMT) (decreased cytokeratin expression and upregulated expression of vimentin) (Kawaguchi et al, 2008). These data further support the idea that Bves is important for maintenance of epithelial sheets, and describes a motif that is necessary for Bves-Bves intracellular interaction and subsequent intercellular adhesion. However, it is still important to understand exactly how Bves functions to elicit cell-cell adhesion. Understanding the precise molecular pathway that results in adhesion and epithelial polarity is crucial in elucidating the significance of Bves protein.

Cell Motility

Although Bves is highly conserved, it shares no structural homology with any identified protein domain that has a defined or associated cell function. Thus, to elucidate the mechanism by which Bves functions, Smith et al. conducted a yeast-two-hybrid screen to identify interacting partners and potentially link Bves

to an established molecular pathway (Smith et al, 2008). Guanine nucleotide exchange factor T (GEFT) was identified as a novel interacting protein in this manner. This remains the only report of a direct or physical interaction between Bves and another protein. GEFs modulate the active state of Rho GTPases by stimulating the exchange of GDP for GTP (Schmidt & Hall, 2002). Specifically, GEFT activates Rho GTPases, Rac1 and Cdc42, to induce lamellipodia and filopodia formation during cell migration. These Rho GTPases are also involved in other cell processes such as proliferation and differentiation, but these functions have not been explored in relation to Bves protein (Bryan et al, 2004; Bryan et al, 2006; Bryan et al, 2005; Guo et al, 2003). GEF distribution is spatially regulated and thus localization at the membrane can potentially be indicative of GEF activation (Schmidt & Hall, 2002). It is intriguing that endogenous Bves and GEFT co-localize primarily at cell-cell borders in both striated and smooth muscle, suggesting this subcellular location (where GEFT is active) is the sight of their interaction. When mutated Bves protein (in this case, the intracellular C-terminus missing the transmembrane domain; also determined to be the minimal GEFT binding domain) is exogenously expressed, cells become more round and are less motile (Smith et al, 2008). These data suggest decreased GEFT and subsequent Rac1 and Cdc42 activation. PAK21 pulldown assays support this hypothesis, demonstrating decreased active Rac1 and Cdc42 protein levels when mutant Bves is expressed, indicating that Bves regulates the activation/inactivation state of GEFT.

Thus, it has been established that Bves and GEFT interact to modulate downstream effector proteins, Rac1 and Cdc42. Still, it is unknown precisely how Bves regulates GEFT. In order to examine this interaction, it is important to consider what is known about the regulation of GEFs (Figure 2.4A). In general, GEFs are modulated in three distinct ways: A) self-regulation through an inhibitory intramolecular association; B) activation via interaction with another protein; C) modulation through subcellular localization (Schmidt & Hall, 2002). Decreased motility upon mutant Bves expression suggests three potential mechanisms: First, Bves may bind and sequester GEFT, such that it is not available for translocation or activation by other proteins (Figure 2.4B). The function of Bves-GEFT interaction may be to negatively regulate the amount of available active GEFT. Over-expression of only the binding portion of Bves may disrupt this balance of activation/inactivation of GEFT, leading to decreased levels of downstream effectors, Rac1 and Cdc42. Second, Bves may directly activate GEFT through binding (Figure 2.4C). In this vein, expression of mutant Bves would act as a dominant-negative, disrupting endogenous localization and function of Bves and disrupting GEFT stimulation. Thus, downstream GEFT effectors are never activated. This would account for the decreased Rac1/Cdc42 activity observed. Finally, Bves may indirectly modulate GEFT activation by localizing or retaining GEFT at the membrane through binding or priming GEFT for activation by other proteins (Figure 2.4D). As mentioned earlier, GEFT may be regulated by its localization within the cell (Schmidt & Hall, 2002). If GEFT cannot be transported to or be retained at its activation site, this would cause a

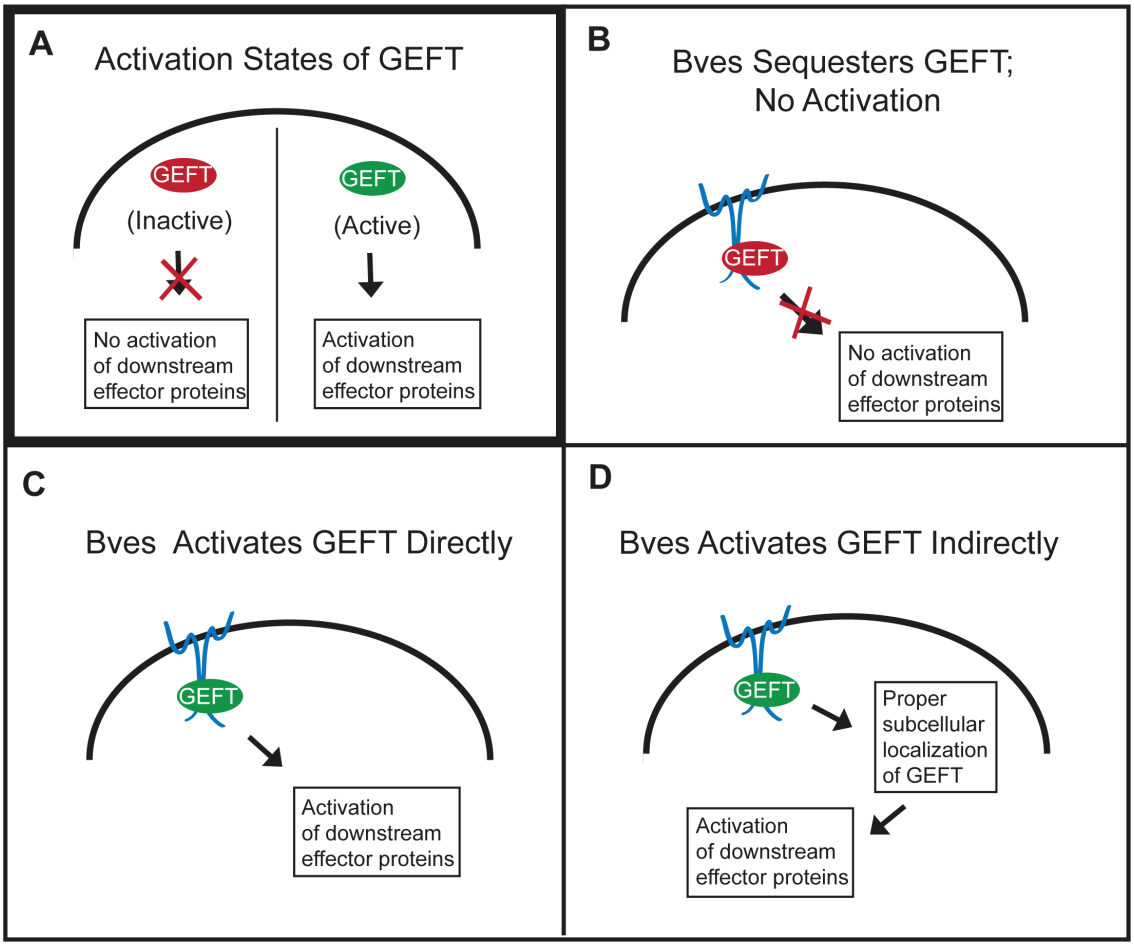


Figure 2.4 Bves regulation of GEFT. In order to induce downstream effectors, GEFT must be activated (A); the mechanism by which this occurs is not specifically known. Bves interaction with GEFT may regulate the activation state of GEFT. Three mechanisms of modulation are outlined: 1) Bves may sequester GEFT, resulting in decreased activation of effector proteins (B). 2) Bves may directly activate GEFT through its interaction (C). 3) Bves may indirectly regulate the activation state of GEFT by localizing it to the membrane (D).

decrease in levels of activated GEFT, and thus a decrease in the activity of downstream modulators. Indeed, preliminary data from our laboratory suggest that Bves may play a significant role in intracellular trafficking (Hager and Bader, unpublished data).

Investigation of these models would bring insight into how GEFT is regulated through activation/inactivation and would reveal the role Bves has in this pathway. Interestingly, identification of the Bves-GEFT interaction may clarify the underlying molecular mechanism of previously seen phenotypes, namely the aberrant cell movement phenotype observed in the *X. laevis*. From a more global perspective, Rho GTPases are involved in a plethora of different cell processes, and it is possible that Bves' role in these biological processes is mediated through this pathway (Braga & Yap, 2005; Malliri & Collard, 2003; Ridley, 2006). This remains to be tested as the molecular function of Bves is revealed.

Bves in Disease

Given the putative role Bves plays in cell adhesion and in maintaining epithelial integrity, it is not unexpected that loss of Bves function could result in abnormal cell behavior and disease. Bves is required for maintenance of E-cadherin at the membrane (Osler et al, 2005), and cells stably transfected with KK-mut Bves (the domain necessary for Bves-Bves interaction) have decreased or mis-localized E-cadherin expression (Kawaguchi et al, 2008). In development after disruption of Bves function, gastrulation of both the *D. melanogastor* and *X.*

laevis are inhibited, suggesting impaired cell adhesion or movement, both of which are dependent upon stable junctions (Lin et al, 2007; Ripley et al, 2006). Therefore, it is plausible that Bves functions to retain, traffic, or attract E-cadherin to the membrane, as it is one of the first proteins localized to points of cell-cell contact. In development or disease, downregulation or mislocalization of E-cadherin is associated with EMT, a cellular process in which cells delaminate from an epithelial sheet to become freely migratory cells (Hirohashi, 1998). EMT is essential for proper development and underlies embryonic processes such as chick gastrulation and coronary vasculature formation (Reese et al, 2002). When spontaneously or aberrantly induced in the adult, EMT is a hallmark of cancer, resulting in loss of epithelial organization and cellular invasion of previously healthy tissue (Yang & Weinberg, 2008). Human cancers of epithelial origin display disorganized histology and decreased cell adhesion due to the loss of E-cadherin (Hirohashi, 1998). In this light, it is interesting to consider that Bves is necessary for cell adhesion and loss of Bves leads to decreased localization of E-cadherin at the membrane and junction formation, with concomitant upregulation of mesenchymal marker proteins. Thus, it is possible that Bves plays a role in tumor suppression and recent evidence supports this idea. Feng et al. reported the DNA methylation levels of 27 genes in non-small cell lung (NSCL) cancer tumors from patients who had undergone surgical resections (Feng et al, 2008). In their study, the authors identified genes that critically mark tumor versus noncancerous tissue based upon methylation levels. *Bves* was identified as a cell adhesion molecule that had ‘some’ methylation in 35% of the

cases and hypermethylation in 24% of the cases. The authors then analyzed genes that were ‘sensitive and specific’ for cancerous tissue; Bves was part of the three-gene panel that identified 51% of cancerous tissue (and only 2% of non-cancerous tissue). This is the first report of a modification of Bves in cancer. Given the known function of Bves as a cell adhesion molecule and its down-regulation in NSCL cancer, investigation of Bves function in the realm of cancer biology is warranted and would be an exciting avenue of study.

Future Studies

Bves was discovered in a screen to identify novel genes, and while the study of Bves function has proved challenging, it has recently progressed. Although unanswered questions remain, general trends are beginning to emerge. Bves plays a role in cell adhesion, epithelial integrity, and cell motility: all interrelated basic processes in cell biology. Bves interaction with GEFT has linked Bves to an established molecular pathway. However, further investigation is needed to understand Bves modulation of GEFT. In order to elucidate all aspects of Bves function in relation to cell adhesion and epithelial cell maintenance, other interacting proteins must be identified and characterized. Particularly, special focus should be given to model organisms displaying only one gene, as these are the key to unlocking Bves function *in vivo.* Similarly, the creation of a Popdc1-3 knockout mouse is necessary if Bves function is to be resolved in higher vertebrates. On a more universal level, it would be exciting to examine the role of Bves in disease, as Bves is important for localization of E-

cadherin to the membrane, and a recent report has linked Bves to NSCL cancer. Furthermore, investigating the underlying mechanism of Bves function in epithelial adhesion and motility in the context of human disease is essential to put in perspective the biological significance of this protein.

CHAPTER III

BVES DIRECTLY INTERACTS WITH GEFT, AND CONTROLS CELL SHAPE AND MOVEMENT THROUGH REGULATION OF RAC1/CDC42 ACTIVITY

This chapter was published under this title in *PNAS* June 17, 2008 (Smith & Hager et al., 2008). Dr. Travis Smith initiated this work and I concluded the study. We are co-first authors on this publication.

Abstract

Bves is an integral membrane protein with no determined function and no homology to proteins outside of the Popdc family. It is widely expressed throughout development in myriad organisms. Here, we demonstrate an interaction between Bves and GEFT, a GEF for Rho-family GTPases. This interaction represents the first identification of any protein that has a direct physical interaction with any member of the Popdc family. Bves and GEFT are shown to co-localize in adult skeletal muscle. We also demonstrate that exogenous expression of Bves reduces Rac1 and Cdc42 activity levels, while not affecting levels of active RhoA. Consistent with a repression of Rac1 and Cdc42 activity, we show changes in speed of cell locomotion and cell roundness also result from exogenous expression of Bves. Modulation of Rho-family GTPase signaling by Bves would be highly consistent with previously described phenotypes occurring upon disruption of Bves function in a wide variety of model

systems. Therefore, we propose Bves as a novel regulator of the Rac1 and Cdc42 signaling cascades.

Introduction

Bves (blood vessel epicardial substance) is the most studied member of the Popdc family, which is a group of evolutionarily conserved transmembrane proteins. *Bves* was discovered by our laboratory in 1999, and is widely expressed throughout development and adulthood in many different species. All three developing germ layers (Osler & Bader, 2004), cardiac muscle (Andree et al, 2000; DiAngelo et al, 2001; Hitz et al, 2002; Reese et al, 1999; Smith & Bader, 2006), skeletal muscle (Andree et al, 2002; Andree et al, 2000; Smith & Bader, 2006), neural tissues (Andree et al, 2000; Osler & Bader, 2004), epicardium (Osler & Bader, 2004; Reese et al, 2002; Reese et al, 1999; Vasavada et al, 2004; Wada et al, 2001; Wada et al, 2003), epithelial components of the eye (Ripley et al, 2004), and smooth muscle (Osler & Bader, 2004; Smith & Bader, 2006) have been demonstrated to express *bves*. While expression of the Bves protein is now resolved, few definitive indications of molecular function exist.

Several possibilities of potential Bves function have been described. Epithelial integrity of cultured corneal cells is severely decreased by knockdown of Bves protein using morpholino oligonucleotides, possibly via an interaction with ZO-1 at the tight junction (Osler et al, 2005). Perturbation of Bves function has also been shown to disrupt proper migration of epithelial components of the

early *Xenopus* embryo (Ripley et al, 2006) and affect wound healing of epithelia in scratch assays (Ripley et al, 2004). Mice null for the *bves* gene are delayed in regeneration of skeletal muscle upon injury (Andree et al, 2002). A recent report demonstrates that Bves knockdown using antisense RNA during *Drosophila* oogenesis results in failure of pole cells to migrate properly to antero-dorsal side of the embryo (Lin et al, 2007). Despite these studies, no direct molecular mechanism for any of these phenotypes exists at this time.

In an effort to ascribe molecular function, we conducted a yeast two-hybrid screen and identified GEFT (guanine nucleotide exchange factor T) (Guo et al, 2003) as a protein that interacts with the cytoplasmic portion of Bves. GEF proteins modulate activity of small GTPases, specifically the Rho family of GTPases, Rac1 and Cdc42, in the case of GEFT (Bryan et al, 2004; Bryan et al, 2006; Bryan et al, 2005; Guo et al, 2003). GEFs stimulate exchange of GDP for GTP, thereby activating Rho small GTPases. The small GTPases, Rac1 and Cdc42, have a myriad of effects on cell behavior; including control of proliferation, differentiation, cell motility, and gene expression (Bishop & Hall, 2000; Etienne-Manneville & Hall, 2002). Given the known functions of GEFs and Bves in regulation of cell differentiation and motility, this relationship was studied in detail.

The present study is the first to demonstrate direct physical interaction of Bves with any protein and linkage to any known molecular pathway. We further describe this interaction by determining that transfection of a truncated version of Bves decreases Rac1 and Cdc42 activity, and that transfection of this Bves

truncation or full-length Bves also decreases motility of NIH 3T3 cells in real-time assays. Modulation of GEFT function by Bves provides molecular explanation of phenotypes previously observed with disruption of Bves and is critical for future investigation of the function of this protein.

Materials and Methods

Yeast two-hybrid screen and deletion analysis

The cytoplasmic portion (Bves-CT, amino acids 115-358) of mouse bves was used to screen a 17.5d mouse heart library. Deletion analysis of Bves-CT and GEFT were standard.

GST-pulldown of Dbl Family Members

mGEFT was amplified from pCMVTag-2b-mGEFT and cloned in frame with GFP of pEGFP-C1 (Clontech) to generate pEGFP-mGEFT. pEGFP-mNudeL1 was generated previously by our laboratory (Soukoulis et al, 2005). pcDNA3-N-myc-Cool1 was a gracious gift of Dr. Richard Cerione, and pC.HA-Vav1 was obtained from Addgene.

COS-7 cells transfected with pEGFP-mGEFT, pC.HA-Vav1, pcDNA3-N-myc-Cool1 or pEGFP-mNudeL1 were grown to confluence in 10-cm dishes. Protein was extracted as described above. Cells were incubated on ice for 30 minutes with gentle agitation, 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 glutathione-Sepharose 4B for 2 hours at 4°C, after which beads were spun down and lysate was removed. Glutathione-Sepharose 4B bound with GST constructs was then added to the lysate and incubated overnight at 4°C. Sepharose conjugates were captured using centrifugation, 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 an 10% SDS-PAGE gel. Western blotting was performed using standard methods. Blots were developed by using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche) and scanned into digital format (Hewlett–Packard).

Rac1/Cdc42 activation assay

COS-7 cells were transfected as described with pEGFP-C3 vector and pEGFP-mBvesCT-myc (aa115-aa358) expression vectors. Cells were then harvested in MLB (Magnesium-containing Lysis Buffer) two days after transfection, lysates were sonicated for five seconds, centrifuged for 30 minutes at 18,000 g at 4°C following manufacturer's specifications for Rac/Cdc42 Assay Reagent Kit (Upstate Cell Signaling) (Taylor & Shalloway, 1996). 10 µg of Rac/Cdc42 assay reagent was added to 600 µL of protein lysate, and gently rocked at 4°C for 30 minutes. PAK-21-agarose conjugates were collected by centrifugation for 5 seconds at 14,000 g at room temperature, washed 3x with 500 µL MLB, and bound protein was eluted in 25 µL SDS-PAGE sample buffer.

Western blotting of these samples, and of 10 μL of the original lysate as a loading control, was performed using standard protocols.

Motility assays

The intracellular C-terminus of Bves (aa115-358) was cloned into a mammalian expression construct (pCMV-myc). NIH 3T3 cells were cotransfected using Lipofectamine 2000 at 95% confluency with pCMV-myc-BvesCT and pEGFP-C1 (as a tracer for transfected cells), GFP-Bves, or with pEGFP-C1 alone. Cells were split to ~10% confluency two days after transfection. For monitoring the velocity of cell motility (total path length/time), cells in 10 cm^2 dishes were placed on the 37° heated stage of a Leica DMIRE2 inverted microscope. Time-lapse images were captured using an Orca-ER camera. Images were captured every 60 seconds over a 45 minute interval using a 10x objective. Quantitative motion analysis was carried out using Dynamic Image Analysis Software (Solltech, Oakdale, IA). The outline of each cell was traced frame by frame, and using these tracings the DIAS software calculated the speed of cell movement by tracking the change in position of the cell centroid for each frame. All data from these experiments were evaluated by ANOVA using Statview (SAS Institute, Cary, NC).

Experiments evaluating the effects of exogenous expression of the carboxyl-terminus of Bves were conducted at the Cell Imaging Shared Resource at Vanderbilt University. Cells were transfected as described, and split to ~10% confluence in 24-well culture plates (Nalgene). Plates were placed on the 37°

heated stage of an inverted Nikon TE300 widefield microscope with automated stage for acquisition of multiple fields or view. Images were captured every two minutes for 30 minutes using a 20x objective. Quantitative motion analysis was carried out as described above using Metamorph software (Molecular Devices, Sunnyvale, CA). Data was evaluated using Microsoft Excel.

Cell roundness assay

In addition to the motility data rendered from the analysis of exogenous expression of GFP-Bves described above, the cell tracings were also used to investigate the relative roundness of cells transfected with either GFP-Bves or GFP alone. The roundness of these cells was calculated using the equation 100 X 4π (area/perimeter^2) (Stites et al, 1998). This equation provides a measurement of how efficiently a given amount of perimeter encloses area: a circle has the largest area for any given perimeter with a roundness of 100%. Accordingly, the greater the number of cell protrusions, the lower the roundness. All data obtained from quantitative assessments were evaluated by ANOVA using Statview as above.

Results

The cytoplasmic C-terminus of Bves interacts with GEFT

A yeast two-hybrid screen was used to isolate Bves interacting proteins from an embryonic mouse heart library. The cytoplasmic carboxyl terminal

portion of Bves (aa115-358) was used for this screen. This region of Bves contains the uncharacterized Popdc domain (Brand, 2005; Breher et al, 2004; Osler et al, 2006). Utilizing a yeast two-hybrid screen with cDNAs expressed in the embryonic mouse heart, we isolated 104 interacting proteins when the carboxyl-terminus (aa115-aa358) of mBves was used as bait. Two independent clones were isolated that contained coding sequence for amino acids 46-344 of the mouse GEFT protein. Both of these clones passed the false positive screening process. As previous experiments have shown defects in cell motility/interaction (Osler et al, 2005; Ripley et al, 2004; Ripley et al, 2006), we chose to pursue this interaction further.

Deletion analysis of interacting domains

In order to determine which regions of the Bves and GEFT proteins were responsible for the interaction revealed by the yeast two-hybrid screen, a deletion analysis further utilizing the yeast two-hybrid method was used. A series of truncations of the cytoplasmic portion of Bves revealed that the portion of the protein between amino acid 250 and amino acid 300 is critical for interaction with GEFT (Figure 3.1A).

The truncation analysis to determine the region of the GEFT protein responsible for interaction with Bves revealed that the portion of the protein between amino acid 300 and amino acid 400 is necessary for interaction with Bves (Figure 3.1A). However, further analysis of the results of these studies revealed that these regions (aa 250-aa 300 of Bves, aa 300-aa 450 of GEFT) are

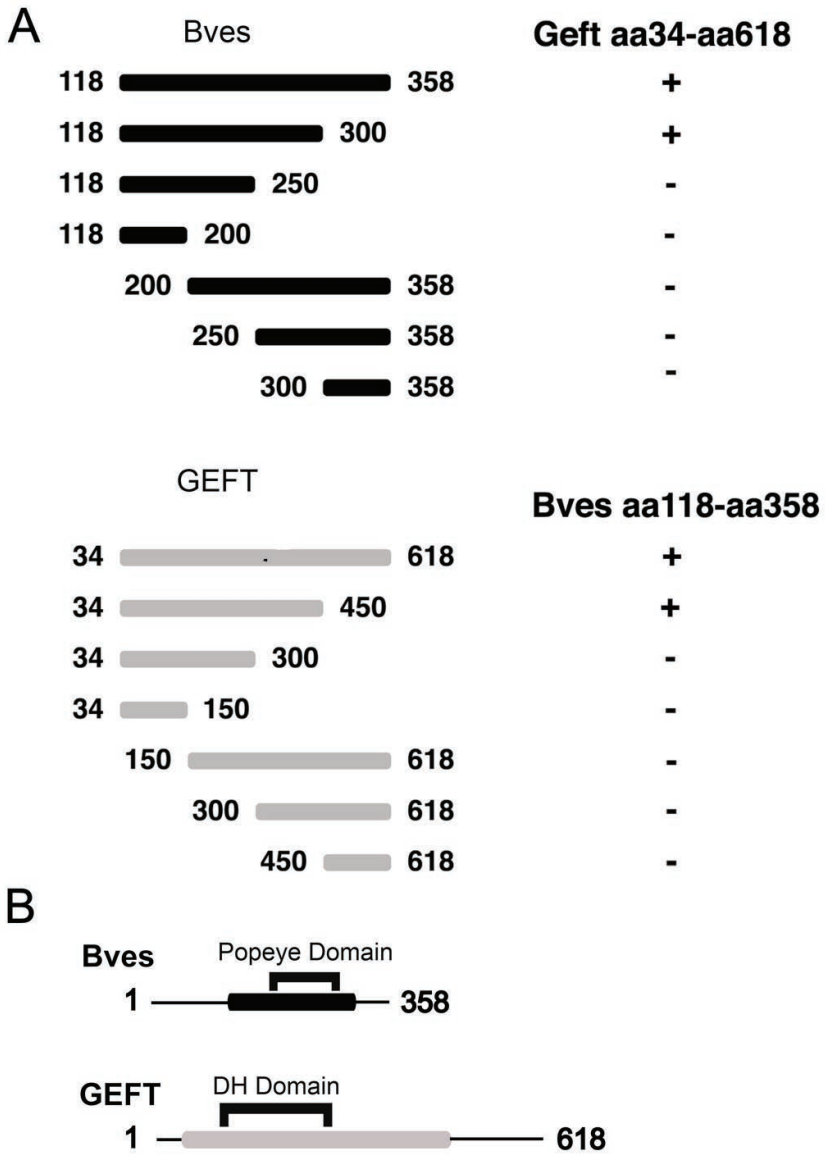


Figure 3.1 Interaction domain analysis. (A) Bves and GEFT truncations were generated using PCR for further definition of interacting domains. Using a yeast two-hybrid strategy, Bves truncations (black bars) were screened against full-length GEFT (aa 34-aa 618) for interaction. GEFT truncations (gray bars) were screened against the intracellular C-terminus (aa 118-aa 358) of Bves. Results of matings listed on right, (+) signifies growth on selective media, while (–) indicates no growth observed upon mating. (B) Minimal interaction domain of Bves (black bar) contains the Popdc domain, while minimal interaction domain of GEFT (gray bar) contains the Dbl homology domain.

not sufficient for the Bves-GEFT interaction to occur as neither aa 250- aa 300 of Bves or aa 300- aa450 of GEFT interacts with the other full-length interacting partner. Thus, the data presented here demonstrate that the aa 250-aa 300 region of Bves and the aa 300-450 region of GEFT are necessary but not sufficient for the interaction between these two proteins.

Bves and GEFT co-localize in muscle cells

As both Bves and GEFT are highly expressed in muscle (Andree et al, 2000; Bryan et al, 2005; Smith & Bader, 2006; Souchet et al, 2002), we next examined their localization in mouse hindlimb muscle, the heart and intestinal smooth muscle. As shown in Figure 3.2 (F, K), Bves and GEFT co-localize at the cell membrane in skeletal muscle. Localization is not complete as GEFT staining is also observed in myofibrils (Souchet et al, 2002) (Figure 3.2K). A similar situation is observed in cardiac muscle where intense overlap of staining is observed at the cell surface with additional GEFT reactivity with the contractile apparatus (Figure 3.2J, C). Finally, intestinal smooth muscle also showed the same intensity of staining at the cell surface (Figure 3.2I). These data indicate that Bves and GEFT co-distribute within cells.

Biochemical verification of mBves-mGEFT interaction

Utilizing a GST-pulldown strategy, our laboratory biochemically confirmed the Bves-GEFT interaction revealed by the yeast two-hybrid screen. Prokaryotic GST-fusion protein expression constructs of Bves were generated, while GEFT-

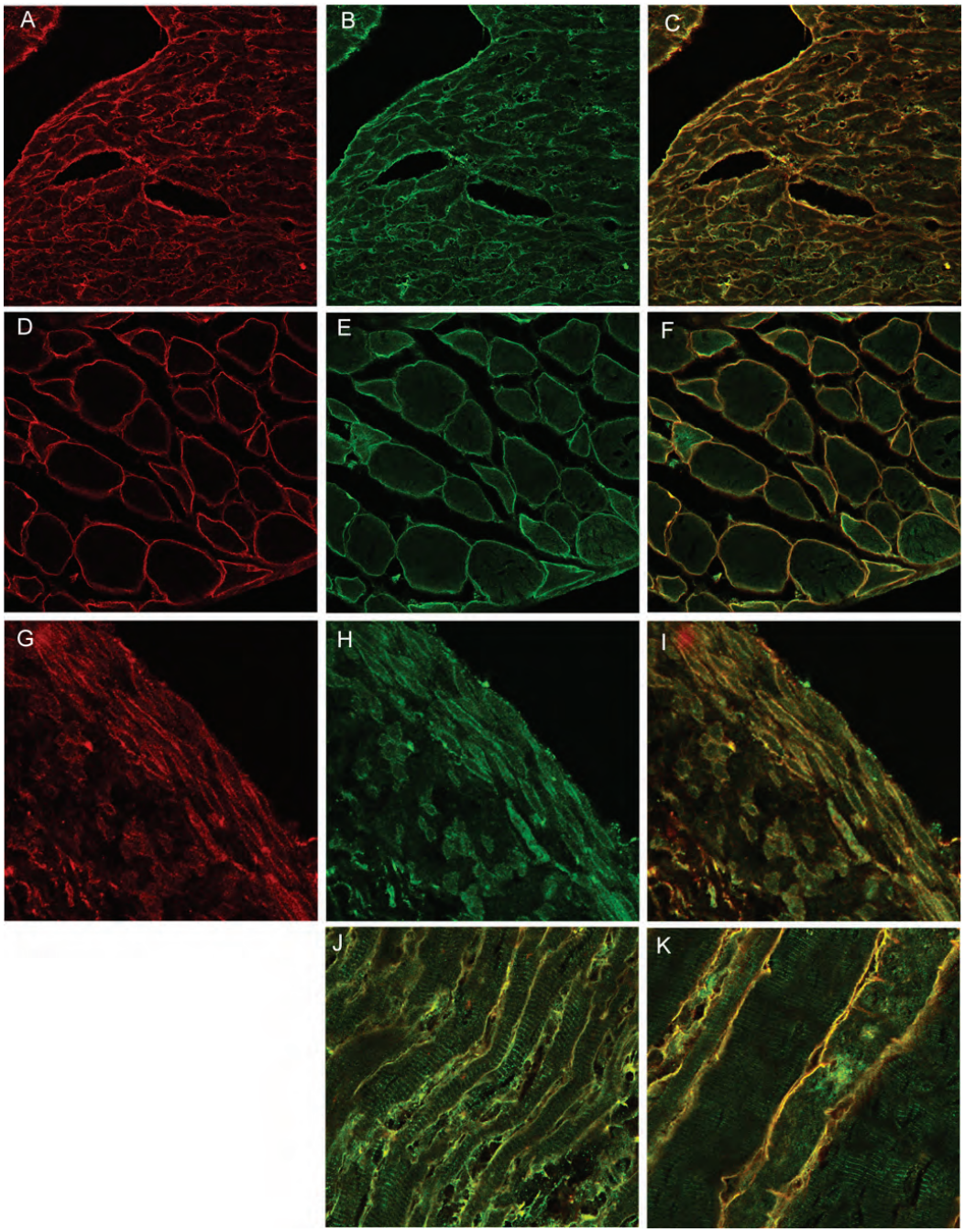


Figure 3.2 Colocalization of Bves and GEFT in cross and transverse sections of mouse cardiac, skeletal, and smooth muscle. Bves, shown in red (A, D, G), is primarily distributed at the cell periphery in cardiac (A-C, J), skeletal (D-F, K), and smooth muscle (G-I). GEFT (B, E, H) also has distribution at the cell membrane in these muscle types, but displays a broader intracellular localization at the myofibrils. Merged images are shown in panels C, F, I, J, K.

GFP fusion protein expression plasmids were generated for use in mammalian cells. COS-7 cells were transfected with the GEFT-GFP expression plasmid, protein was harvested and incubated with GST-mBves Sepharose. Figure 3.3B demonstrates that mBves specifically pulls down GEFT protein while no interaction is detected using GFP-NudeL protein as a negative control. NudeL is a microtubule-binding protein unrelated to Bves function (Li et al, 2005; Liang et al, 2004). It should also be noted that the Dbl homology (DH) domain (Hoffman & Cerione, 2002), which is the domain of GEFT responsible for nucleotide exchange activity with GTPases, falls within the region that we have found to be necessary for GEFT-Bves interaction. This domain is present in all Dbl family members, thus we tested two other members of this family, Vav1 and Cool1 for interaction with Bves using the GST-pulldown method. We were unable to precipitate Vav1 above background (Figure 3.4A). However, we were able to pull down Cool1 above background, but not to the degree that GEFT was reactive (Figure 3.4B). Thus our results indicate that Bves interacts preferentially with mGEFT in this assay and corroborate the yeast two-hybrid analyses.

Exogenous expression of mBves affects activation of Rac and Cdc42

Having demonstrated that mBves interacts with mGEFT, we next sought to determine if mBves expression changes activity levels of the Rac and Cdc42 GTPases. As the PAK-21 protein binds to only activated (GTP-bound) forms of active GTPases (Benard et al, 1999; Chiang et al, 2001), we utilized a PAK-21 pulldown approach to assay for GTPase activity upon transfection of mBves

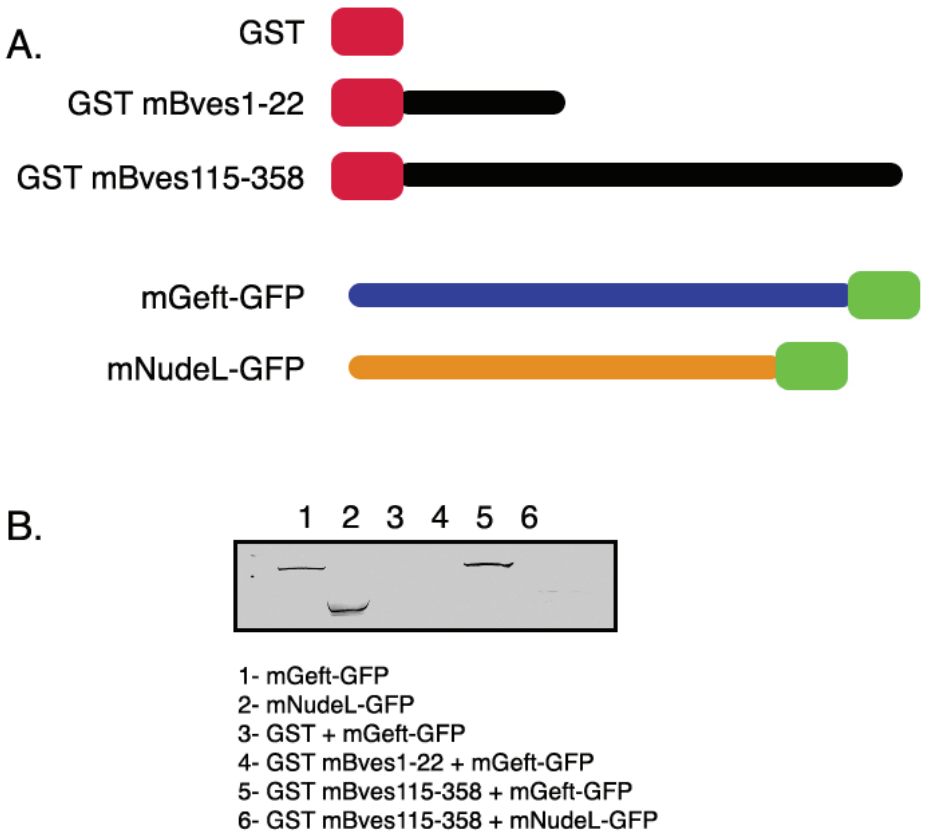
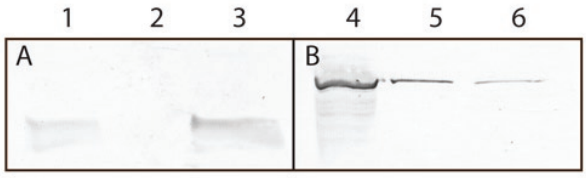


Figure 3.3 GST-Bves pulldown of GEFT. GST-Bves fusion proteins representing the extracellular N-terminus and cytoplasmic C-terminus of Bves were tested for interaction with GEFT-GFP and NudeL-GFP. Representative mobilities of mGEFT-GFP and mNudeL-GFP are provided (lanes 1 and 2). No reactivity is observed in lanes containing isolates from GST/mGEFT-GFP (lane 3), GST-mBves1-22/mGEFT-GFP (lane 4), or GST-mBves 115-358/mNudeL-GFP pulldowns (lane 6), indicating these proteins do not interact. A band representing mGEFT-GFP is clearly seen in the lane containing isolate from the GST-mBves115-358/mGEFT-GFP pulldown (lane 5).



1 - pcDNA3-N-myc-Cool1
2 - GST
3 - pcDNA3-N-myc-Cool1 + GST mBves 115-358
4 - pC.HA-Vav1
5 - GST
6 - pC.HA-Vav1 + GST mBves 115-358

Figure 3.4 GST-Bves pulldown of Dbl family members. GST-Bves fusion proteins, as depicted in Figure 3.3, were tested for interaction with Cool1-myc (A) and Vav1-HA (B). Lanes 1 and 4 represent loading controls, while lanes 2 and 5 indicate interaction with GST protein alone. Cool1 showed no interaction with GST, yet, Cool1 does interact with GST-Bves 115-358. However, this result was inconsistent. Vav1 shows no interaction with GST-Bves 115-358 above background.

constructs. NIH 3T3 cells were transfected with pEGFP-mBvesCT-myc or pEGFP-C3 vector as a control. Lysates were harvested and subjected to PAK-21 pulldown. Amounts of GTP-bound Rac1, Cdc42, and RhoA were determined by SDS-PAGE followed by immunoblotting using published methodologies. Whole cell lysates from each sample were also immunoblotted to verify that similar amounts of protein were used for each pulldown experiment, and each assay was performed in triplicate. As seen in Figure 3.5, transfection of mBves-CT markedly reduces the amount of active Rac1 and Cdc42 while the amount of active RhoA remains unchanged. As GEFT has previously been shown to bind and preferentially activate Rac1 and Cdc42 as opposed to RhoA (Guo et al, 2003), this result is consistent with Bves modulation of Rho-family GTPase activity through an interaction with GEFT.

Bves decreases movement speed of NIH 3T3 cells and increases cell roundness

Having determined that expression of the Bves-CT reduces the amount of active Rac1 and Cdc42 in NIH 3T3 cells, we next sought to determine whether transfection of Bves-CT has an effect on cell motility. Previous studies have determined that reduction of Rac1 and Cdc42 activity results in a decrease in cell movement (Etienne-Manneville & Hall, 2001; Itoh et al, 2002; Kraynov et al, 2000). Time lapse imaging was carried out with cells co-transfected with a GFP marker plasmid together with a plasmid expressing Bves-CT. Parallel transfections with GFP marker plasmid alone were carried out as controls. As summarized in Figure 3.6A, cells expressing Bves-CT showed markedly (~45%)

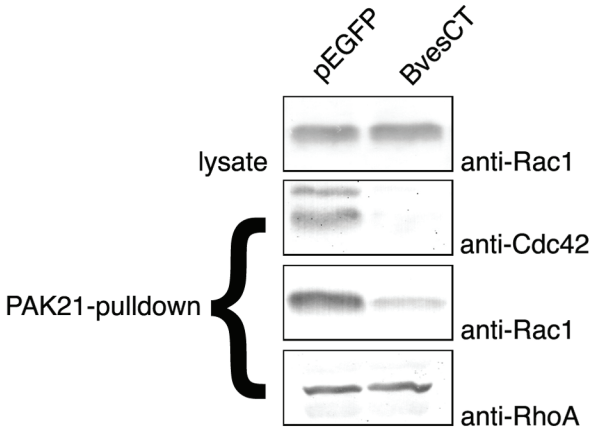


Figure 3.5 Transfection of the carboxyl-terminus of Bves reduces Rac1 and Cdc42 activity in NIH 3T3 cells, but does not affect the amount of active RhoA. Cells were transfected with either pEGFP (control) or pEGFP-BvesCT (amino acids118-358 of mouse Bves). Lysates were harvested, and PAK-21 pulldowns were performed. Samples from pulldowns were loaded and blotted with "-Rac1, "-Cdc42, and "-RhoA antibodies to determine relative amounts of isolated active proteins. Amount of isolated Rac1 and Cdc42 is significantly reduced upon truncated Bves expression, while amount of active RhoA remains unchanged. Cell lysates were loaded and blotted with "-Rac1 to verify equivalent amounts of total Rac1 was present in samples used for assay.

reduced speed of cell locomotion (total path length/time) when compared to control cells transfected with a GFP-only expression plasmid. In contrast, there was no significant change in the directionality of cell movement (net path length/total path length) with the transfection of Bves-CT (data not shown).

As Rac1 and Cdc42 are known to regulate lamellipodial and filopodial cell protrusions (Etienne-Manneville & Hall, 2002), we further examined cell protrusive activity by using time lapse imaging to measure positive and negative cytoplasmic flow, and quantify the overall roundness of the cell. Positive cytoplasmic flow is the net area of cell protrusions, whether from lamellipodial and/or filopodial extensions, while negative cytoplasmic flow represents the net area of cytoplasmic retractions. Our analyses showed no net change in positive or negative cytoplasmic flow, indicating no net change in the overall level of cell protrusive activity. Nevertheless, Bves transfected cells showed an increase in roundness (Figure 3.6B). Roundness, which is calculated from the measured area and perimeter length of a cell, quantifies how efficiently the measured perimeter encompasses the cellular area, with maximum roundness corresponding to a perfect circle. We found that cells expressing exogenous Bves-CT were ~25% more round than cells transfected with a GFP marker plasmid alone (Figure 3.6B).

Subsequent experiments conducted at Vanderbilt by our laboratory further support our findings. Disruption of Bves/GEFT function inhibits or delays the differentiation of skeletal myogenic cells in vitro (Figure 3.7). Taken together,

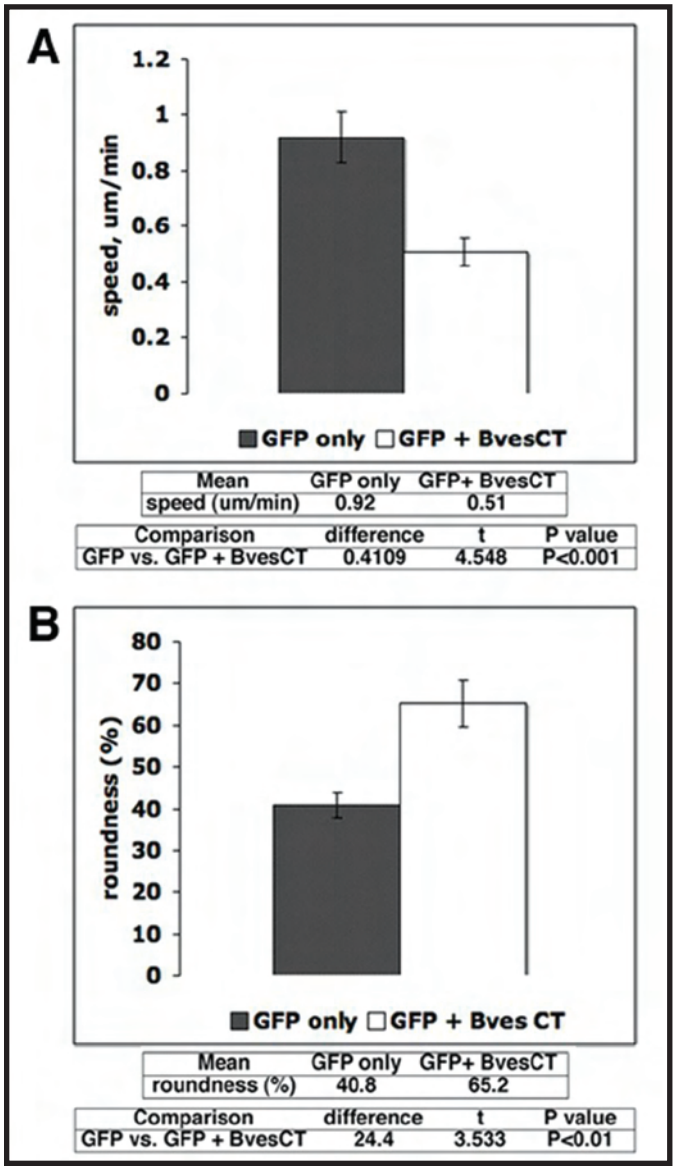


Figure 3.6 Transfection of Bves-CT reduces motility and increases roundness of NIH 3T3 cells. (A) Upon transfection of Bves-CT, a significant reduction in motility speed (white bar) is observed in comparison to cells transfected with a GFP only expressing plasmid (gray bar). (B) Roundness of cells measured in real-time as described in Materials and Methods. Software analysis of cellular area and perimeter allows determination of cell roundness. Upon transfection of Bves-CT, an increase in roundness (white bar) of ~25% is observed in comparison to cells transfected with GFP only (gray bar). Error bars represent SEM, significance determined using standard Student-T test.

these findings demonstrate that exogenous expression of full-length Bves or the cytoplasmic carboxyl-terminus of Bves negatively regulates cell movement.

Discussion

Bves is a protein expressed in a variety of tissue types throughout development. Several phenotypes have been reported in vivo and in vitro when Bves protein levels are decreased, but no molecular mechanism for these observations has been determined to this point. The present data are the first to establish a direct physical interaction with any protein and link Bves to an established molecular pathway.

Upon knockdown of Bves expression in gastrulating *Xenopus laevis*, defects in epithelial morphogenesis and cell movements have been observed (Ripley et al, 2006). Global inactivation of the murine Bves gene leads to defects in skeletal muscle repair by satellite cells (Andree et al, 2002), while knockdown of Bves in cultured epithelia results in defects in wound healing (Ripley et al, 2004). Finally, RNA interference analysis in *Drosophila* inhibits germ cell migration (Lin et al, 2007). The described interaction with a component of the Rac1/Cdc42 signaling pathway may provide the first molecular mechanism to explain these cellular/embryonic phenotypes observed upon alteration of Bves expression levels previously described in the literature.

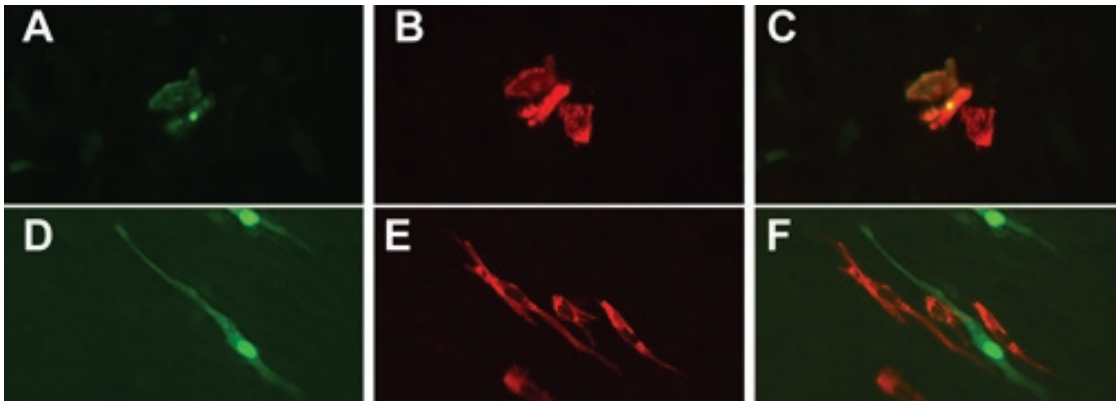


Figure 3.7 Expression of mutated Bves inhibits C2C12 differentiation. Co-transfection of GFP and the C-terminus of Bves (D) is visualized in C2C12 cells. Colocalization with MF20, a marker of myogenic differentiation (E), is not observed (merge, F). Control GFP (A) and MF20 staining (B) is readily observed in the merge (C).

Bves interacts with GEFT, a modulator of Rho GTPase signaling

Bves interacts with GEFT, which has previously been shown to affect cell proliferation, foci formation (Guo et al, 2003), neurite outgrowth (Bryan et al, 2004; Bryan et al, 2006), differentiation, and skeletal muscle regeneration (Bryan et al, 2005); presumably through modulation of the Rho GTPase activity. The motility of cells is controlled by Rho GTPases through regulation of filopodial and lamellipodial extension, as well as polymerization of actin (Etienne-Manneville & Hall, 2002). Here, we show that when a Bves truncation is transfected into NIH 3T3 cells, movement and roundness of these cells is dramatically affected. We also show here that exogenous overexpression of truncated Bves reduces the amount of active Rac and Cdc42 when expressed in NIH 3T3 cells. These results strongly support our hypothesis that Bves modulates the Rac/Cdc42 activity through an interaction with GEFT.

Modulation of Rac/Cdc42 activity by Bves is consistent with observed Bves knockdown/knockout phenotypes

Control of GTPase activity via an interaction with GEFs could provide an explanation for previously observed phenotypes that currently lack mechanistic explanation. Numerous studies have demonstrated the critical role for Rac/Cdc42 during gastrulation and convergent extension of *Xenopus* (Habas et al, 2003; Kwan & Kirschner, 2005; Miyakoshi et al, 2004; Ren et al, 2006a; Tahinci & Symes, 2003). Our laboratory previously described a defect in epithelial migration upon knockdown of Bves expression using morpholino oligonucleotides on developing *Xenopus* embryos. Specifically, Bves depletion results in

randomization of cell movements during convergent/extension. This process is largely dictated by changes in cell shape, as cells radially intercalate, converge towards the midline, and extend towards the blastopore. Cells unable to alter their morphology, remain rounded and fail to undergo gastrulation properly. Perturbation of Rac/Cdc42 activity by Bves knockdown would seem a plausible explanation for this phenotype.

Similarly, previous work has shown that knockdown of Bves expression in cultured corneal epithelial cells disrupted epithelial integrity and delayed healing of epithelial sheets upon wounding via scratch assay. Rac1 and Cdc42 are required for wound healing and epithelial sheet integrity (Fenteany et al, 2000; Kimura et al, 2006; Kofron et al, 2002; Malliri et al, 2004; Nobes, 2000; Stramer et al, 2005; Woolner et al, 2005). Again, the phenotypes observed upon disregulation of normal Bves levels are consistent with a role for Bves in control of Rac/Cdc42 signaling.

Additionally, the Brand laboratory noted that in Bves-null animals, skeletal muscle regeneration is delayed upon injury. Rac/Cdc42 has been shown to affect skeletal muscle regeneration (Bryan et al, 2005; Chen et al, 2003) and regeneration is dependent on process extension and myoblast motility (Carlson, 1973; Carlson & Faulkner, 1983). Inhibition of myogenesis after expression of mutated Bves (Figure 3.7) is consistent with these published findings.

Potential mechanisms of Bves modulation of Rac/Cdc42 activity

The discovery of an interaction between Bves and GEFT leads us to potential models for Bves function. Future investigations by our laboratory will attempt to determine which, if any of these current models represent the actual mechanism through which Bves generates the previously observed phenotypes.

In the first model, Bves would control the nucleotide binding ability of GEFT. As shown previously, Bves preferentially localizes to the plasma membrane (Osler et al, 2005; Smith & Bader, 2006; Wada et al, 2001). GEFT contains a pleckstrin homology domain (PH), which has been demonstrated to localize Dbl family GEFs to the membrane (Russo et al, 2001; Vanni et al, 2002). As demonstrated here, the intracellular carboxyl terminus of Bves interacts with the DH domain of GEFT, which is the portion of GEFT responsible for interaction with the nucleotide-binding pocket of GTPases. This GEF-GTPase interaction leads to a conformational change in the nucleotide-binding pocket of the GTPase, which stimulates GDP release (Rossman et al, 2002). A Bves/GEFT interaction may serve as a negative regulator of GEFT activity, thereby leading to decreased activation of GTPase signaling. Thus, the expression of truncated Bves may lead to aberrant blockage of this active site, causing the experimental results presented here.

Another potential model for Bves regulation of GTPase signaling through GEFT interaction is one in which Bves controls the proper localization of GEFT to active sites of GTPase activity. As Rac and Cdc42 activity have been previously reported to be highest at the leading edges of motile cells (Etienne-Manneville &

Hall, 2002), proper localization of GEF proteins to the plasma membrane in these areas is critical for proper control of cellular motility. Bves may serve to localize GEFT to this leading edge, allowing them to catalyze nucleotide exchange of GTPases. This model is also consistent with the results described here. Exogenous expression of truncated Bves may disrupt this controlled localization, resulting in a decrease in overall GTPase activity.

In summary, we have determined that Bves interacts with GEFT, a member of the Dbl family of GEFs and that exogenous expression of Bves leads to a decrease in active levels of Rac1 and Cdc42. This represents the first direct molecular interaction elucidated for the Bves protein, and provides the first and only current link to a characterized cellular pathway. These results are consistent with previously observed phenotypes and provide a molecular context for future investigation of Bves function.

CHAPTER IV

IDENTIFICATION OF A NOVEL BVES FUNCTION: REGULATION OF VESICULAR TRANSPORT

This chapter was accepted into *EMBO Journal* on November 6th, 2009 and is currently in press.

Abstract

Blood vessel/epicardial substance (Bves) is a transmembrane protein that influences cell adhesion and motility through unknown mechanisms. We have discovered that Bves directly interacts with VAMP3, a SNARE protein that facilitates vesicular transport and specifically recycles transferrin and β-1 integrin. Two independent assays document that cells expressing a mutated form of Bves are severely impaired in the recycling of these molecules, a phenotype consistent with disruption of VAMP3 function. Using Morpholino knockdown in *Xenopus laevis,* we demonstrate that elimination of Bves function specifically inhibits transferrin receptor recycling, and results in gastrulation defects previously reported with impaired integrin-dependent cell movements. Kymographic analysis of Bves-depleted primary and cultured cells reveals severe impairment of cell spreading and adhesion on fibronectin, indicative of disruption of integrin-mediated adhesion. Taken together, these data demonstrate that Bves interacts with VAMP3 and facilitates receptor recycling both *in vitro* and during early

development. Thus, this study establishes a newly identified role for Bves in vesicular transport and reveals a novel, broadly applied mechanism governing SNARE protein function.

Introduction

Vesicular transport is a conserved process where membrane bound vesicles transfer material within the cell and to the cell surface. Protein trafficking and recycling through vesicles is crucial for a myriad of processes including membrane receptor localization and cell motility. Membrane trafficking consists of both the endocytic and exocytic pathways that modulate receptors, ligands, and molecules that are present on the cell surface. While much is known about this process, identification of novel regulators is essential for a comprehensive understanding of the role vesicular transport plays in a broad spectrum of cell functions.

There are four essential steps of membrane trafficking: vesicle budding, transport, tethering, and fusion (Cai et al, 2007; Grosshans et al, 2006). Coat proteins and adaptor proteins select vesicle cargo and facilitate the initial step of vesicle budding (Cai et al, 2007; Mellman & Nelson, 2008). Rab GTPases and motor proteins primarily transport vesicles to the target membrane; however there is accumulating evidence that Rab GTPases participate in every aspect of protein trafficking from budding to docking (Grosshans et al, 2006; Pfeffer, 2007; Segev, 2001). Vesicle tethering is carried out by a diverse set of multi-subunit proteins (Grosshans et al, 2006). The final step in membrane trafficking is carried

out by SNARE proteins, which facilitate fusion of intracellular vesicles to the membrane (Brunger, 2005; Jahn & Scheller, 2006; Leabu, 2006). There are three families of SNARE proteins: vesicle-associated membrane proteins (VAMPs); membrane proteins located on the target membrane (syntaxins); and target membrane localized synaptosomal associated proteins (SNAPs) (Brunger, 2005; Jahn & Scheller, 2006; Leabu, 2006). These proteins interact to form a SNARE complex via their coiled-coil SNARE domains (Brunger, 2005; Leabu, 2006).

Vesicle-associated membrane protein 3 (VAMP3) is a ubiquitously expressed vesicular SNARE protein that binds syntaxin 4 in the basolateral region of epithelial cells to tether vesicular cargo to the membrane (Fields et al, 2007). VAMP3 recycles specific receptors to and from the plasma membrane through the recycling endosome (RE) (Borisovska et al, 2005; Breton et al, 2000; Galli et al, 1994; Polgar et al, 2002). VAMP3 has an established role in the recycling of transferrin and low-density lipoprotein receptor (LDLR), and is required for specific sorting through Adaptor Protein, 1B (Fields et al, 2007; McMahon et al, 1993). Disruption of VAMP3 results in the aberrant localization of both transferrin and LDLR. Overall, VAMP3 is necessary for efficient transport of cargos, which subsequently act to mediate distinct cellular functions. Several studies have shown that VAMP3 is also required for cellular movement through the trafficking of β-1 integrins. Disruption of VAMP3 results in reduced migration rate in wounded epithelial cells and slower cellular spreading on different substrates, as β-1 integrin recycling is impaired (Luftman et al, 2009; Proux-Gillardeaux et al, 2005a; Skalski & Coppolino, 2005; Tayeb et al, 2005). Integrins

stabilize lamellar protrusions through interactions with the ECM, and thus must be trafficked to the leading edge of the cell during migration (Caswell & Norman, 2008; Caswell & Norman, 2006). In development, integrin-based cell motility is imperative for proper morphogenesis. This is evident in gastrulation of *Xenopus laevis* (*X. laevis*), where integrin adhesion to fibronectin (FN) underlies mesodermal migration and subsequent formation of the head and trunk (Ramos & DeSimone, 1996).

Blood vessel/epicardial substance (Bves) is a highly conserved member of the Popdc family of proteins (Hager & Bader, 2009). Bves exists as a dimerized three-pass transmembrane protein with an extracellular glycosylated N-terminus and an intracellular self-associating C-terminal tail (Kawaguchi et al, 2008; Knight et al, 2003). Within the C-terminus is the highly conserved Popeye domain, although no specific function has been linked to this motif (Brand, 2005; Osler et al, 2006). A wide variety of adherent tissues express Bves including all three muscle types and various epithelia (Andree et al, 2000; McCarthy, 2006; Osler & Bader, 2004; Osler et al, 2006; Ripley et al, 2004; Smith & Bader, 2006; Torlopp et al, 2006; Vasavada et al, 2004). Bves localizes to the lateral region of the plasma membrane of epithelial cells, overlapping the distribution of junctional molecules such as E-cadherin, ZO-1, and Occludin (Osler et al, 2005; Osler et al, 2006). In smooth, skeletal, and cardiac muscle Bves is observed around the circumference of cells (Smith et al, 2008). Intracellular punctate distribution is also observed in both polarized monolayers and unpolarized individual cells *in vitro*.

Disruption of Bves leads to a wide range of cell phenotypes in both vertebrates and invertebrates, the majority of which remain poorly understood at the molecular level. The trans-epithelial resistance of polarized epithelial cells is significantly decreased when Bves protein is knocked down, and junctional proteins such as E-cadherin, ZO-1, and β-catenin fail to traffic to points of cell-cell contact (Osler et al, 2005). Although Bves may play a role in localizing or stabilizing proteins at the membrane, the mechanism by which Bves functions in epithelial biogenesis remains completely unknown.

Most recently, Bves has been shown to interact with a Rho GEF, Guanine Nucleotide Exchange Factor T (GEFT) (Smith et al, 2008). GEFT specifically activates Rac1 and Cdc42 to initiate filopodia and lamellipodia extension through rearrangement of the actin cortical network (Bryan et al, 2004; Bryan et al, 2006; Guo et al, 2003). Disruption of Bves function results in increased cell roundness coupled with decreased activity of Rac1 and Cdc42, indicating decreased protrusion extension. Bves disruption also results in decreased cell movement, which is consistent with decreased Rac1 and Cdc42 activity (Smith et al, 2008). However, the exact mechanism by which Bves regulates GEFT remains unexplained.

Finally, Bves has been studied in the context of *X. laevis* gastrulation, where Bves is the only Popdc family member expressed (Hager & Bader, 2009; Ripley et al, 2006). Frog gastrulation is highly dependent upon cellular migration via integrin recycling (Davidson et al, 2006; Marsden & DeSimone, 2001; Ramos & DeSimone, 1996; Ramos et al, 1996), and protein knockdown suggests that

Bves is necessary for cell movement (Ripley et al, 2006). Again, no prior data demonstrate the precise molecular and cellular mechanism underlying Bves function in *X. laevis*.

An emerging theme is that vesicular transport may underlie the essential biological processes in which Bves is involved: cell-cell adhesion, movement, and epithelial biogenesis. However, little is known about the mechanism by which Bves functions in these diverse yet fundamental processes. As inhibition of Bves function disrupts vital membrane functions and possibly vesicular transport, we conducted a split-ubiquitin screen to identify potential protein-protein interactions at the cell membrane. Here we report that Bves interacts with the SNARE protein, VAMP3, and that disruption or depletion of Bves results in impaired VAMP3-mediated vesicular transport. From these data, we hypothesize that Bves influences VAMP3 function to affect multiple cellular behaviors and suggest that a role for Bves in the general process of vesicular transport may explain the varied nature of previously reported phenotypes.

Materials and Methods

For all assays below data was analyzed with Microsoft Excel and error bars indicate standard deviation (SD); student t-tests were standard.

Antibodies, Constructs, Cell Lines, Tissue Processing, and Protein Harvest

VAMP3-GFP and VAMP2-GFP were generous gifts from Dr. W Trimble (University of Toronto) (Bajno et al, 2000). Rescue RNA that is mutated in the

MO binding site was used for *X. laevis* experiments as reported (Ripley et al, 2006). Rat VAMP3 was cloned into pCMV-3tag-4A, and RNA synthesized with mMESSAGE mMACHINE (Ambion). Bves antibodies SB1 and B846 were reported (Smith & Bader, 2006; Wada et al, 2001). All other antibodies and reagents were obtained commercially as follows: Anti-VAMP3 (Novus Biologicals, NB300-510 and Santa Cruz, sc-18208, clone N-12); Anti- β-1-FITC (BD Pharmingen, clone Ha 2/5 555005); Anti-CD29 (BD Transduction Laboratories, clone 18, 610468); Anti-CD29 (BD Transduction Laboratories, clone Ha 2/5, 555003); Anti-8C8 supernatant (*X. laevis* β-1 integrin) (Developmental Studies Hybridoma Bank); Anti-GFP (Clonetech, JL8); Anti-myc (Sigma, M4439 and C3956); Anti-GST (GE Healthcare); and Phalloidin-488 and 568 (Molecular Probes). MDCK cells were obtained from ATCC. Tissue processing and western blotting followed standard protocols (Ripley et al, 2006).

Split-Ubiquitin Screen

A split-ubiquitin screen was conducted by Dualsystems (Zurich, Switzerland). Full-length mouse Bves was cloned into pCCW-Ste and screened against a mouse adult heart library cloned into pDSL-Nx. VAMP3 passed all selection tests.

GST-pulldown and Co-IP

Lysates were prepared as follows: COS-7 cells were transfected with either VAMP3-GFP alone or both VAMP3-GFP and Bves-myc and confluent

monolayers harvested by rocking at 4° for one hour in CHAPS buffer (50mM Tris HCl pH 8.0, 150mM NaCl, 10mM EDTA, 1% CHAPS) plus protease inhibitors (Roche, 11697498001). Lysates were collected and spun down at 18,000 x g for 30 minutes at 4° C. Co-IPs were conducted using Protein G Magnetic Dynabeads and a Dynal MPC Magnet as per manufacturer's instructions (Invitrogen). Bves-GST-Pulldowns of VAMP-GFP proteins were conducted as previously published (Smith et al, 2008); western blots were standard.

Generation of Stable Cell Lines

The extracellular and transmembrane domains of mouse Bves (amino acids 1-118; referred to as Bves118) were cloned in frame into pCMV-3myc-4A (Stratagene). Bves118 was nucleofected into MDCK cells according to manufacturer's specifications (Amaxa). Three individual clones were selected and then maintained in 400µg/mL of G418. RT-PCR and immunofluorescence confirmed the expression of stably transfected tagged proteins (Figure 4.18). Cell lines expressing wildtype or mutant Tetanus toxin, WT TeNT and mut-TeNT, respectively have been described (Proux-Gillardeaux et al, 2005a).

Transferrin Assays

MDCK cells: Uptake of Alexa-633 or Alexa-488 labeled transferrin (Invitrogen, T23362, T13342) was assessed using flow cytometry. Briefly, cells that had been passaged three times were incubated in DMEM and 0.2% BSA for 2 hours at 37° C and then incubated for 30 minutes with 50ug/mL of labeled

transferrin at 4° C in the dark. Cells were allowed to internalize labeled transferrin at 37°C for the indicated times, and then washed 4X with ice cold PBS on ice. Cells were probed for Mean Fluorescence Intensity (MFI) using a BD FACS Canto II; data were acquired with Diva 6.0, analyzed with WinList, and are reported as the average MFI from four different experiments.

Animal Caps: Internalization of labeled transferrin-633 (Invitrogen T23362) in animal caps was measured in a T-format spectrofluorometer (PTI Quantamaster 2000-7SE). Animal caps were dissected at stage 9-10 in 1X Modified Barth's saline (MBS) and 0.1% BSA and then serum starved in agar-coated dishes for 2 hours in 1X MBS. Caps were incubated with 50µg/mL of transferrin-633 for 30 minutes at 4° C in the dark, and then allowed to uptake transferrin-633 for 25 minutes (first five minutes at 37° C; last 20 minutes at room temperature with gentle agitation). Caps were washed 5X with cold 1X MBS on ice, briefly spun down at 8,000 x g at 4° C, and then 4 caps/well were solubilized by vortexing for 15 seconds in 0.2 mL of 0.25% Triton X-100 in PBS. Protein was harvested by centrifuging at 15,000 x g for 5 minutes, and the supernatant was analyzed for fluorescence (excitation = 488; emission = 633). Protein concentration of the animal caps was determined using a BCA assay (Thermo Scientific 23227) and fluorescent units/µg of protein determined. For side by side comparison of control and experimental models, Bves MO, Bves MOR, VAMP3 MO and VAMP3 MOR data are reported as a percentage of the control (COMO).

Scratch Assay

A scratch assay was performed and scored for the amount of internalized β-1 integrin according to Proux-Gillardeaux et al. For scratch assays that directly compared TeNT cells and Bves118 cells, CD29 was conjugated to Alexa-564 (Molecular Probes) and conducted in triplicate.

Cell Spreading Assay in MDCK Cells

Cell spreading was defined as the increase of cell area over time prior to polarized cell movement. One day before cell spreading analysis, cells were plated at single cell density so that they would be contact naïve at the time of the assay. On the day of image acquisition, cells were trypsinized and plated at single cell density on MatTek dishes (MatTek Corporation) coated with 25μg/mL of FN Sigma (F4759). Attached yet rounded cells were chosen 45 minutes after plating and DIC images acquired with a temperature and CO_2-controlled WeatherStation as part of a DeltaVision platform (Precision Control). Images were obtained with an Olympus IX71 inverted microscope and a CoolSNAP-HQ2 CCD camera using the 40X objective at intervals of one minute for one hour. Image deconvolution was carried out with the SoftWorx software. Metamorph 6.0 was used to determine cell area and construct kymographs from DIC images every tenth minute.

X. laevis Embryos

Female *X. laevis* were obtained from Nasco, primed, and fertilized by standard methods (Ripley et al, 2006). Images of appropriately staged embryos (Nieuwkoop & Faber, 1994) were captured with Magnafire (Olympus America Diagnostics).

Microinjection and Morpholino Treatment

Embryos were microinjected with 5nL into both cells at stage 2; embryos were injected in 5% Ficoll in 1X Steinberg's Solution (SS), then switched to 0.1X SS before gastrulation. Bves MO, VAMP3 MO, or COMO were injected into sister embryos along with mGFP or mRFP (1.5ng) as a tracer at a concentration of 20ng (stage 35-42 analysis only) or 40ng/embryo (Gene Tools, LLC) (Ripley et al, 2006; Wallingford et al, 2000). For transferrin assays, 100pg of Rescue RNA (described above) was co-injected along with Bves MO or VAMP3 MO. Xbves Rescue RNA has been reported previously (Ripley et al, 2006) and is mutated in the MO binding sequence, thus not recognized by Bves MO. The most successful MO for knockdown of *X. laevis* VAMP3 was designed against the 5' UTR, approximately 20 base pairs upstream from the ATG site: GGACACCGGTCCGACTTTACTC (Gene Tools, LLC). Note this sequence is perfectly conserved with *Xenopus tropicalis*, but has no conservation with rat VAMP3 (EST databases).

SEM

Embryos were fixed and processed for SEM with standard methods and HM was dissected out (the overlying BCR was peeled away from this region to expose cells that are attached to FN) with eyebrow knives. SEM images were quantified as follows: cells were chosen from random fields from ten different embryos and measured for cellular overlap and polarity using the leading edge as a reference point. Overlap was defined as the number of overlapping cell bodies or lamellipodia for each selected cell. Polarity was determined by defining the length/width axis (longest axis of the cell intersected perpendicularly by the widest part of the cell), measuring the deviation of this axis from a set point, and then averaging the standard deviations from the defined point.

X. laevis Microdissections, Adhesion Assays, and Spreading Assays

Microdissections of *X. laevis* embryos were carried out according to (Ren et al, 2006b). Explants were disassociated in Ca^{2}+ Mg^{2}+ free MBS and single cells were plated in MBS on slides (for adhesion assays; LabTek) or on Mat Tek dishes (for spreading assays) coated with 200µg/mL of FN (Sigma F4759). Explants from several embryos (control or experimental) were pooled and plated for either adhesion or spreading assays.

Adhesion assays on FN were conducted and scored as described (Ramos & DeSimone, 1996). Cells were cultured for two hours, washed three times in MBS to remove non-adhered cells, fixed in PFA overnight at 4° C, labeled with Phalloidin, and imaged with a Zeiss Inverted LSM 510 Confocal Microscope

using a 40X objective. Round cells are attached to the plate and spherical, while spread cells are elongated and had two or more lamellipodia as previously defined (Ramos & DeSimone, 1996).

For spreading analysis, time-lapse images were obtained with the DeltaVision platform. Movies of mGFP or mRFP labeled cells were imaged over a 35 minute timeframe with fluorescent and DIC images being collected every minute. The point of cell/matrix interaction was used as the focal point in obtaining these images. Quantification of lamellipodia and cell spreading are as follows: for at least 12 different cells, the average number of lamellipodia (defined here as 10 μm extensions devoid of yolk granules with distinct matrix attachment sites) was determined for ten time points at three minute intervals over the period of culture for both control and experimental groups. Metamorph 6.0 was used to analyze every third frame to determine the cell area and construct kymographs.

Results

Bves interacts with VAMP3

Given the protein distribution of Bves, and previously reported phenotypes, we conducted a split-ubiquitin screen to detect Bves-interacting membrane proteins with characterized functions in cell movement (Dunnwald et al, 1999). VAMP3, a SNARE protein that facilitates the fusion of apposing membranes during vesicular transport (McMahon et al, 1993), was identified as a binding partner in this screen. Importantly, VAMP3 transports membrane

proteins, and is required for the vesicular transport underlying cell motility (Galli et al, 1994; Proux-Gillardeaux et al, 2005a; Skalski & Coppolino, 2005; Tayeb et al, 2005). As Bves is also required for cell motility, and VAMP3 has a known function in this process, we chose to probe this interaction further.

To confirm this result, we determined if Bves and VAMP3 proteins interact biochemically using co-immunoprecipitation (co-IP) and GST-pulldown assays. As seen in Figure 4.1A, VAMP3 was precipitated with Bves. Additionally, GST-pulldown assays demonstrated a specific interaction between Bves and VAMP3, localized to the intracellular C-terminal Popeye domain (Figure 4.1B). Interestingly, Bves also interacts with VAMP2 via GST-pulldown (Figure 4.2) VAMP2 and VAMP3 are highly homologous, with only one amino acid difference in their SNARE binding domain (McMahon et al, 1993), suggesting a conserved interaction between Bves and SNARE proteins. Taken together, these data confirm the direct interaction of Bves through its cytoplasmic tail with VAMP3.

Bves and VAMP3 co-localize

Bves and VAMP3 exhibit similar dynamic distributions that are both at the cell periphery and in intracellular compartments (Hager & Bader, 2009; Osler et al, 2005). Co-localization in Madin-Darby Canine Kidney (MDCK) cells of endogenous Bves (Figure 4.3A) and exogenously expressed VAMP3-GFP (Figure 4.3B) is readily observed both at the cell periphery and in intracellular vesicles (merge in Figure 4.3C, arrows). While overlap is extensive, it is not complete, as some intracellular vesicles labeled with Bves are not co-labeled

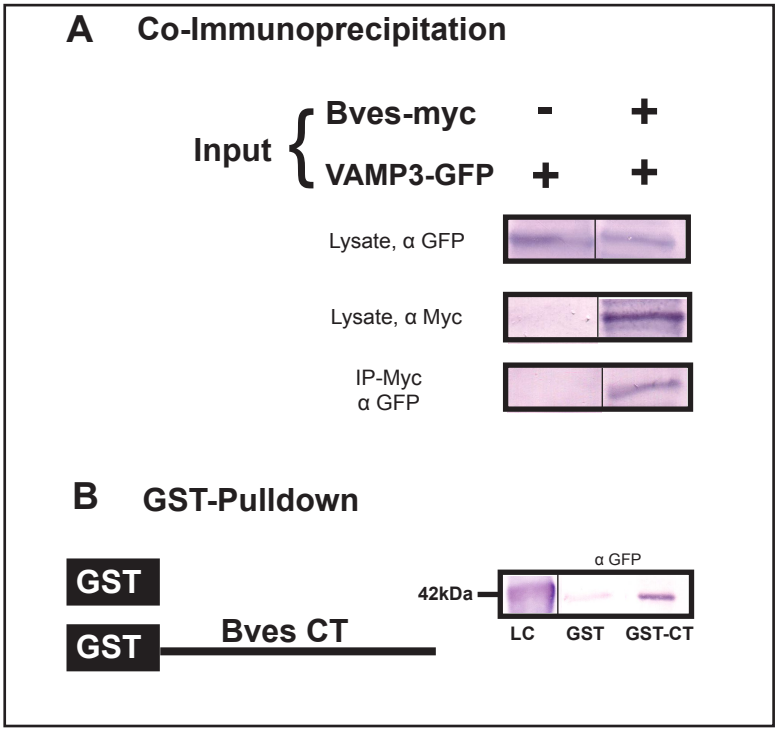


Figure 4.1 Bves and VAMP3 interact. For Co-IP (A), COS-7 cells were transfected with tagged proteins: VAMP3-GFP alone, or VAMP3-GFP and full length Bves-myc. Cell lysates were pulled down with myc and blotted for GFP. In GST pulldown assays (B), the C-terminus of Bves fused to GST (GST-CT) was sufficient to pulldown transfected VAMP3-GFP from COS-7 cell lysate. Loading controls (lysate in A and LC in B) are shown for comparison.

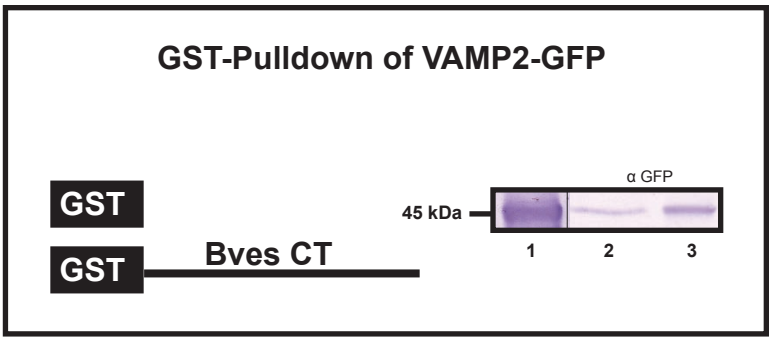


Figure 4.2 GST pulldown demonstrates Bves and VAMP2-GFP interact biochemically. Loading control (Lane 1) detects the mobility of VAMP2 by immunoblotting. GST alone does not interact with VAMP2 above background (Lane 2). The C-terminus of Bves fused to GST was sufficient to pulldown transfected VAMP2-GFP from COS-7 cell lysate (Lane 3), demonstrating this intracellular portion of the protein binds VAMP2-GFP, in addition to VAMP3-GFP. Lane 1. VAMP2-GFP Loading Control ; Lane 2. GST/VAMP2-GFP; Lane 3. GST-CT/VAMP2-GFP.

with VAMP3. Examining endogenous localization in confluent epithelial sheets, Bves (Figure 4.3D) and VAMP3 (Figure 4.3E) co-localized in the lateral portion of MDCK cells and staining is also observed in intracellular vesicles (merge in Figure 4.3F, arrows). These data confirm that overlap of these two proteins is consistent with their previously reported endogenous distribution profile (Fields et al, 2007; Osler et al, 2005). Disruption of VAMP3 function (detailed below) resulted in a significant decrease in the presence of Bves at the cell membrane (Figure 4.4) while expression of a truncated Bves lacking the VAMP3 binding domain (also detailed below) produced only minor changes in protein distribution.

While VAMP3 is ubiquitously expressed across all non-neuronal tissue types, Bves is present at high levels in muscle as well as in other adherent or excitable tissues (Hager & Bader, 2009; McMahon et al, 1993). Thus, we characterized endogenous protein distribution in mouse heart and skeletal muscle to probe for co-localization. Significant co-localization was observed in both muscle types, although overlap was not absolute (Figure 4.5). Co-localization was seen primarily at the circumference of the myocytes, with certain muscle cells demonstrating more intense labeling (Figure 4.5C, G, white arrows). Intensity profiles (Figure 4.5D, H) of both Bves and VAMP3 signal demonstrate the high degree of co-localization (Figure 4.5C, G; red arrows indicate area of intensity profile). As Bves and VAMP3 both interact and co-localize, we next focused on functional assays to determine the potential importance of this interaction.

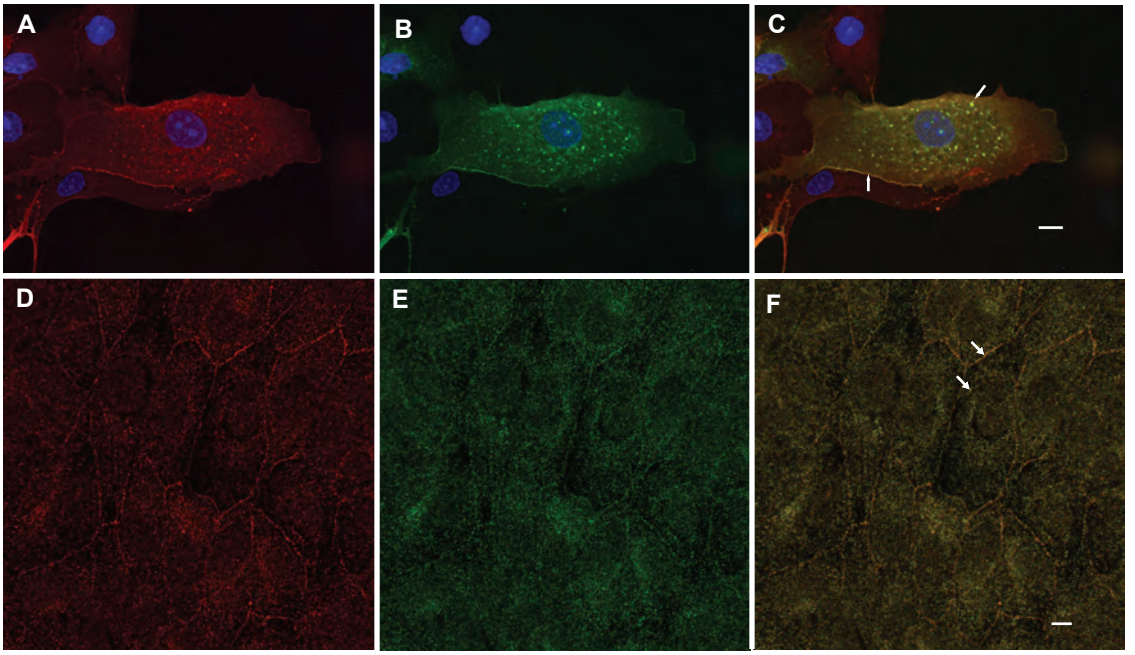


Figure 4.3 Bves and VAMP3 co-localize in MDCK cells. Both endogenous Bves (A) and transfected VAMP3-GFP (B) are observed at the membrane and in vesicles. The endogenous distribution of both proteins also demonstrates this same localization pattern (Bves, D; VAMP3, E), and Bves and VAMP3 co-localize at both of these subcellular locations (arrows, C, F). Scale bars are 5μm.

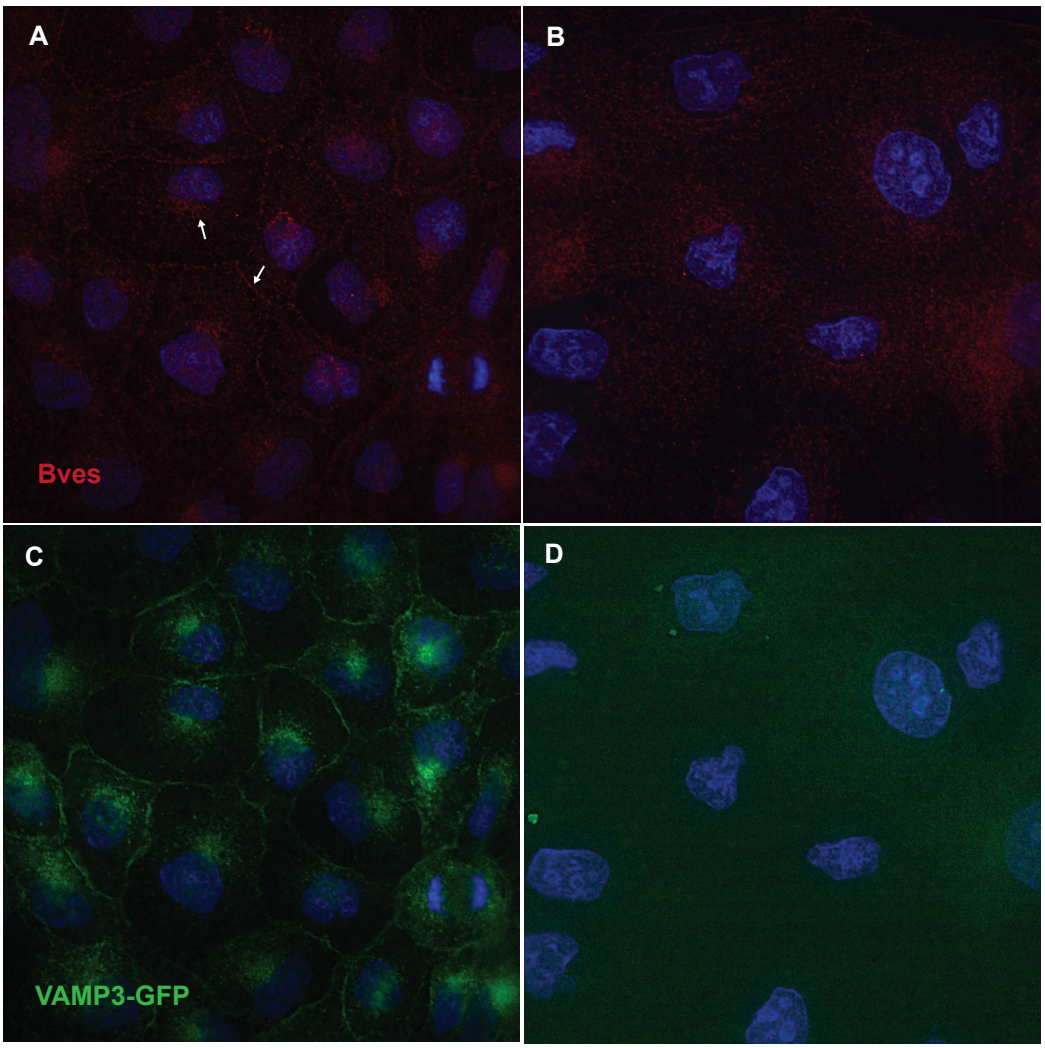


Figure 4.4 Bves localization in TeNT cell lines. In mut-TeNT cells, which express VAMP3-GFP (C), Bves (red) localization is seen intracellularly and at the membrane in confluent epithelial sheets (A, arrows). However, when VAMP3-GFP is cleaved by wildtype Tetanus toxin (D), Bves localization is reduced at the membrane, although intracellular labeling is still visible (B).

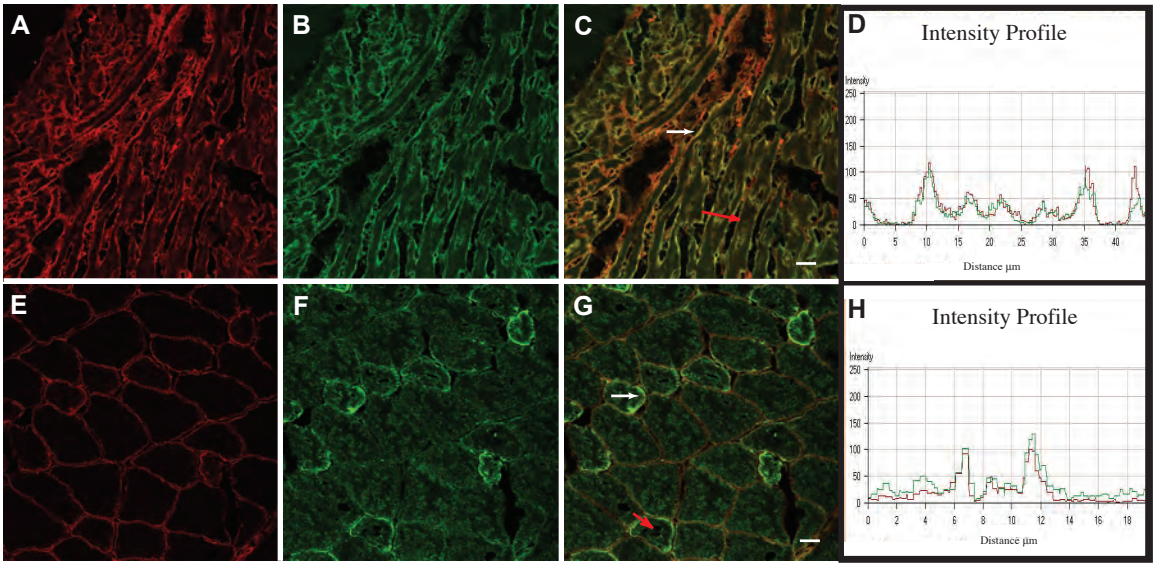


Figure 4.5 Endogenous Bves and VAMP3 co-localize in muscle. Bves (A, E) and VAMP3 (B, F) are seen at the cell periphery in adult cardiac (A-C) and skeletal muscle (E-G). Areas of intense co-localization are denoted by the white arrows (C, G). Red arrows indicate the area of the fluorescent intensity profile for cardiac (D) and skeletal muscle (H). Scale bars are 20µm.

Transferrin recycling is attenuated in cells with disrupted Bves function

VAMP3 is required for recycling of transferrin, as cleavage of VAMP3 disrupts vesicular transport of this receptor (Galli et al, 1994; McMahon et al, 1993). To identify the potential role of Bves in VAMP3-dependent recycling using a standard transferrin uptake assay, we developed an MDCK cell line that stably expresses only the first 118 amino acids of Bves (Bves118). Bves118 lacks the intracellular VAMP3-binding domain and contains only the short extracellular and transmembrane domains. Transferrin endocytosis was analyzed at 5, 10, and 20 minutes (Figure 4.6A), and the mean fluorescence intensity (MFI) was analyzed in MDCK and Bves118 cells. At 5 minutes internalization, average MFI (which directly correlates to the amount of endocytosed transferrin) for MDCK cells was 46.53, whereas Bves118 cells had an average MFI of 16.41, demonstrating decreased uptake of labeled transferrin. This trend continued at 10 and 20 minutes internalization with Bves118 cells having severely decreased internalization of labeled transferrin relative to MDCK cells (5 minutes: $p < 0.001$; 10 minutes: $p < 0.003$; 20 minutes: $p < 0.0036$). Disruption of transferrin kinetics was also demonstrated by a recycling assay, as labeled transferrin was exocytosed from Bves118 cells more slowly compared to controls (Figure 4.7). This phenotype is consistent with disrupted VAMP3 function and supports the hypothesis that Bves-VAMP3 interaction is necessary for VAMP3-mediated vesicular transport.

To corroborate and extend these studies with an *in vivo* model and directly compare the effects of transferrin recycling between Bves- and VAMP3-depleted

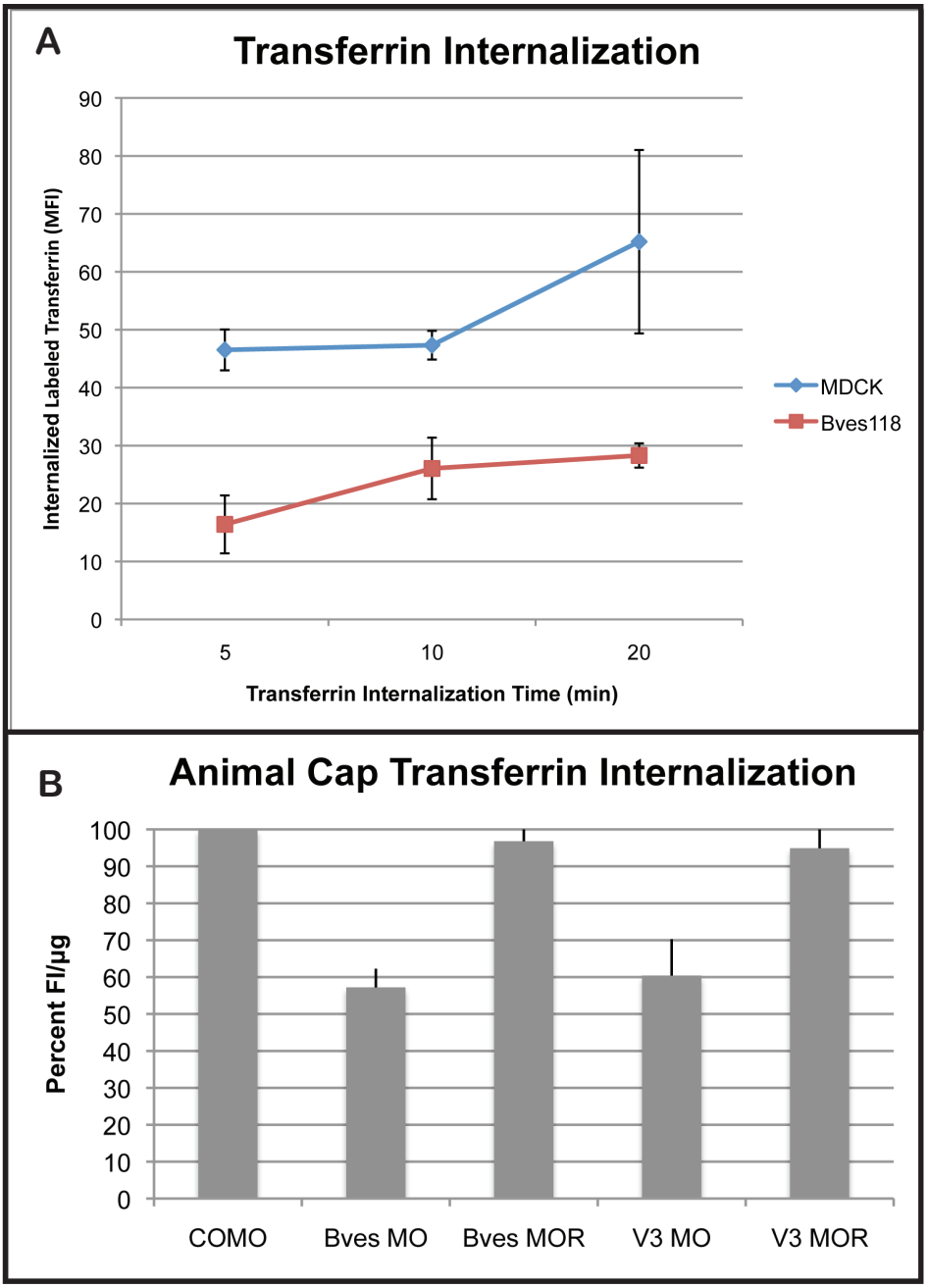


Figure 4.6 Transferrin uptake is attenuated when Bves is disrupted. (A) MDCK and Bves118 cells internalized labeled transferrin for 5, 10, or 20 minutes. Transferrin uptake, as measured by the MFI, was significantly decreased in Bves118 cells at all time points. (B) When normalized with COMO values (100%), Bves MO and VAMP3 (V3) MO treated caps were impaired in internalization of labeled transferrin/µg of total protein. Transferrin uptake is restored when Bves MO or V3 MO are co-injected with rescue Bves (Bves MOR) or VAMP3 (V3 MOR) RNAs, demonstrating that this phenotype is specific to depletion of the respective proteins.

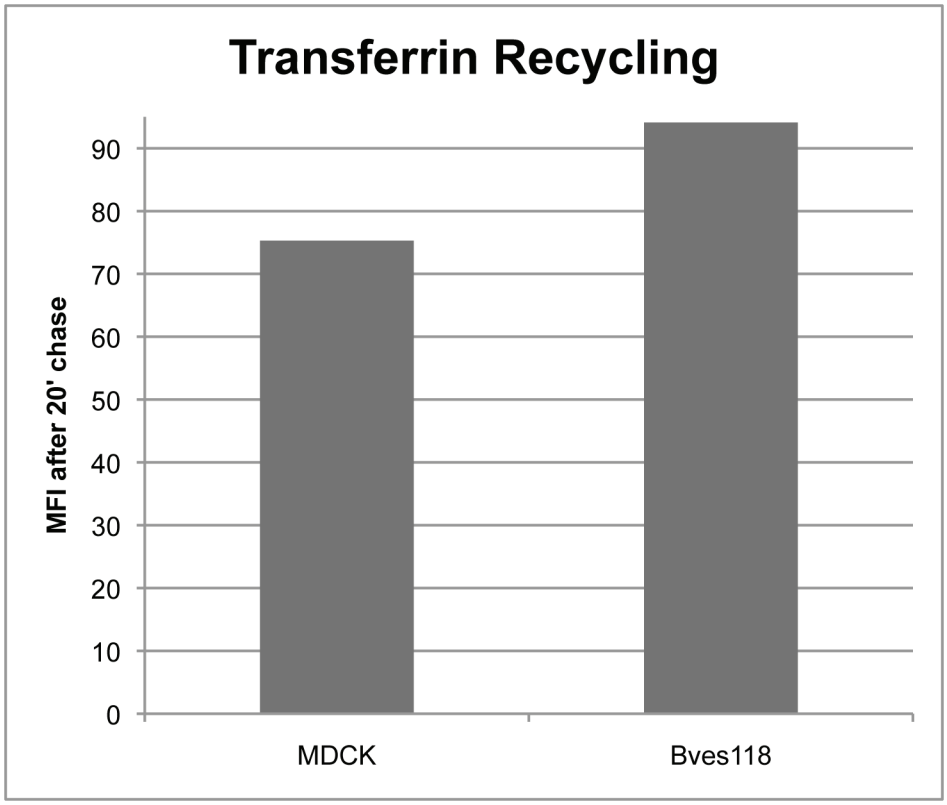


Figure 4.7 Transferrin recycling in Bves118 cells. MDCK or Bves118 cells were serum starved for three hours in DMEM plus 0.2% BSA at 37° C, and then allowed to uptake Transferrin-633 (25µg/mL) for 60 minutes at 37° C. Cells were then washed 4X on ice with cold PBS with 0.1% BSA and incubated in DMEM plus 0.2% BSA with 5mg/mL of unlabeled APO transferrin for 20 minutes at 37° C. After the twenty minute chase with unlabeled transferrin, cells were probed for MFI using a MACSQuant flow cytometer (Miltenyi Biotech). It was found that Bves118 cells had a higher MFI (94.1±33) than MDCK cells 75.3±28, indicating that exocytosis of transferrin, a process mediated by VAMP3, was impaired in cells expressing mutated Bves.

embryos, we used a Morpholino (MO) knockdown and rescue strategy in *X. laevis* (Ripley et al, 2006). Embryos were injected with Bves MO, VAMP3 MO, or Control MO (COMO); alternatively, sister embryos were co-injected with Bves MO or VAMP3 MO and their respective rescue RNAs (Bves MOR, VAMP3 MOR). Isolated animal caps, which express the transferrin receptor (NCBI, EST databases), were allowed to endocytose transferrin-633 for 25 minutes and the fluorescent intensity (FI)/µg of animal cap protein was determined. As seen in Figure 4.6B, recycling of labeled transferrin in Bves MO and VAMP3 MO treated animal caps is severely reduced relative to COMO treated caps. When normalized against COMO treated caps (100%), Bves MO treated animal caps display only 57.2±5.06% of FI/µg, demonstrating that recycling of labeled transferrin is inhibited when Bves is depleted (Table 1). Similarly, VAMP3 MO treated caps internalize 60.4±9.9% of labeled transferrin relative to COMO (Table 1). This reduction in recycling of labeled transferrin in animal caps is completely dependent upon knockdown of Bves or VAMP3, as these phenotypes are rescued in caps co-injected with Bves rescue RNA along with Bves MO, or VAMP3 rescue RNA along with VAMP3 MO (Bves MOR: 96.8±4.57% and VAMP3 MOR: 94.9±12.14% of FI/µg relative to COMO treated caps; Table 1). Taken together, these two independent methods demonstrate that recycling of transferrin is attenuated after Bves disruption, suggesting that VAMP3-mediated transport is dependent on Bves function.

Table 1

Figure 4.6

Transferrin Internalization in Animal Caps

% FI/µg of COMO

COMO	Bves MO	Bves MOR	VAMP3 MO	VAMP3 MOR
100	57.2±5.06	96.8±4.57	60.4±9.9	94.9±12.14
P Value < 0.0001	P Value < 0.2111		P Value < 0.0002	P Value <0.4340

VAMP3-mediated recycling of β-1 integrin is impaired in cells expressing mutated Bves

VAMP3 function is necessary for the recycling of β-1 integrin during cell movement (Luftman et al, 2009; Proux-Gillardeaux et al, 2005a; Skalski & Coppolino, 2005; Tayeb et al, 2005). Proux-Gillardeaux et al. have reported an *in vitro* scratch assay that directly tests VAMP3-mediated recycling of β-1 integrins by quantifying its recycling over time; we adapted this method by using β-1 integrin labeled with FITC. In wildtype MDCK cells, 59.6 ±5% of cells at the free edge of the wound were positive for labeled integrin (Figure 4.8A-C; Table 2). Bves118 cells showed a dramatic decrease in endocytosed FITC-labeled integrins (Figure 4.8D-F). Note the limited number of Bves118 cells with internalized FITC-labeled integrin (35.5±5%) as compared to wildtype MDCK cells (Figure 4.8G; Table 2, $p < 0.0001$) even though β-1 integrin protein levels remain the same (Figure 4.8H). To confirm that disruption of Bves does in fact phenocopy disruption of VAMP3, we repeated this assay using β-1 integrin antibody (CD29, clone Ha 2/5) conjugated to Alexa-564, and directly compared cells expressing wildtype Tetanus toxin (WT-TeNT, which effectively cleaves VAMP3, rendering it unable to transport integrin) with Bves118 cells. Using the exact cell line in which disrupted integrin recycling upon VAMP3 knockdown was first reported (Proux-Gillardeaux et al, 2005a), along with the control cell line that expresses mutant inactive Tetanus neurotoxin (mut-TeNT), we determined that in a side-by-side comparison, 37.8±7 % of Bves118 cells and 40.8±2% WT TeNT cells internalized labeled integrins, while 62.4±16% of mut-TeNT cells were positive for integrin internalization (Figure 4.8G; Figure 4.9). Additionally,

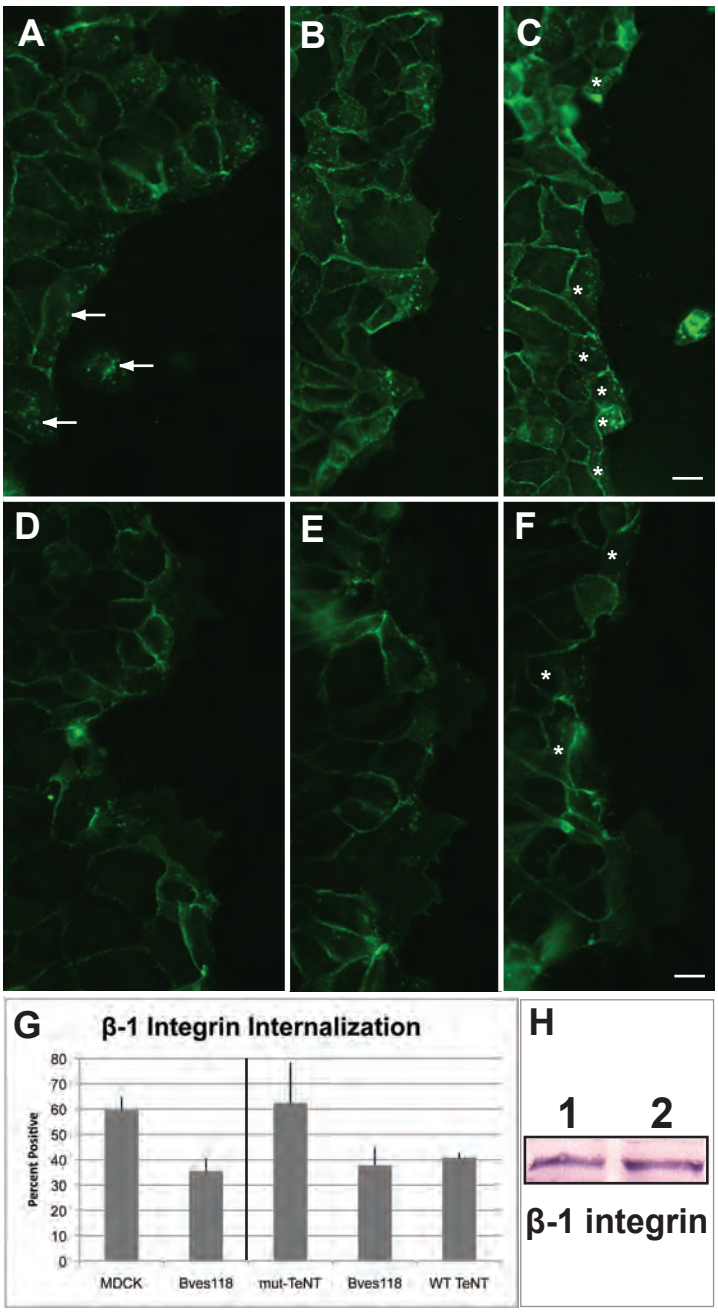


Figure 4.8 Cells stably expressing mutated Bves have decreased integrin recycling. A wounded monolayer of MDCK cells (A-C) internalized FITC labeled β-1 integrin antibody (panel A, arrows: intracellular labeling) as cells migrated to close the wound. β-1 integrin recycling is visualized by the presence of FITC-labeled protein in intracellular compartments. In Bves 118 cells, integrin recycling was attenuated (D-F), as seen by decreased intracellular punctate labeling, although integrin expression levels of Bves118 cells are consistent with MDCK cells (H). This decrease in integrin internalization is also seen when directly compared to WT TeNT and mut-TeNT cells (G; SI Fig 6). Cells marked with an asterisk (C, F), were counted as integrin positive in quantification (G, Table II). Scale bars are 20μm.

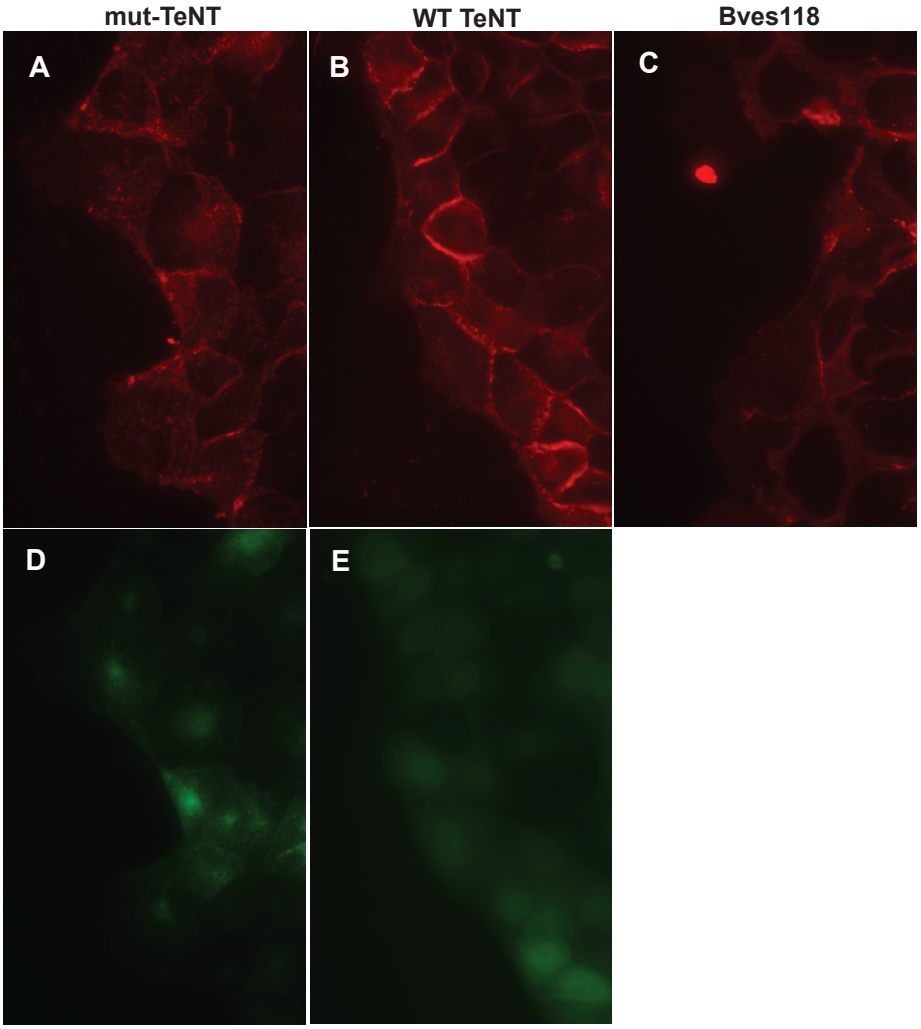


Figure 4.9 Integrin internalization in cells expressing mutated Bves and VAMP3. As demonstrated in Figure 4.8, Bves118 cells have decreased internalization of β-1 integrin-FITC when induced to migrate. To verify this result, we directly compared Bves118 cells uptake of β-1 integrin-Alexa-564 with cells without VAMP3. As Tetanus toxin selectively cleaves VAMP1-3 (McMahon et al., 2003), Proux-Gillardeaux et al., 2005 utilized this property to create cell lines without VAMP3, which we used here. In our hands, 62.4±16% of MDCK cells expressing VAMP3-GFP and mutated and inactive form Tetanus toxin (mut-TeNT were positive for internalized β-1 integrin; visualized by the punctate intracellular labeling of VAMP3-GFP, panel D). Note that the mutated toxin serves as a positive control as VAMP3 remains intact. In contrast, MDCK cells expressing VAMP3-GFP and wild type Tetanus toxin (WT TeNT) display a diffuse intracellular labeling due to the of cleavage of VAMP3-GFP (panel E). When expressing the WT TeNT, only 40.8±2% of cells were positive for internalized β-1 integrin (B). This decrease in integrin internalization was reported previously in Proux-Gillardeaux et al., 2005. In a side-by-side comparison, 37.8±7% of Bves118 cells were positive for intracellular integrin labeling (panel C), demonstrating that β-1 integrin internalization is attenuated in Bves118 cells, with similar internalization rates as cells without VAMP3.

Table 2

Figure 4.8

β-1 Integrin Recycling

	MDCK	Bves118
% Positive	59.6±5	35.5 ±5
Total Cells	1955	2597
		P Value < 0.0001

	mut-TeNT	Bves118	WT TeNT
% Positive	62.4±16	37.8±7	40.8±2
Total Cells	733	768	816
		P Value < 0.004	P Value < 0.008

internalized β-1 integrin co-localized with both Bves and VAMP3 antibodies (Figure 4.10), supporting a role for Bves and VAMP3 in recycling of integrins. These data demonstrate that disruption of Bves and VAMP3 result in similar phenotypes and further supports the hypothesis that intact Bves function is required for proper VAMP3-mediated recycling of different molecules.

Expression of mutated Bves or TeNT disrupts cell spreading

As Bves118 and WT TeNT cells have impaired integrin uptake during cell movement, we determined if another integrin dependent function, cell spreading, was also disrupted. Cells were plated on FN at single cell density and allowed to adhere for 45 minutes prior to time-lapse analysis. At time 0, all four cell types, MDCK (Figure 4.11A), Bves118 (Figure 4.11C), mut-TeNT (Figure 4.11E), and WT TeNT (Figure 4.11G) displayed similar areas and it was evident that cell protrusions were beginning to form. However, after one hour of image acquisition, MDCK and mut-TeNT cells (Figure 4.11A, E; Time 60) greatly increased cellular areas (Figure 4.11J, Table 3), 73% and 87% respectively, while Bves118 and WT TeNT cells (Figure 4.11C, G; Time 60) only increased cell areas by 16% and 13%, respectively (Figure 4.11J; Table 3). Kymographs of cell spreading over time reveal significant differences in cell spreading between MDCK cells (Figure 4.11B) and Bves118 cells (Figure 4.11D); as well as between mut-TeNT cells (Figure 4.11F) and WT TeNT cells (Figure 4.11H; Table 3). It should be noted that in a direct comparison of the area of cell spreading (Figure 4.11I), both experimental cell lines have similar areas of cell spreading

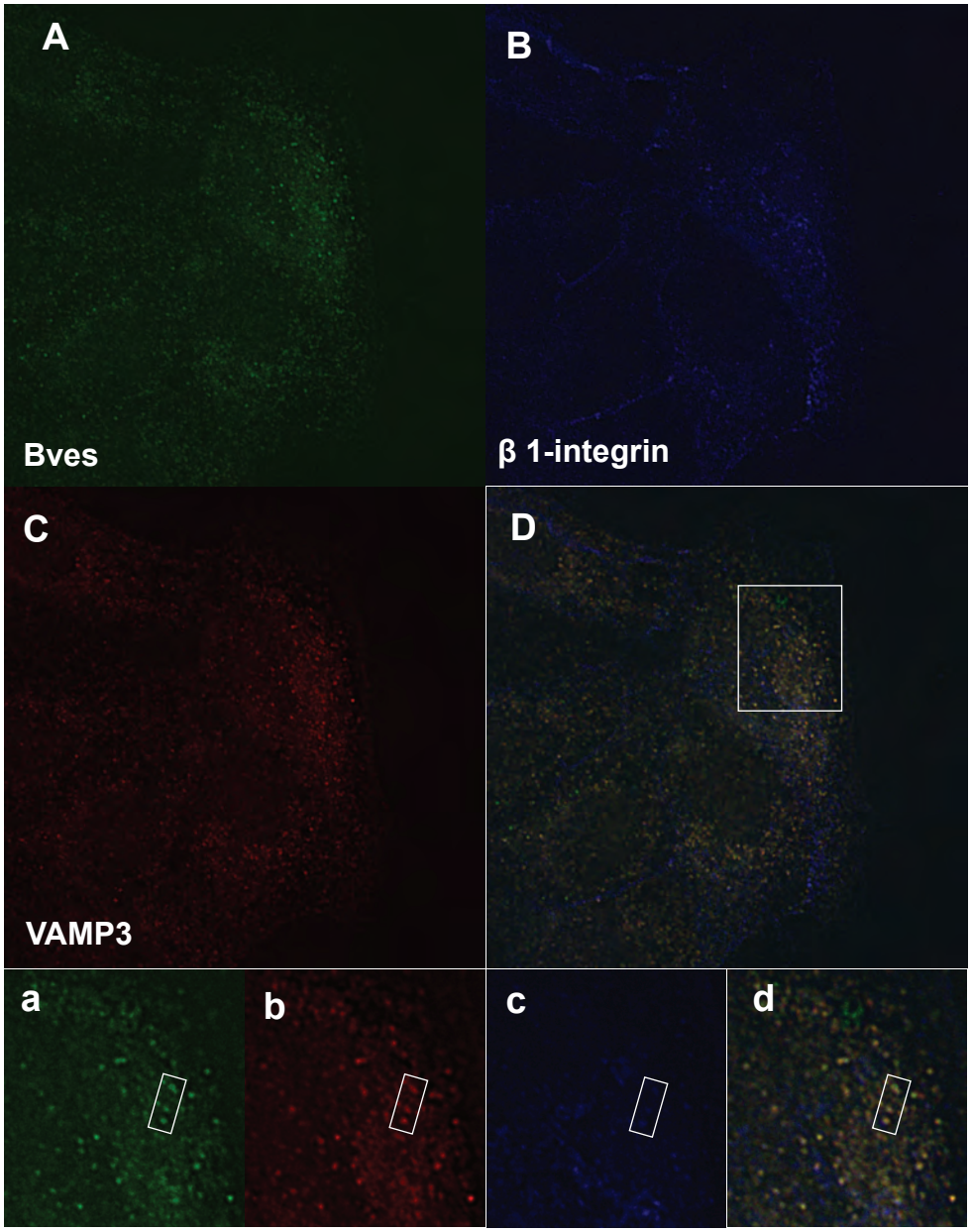


Figure 4.10 Co-localization of Bves, VAMP3, and β-1 integrin. Confluent MDCK cells were wounded via scratch and allowed to internalize β-1 integrin-FITC (B) as described in the methods. Antibody labeling of Bves (A) and VAMP3 (C) revealed these proteins co-localize with endocytosed integrins in individual vesicles as seen in the merged image (D, white vesicles). For better visualization, corresponding magnified views of panels A, B, and C of the area outlined in D are seen in panels a, b, c, and d. The white box surrounds three separate vesicles, all of which are positive for Bves, VAMP3, and β-1 integrin.

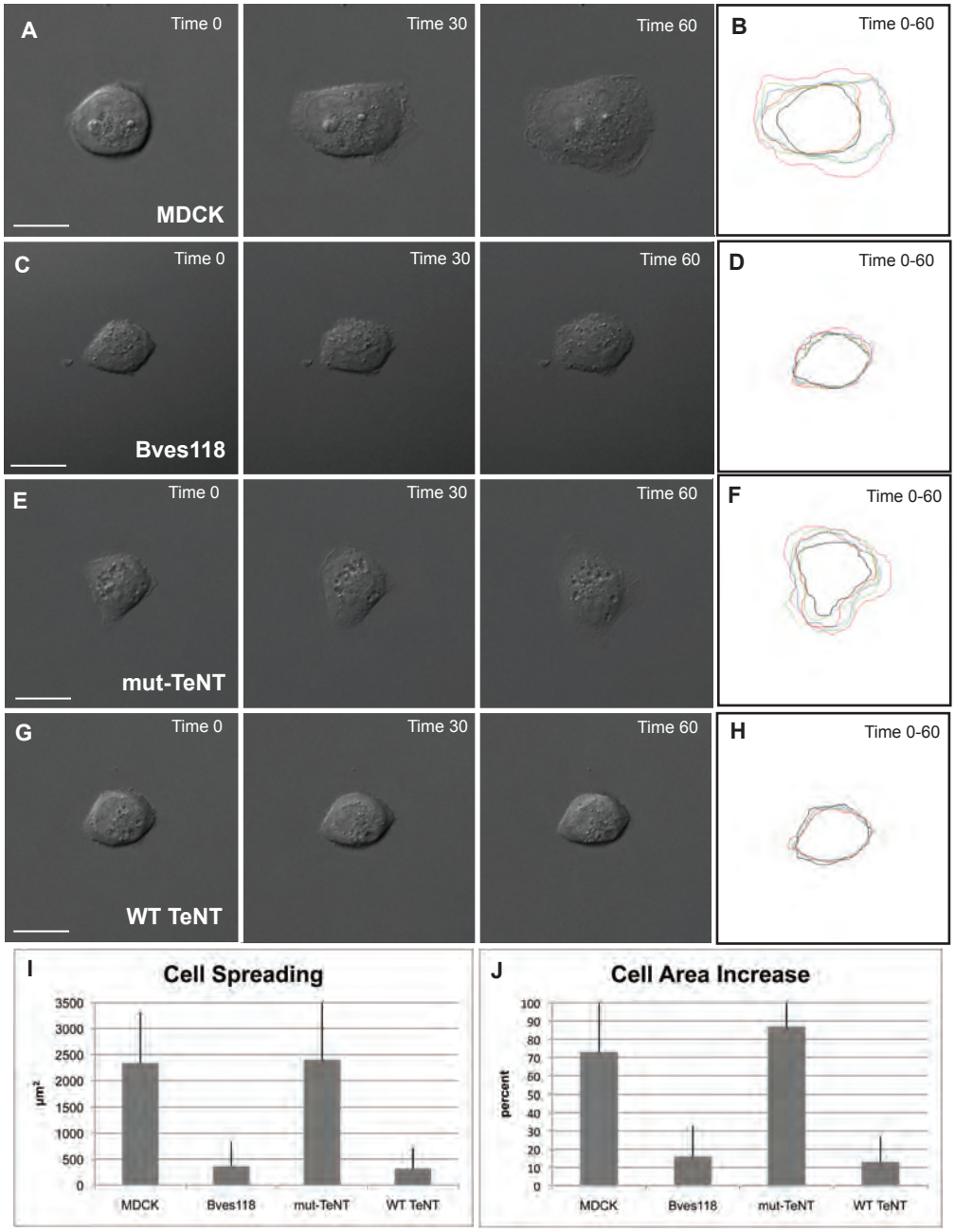


Figure 4.11 Cell Spreading is attenuated with disruption of Bves or VAMP3 function. Time-lapse analysis indicates that cell spreading, or the increase of area prior to polarized cell movement, is decreased in Bves118 cells (C) compared to MDCK cells (A). Similarly, WT TeNT (G) cells have less cell spreading than mut-TeNT cells (E). Kymographs of cell spreading over time demonstrate the difference in the degree of cell spreading between control and experimental groups (MDCK, panel B vs Bves118, panel D; and mut-TeNT, panel F vs WT TeNT, panel H). Note the similarity in cell areas (I) and percent increase of cell area (J) between experimental groups and control groups as determined from composite kymographs (B, D, F, H). Scale bars are 20µm.

Table 3

Figure 4.11

MDCK Cell Spreading Quantification

	MDCK	Bves118	mut-TeNT	WT TeNT
% Area Increase	73±30	16±17	87±49	13±14
P Value <		0.0001		0.0007

	MDCK	Bves118	mut-TeNT	WT TeNT
Cell Spreading (μm^2)	2,337±979	363±466	2,401±1111	318±396
P value		0.0001		0.0002

(Bves: 363μm^2; and VAMP3: 318μm^2), which are significantly reduced from the areas observed in both control cell lines (MDCK: 2,337μm^2; and mut-TeNT: 2,401μm^2). Individual frames of composite kymographs seen in Figure 4.11B, D, F and H are given in Figure 4.12. These data, which are corroborated using the *X. laevis* system (see below), demonstrate that cell spreading is significantly impaired in cells with mutated Bves or VAMP3, suggesting that interaction of these two proteins is important for integrin-mediated processes.

Morphological defects are observed in Bves- and VAMP3-depleted *X. laevis* embryos

Having established that Bves is required for VAMP3-mediated vesicular transport *in vitro*, we next determined the *in vivo* significance of this interaction. Gastrulating *X. laevis* embryos undergo extensive integrin-dependent cellular rearrangement, hence this is an advantageous system in which to analyze Bves function in development (DeSimone et al, 2005; Keller, 1980). Bves-depleted embryos (via aforementioned MO knockdown) exhibited delayed closure of the blastopore during gastrulation, which is indicative of disrupted cellular movement (Figure 4.13B) (Johnson et al, 1993; Marsden & DeSimone, 2001; Marsden & DeSimone, 2003; Ramos & DeSimone, 1996; Ramos et al, 1996). Similarly, embryos injected with VAMP3 MO, displayed a delay in blastopore closure (Figure 4.14B), although this phenotype was less penetrant when compared to the Bves phenotype. It is interesting that similar defects are seen at this stage, as this is when integrin-mediated adhesion is important for migration across the blastocoel roof (BCR), which results in blastopore closure (Marsden &

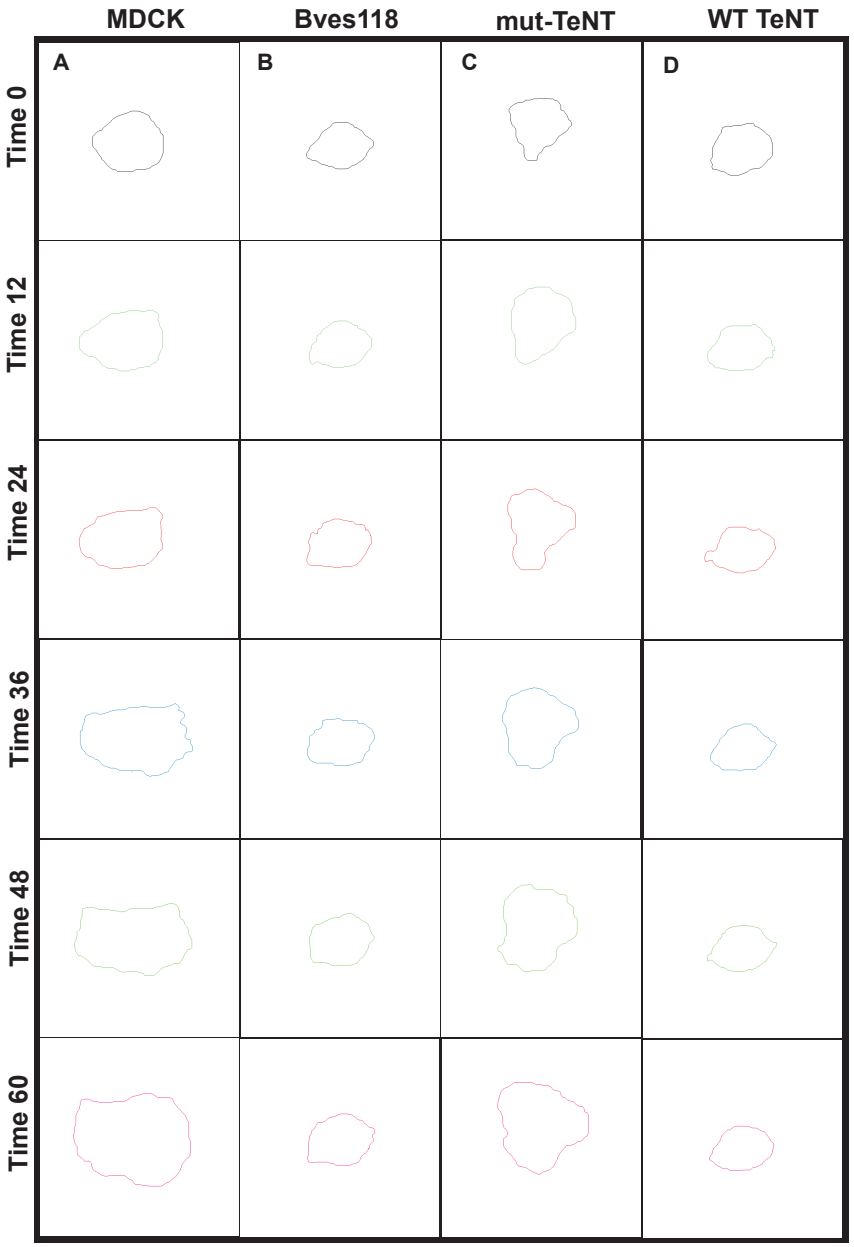


Figure 4.12 Individual frames of time-lapse imaging.
As seen in Figure 4.11, Bves118 and WT TeNT cells have decreased cell spreading on FN. Cell tracings from different time points demonstrate that although cell area is similar in all four cell lines at the onset of image acquisition (panels A-D, Time 0), during the period of analysis, MDCK and mut-TeNT cell lines increase their cellular area more quickly than Bves118 and WT TeNT cell lines (panels A-D, Time 60). Individual cell tracings throughout time are shown here and are displayed as a composite in Figure 4.11, panels B, D, F, and H.

DeSimone, 2001). The BCR intercalates to become two to three cell layers thick in COMO injected embryos (Figure 4.13D, arrow), but remained thickened in Bves-depleted embryos upon histological analysis (Figure 4.13E, arrow) (Keller, 1980). Additionally, the involuting mesoderm is disassociated from the BCR in Bves-depleted embryos, suggesting decreased cell-matrix adhesion (Figure 4.13E, asterisk). Interestingly, *X. laevis* embryos injected in one of two cells with a lower dose of Bves MO (20ng), display anterior defects, characterized by disrupted morphogenesis of head structures and ectodermal outgrowths on the injected side (Figure 4.13C, arrows). These phenotypes are completely dependent upon inhibition of Bves function as total rescue is achieved by co-injecting Bves MO with 100 pg of *X. laevis* Bves mRNA (Figure 4.15). Conversely, VAMP3 MO treated embryos did not display overt defects in the anterior region at the tadpole stage and generally had a less severe phenotype compared to Bves MO treated embryos that was characterized by a shorter Anterior-Posterior (AP) axis and moderate to severe edema (Figure 4.14).

In *X. laevis*, anterior structures are the progeny of the involuting head mesoderm (HM), thus, we further analyzed this population of cells (region denoted by the asterisk in Figure 4.13E) (Kumano & Smith, 2002). Involuting HM utilizes integrin adhesion to migrate along a FN gradient that is distributed on the BCR (Smith et al, 1990). These cells are directionally polarized towards the leading edge and extend lamellipodia (Winklbauer & Nagel, 1991). Scanning electron microscopy (SEM) and quantitative morphometrics of this region revealed significant changes in cell polarity and overlap in experimental embryos

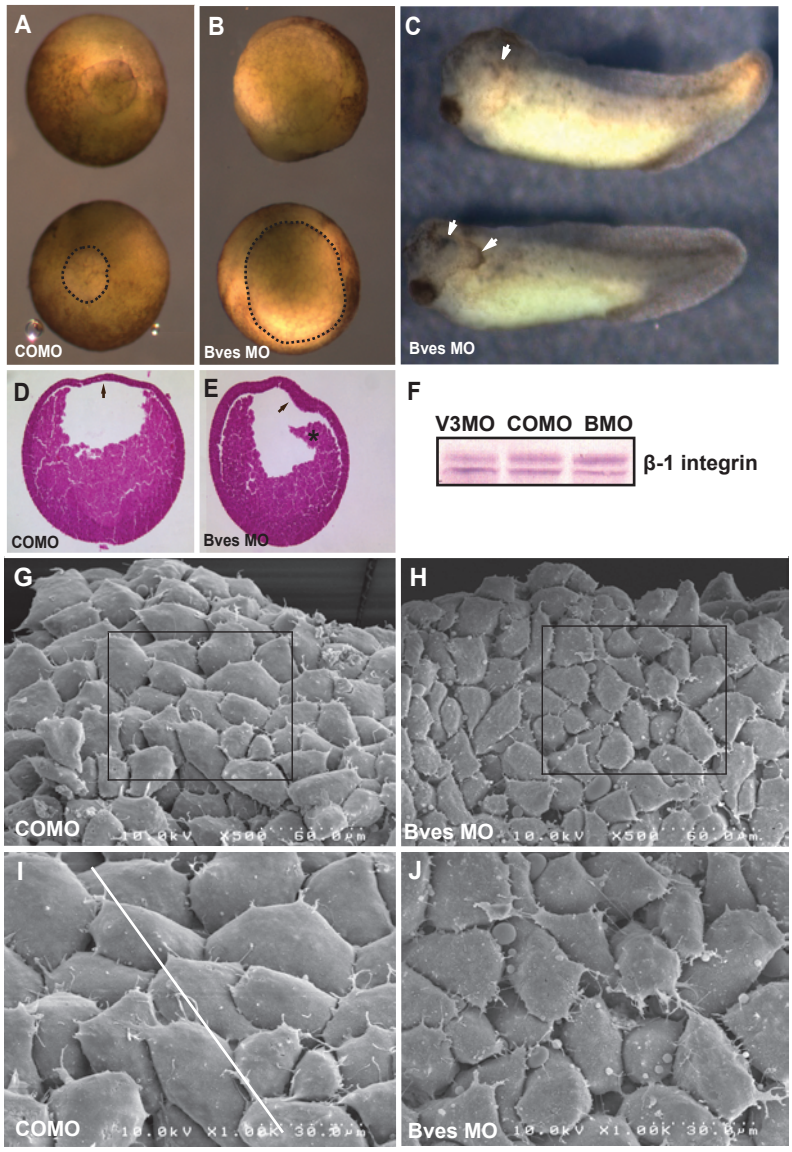
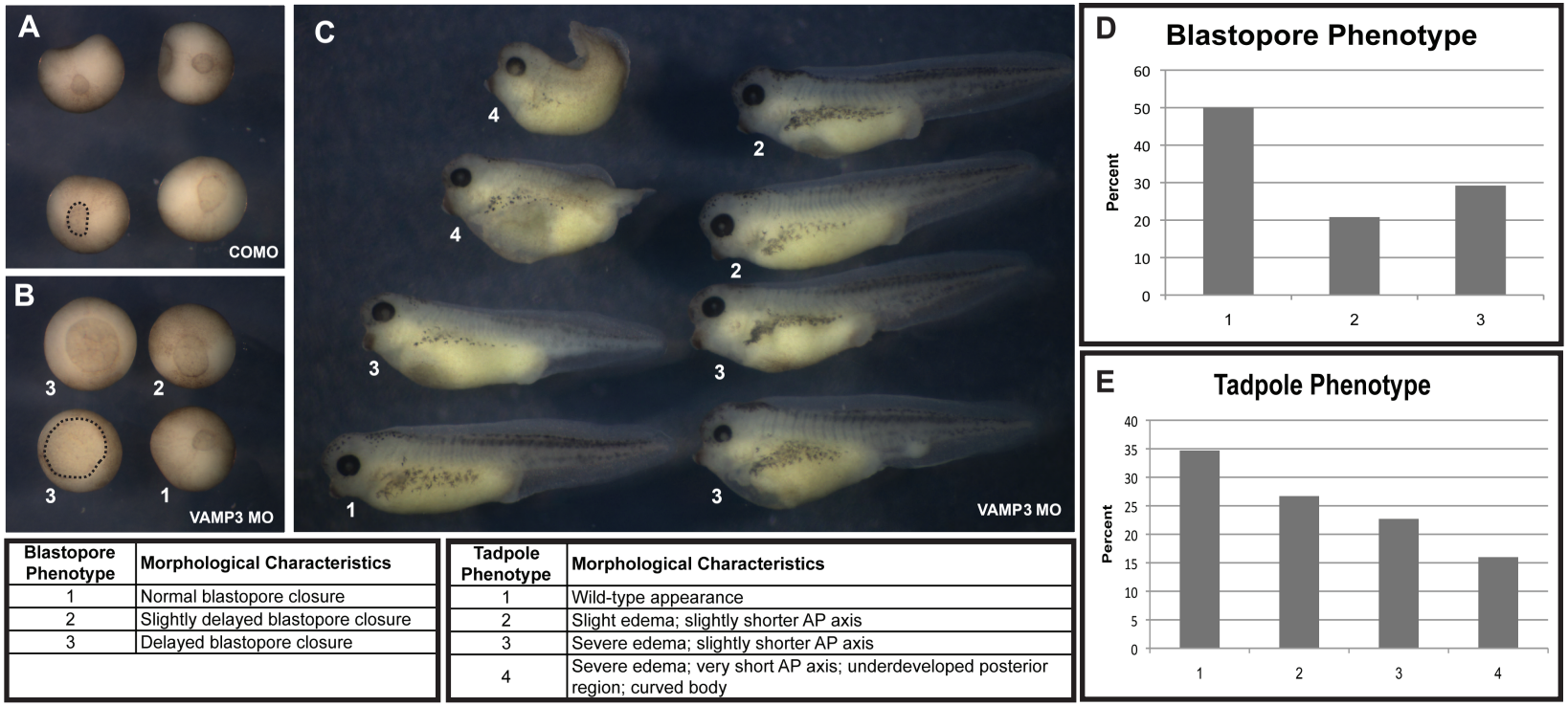


Figure 4.13 Bves depletion in X. laevis embryos. Blastopore closure in embryos injected with Bves MO was dramatically decreased (B) in comparison to embryos injected with COMO (A). The blastopore is outlined in the bottom embryo in panels A and B for better visualization. Anterior defects are observed in Bves-depleted stage 35 embryos (C), characterized by disrupted eye morphogenesis and ectodermal outgrowths (arrows). Histological staining demonstrates the BCR has failed to intercalate properly, and remains thickened (E, arrow), whereas the BCR of control embryos has thinned (D, arrow). Also, the involuting HM has become detached from the BCR in Bves depleted embryos (E, asterisk). Integrin levels in Bves MO treated embryos (and in VAMP3 treated embryos) are similar to COMO treated embryos (F). In SEM analysis of HM, COMO injected embryos display a distinct pattern of tightly overlapped and polarized cells (G, I, white line indicates direction of polarity), whereas Bves MO injected embryos lack directionality, have increased spaces between cells, and exhibit irregular cell shapes (H, J; quantified in Table IV). Panels I and J show magnified views of the boxed areas in panels G and H, respectively.



Blastopore Phenotype	Morphological Characteristics
1	Normal blastopore closure
2	Slightly delayed blastopore closure
3	Delayed blastopore closure

Tadpole Phenotype	Morphological Characteristics
1	Wild-type appearance
2	Slight edema; slightly shorter AP axis
3	Severe edema; slightly shorter AP axis
4	Severe edema; very short AP axis; underdeveloped posterior region; curved body

Figure 4.14: Depletion of VAMP3 in X. laevis. Embryos were injected with 40 ng of VAMP3 MO at stage two and then fixed at different time points. In stage 11.5 embryos, blastopore closure was delayed in 50% of embryos (Panel B, D; 2,3), while the remaining 50% of embryos had normal blastopore closure (Panel B, D; 1) relative to COMO injected embryos (Panel A). This represents a similar, yet less severe phenotype in relation to Bves MO injected embryos (see Figure 4.13B). It is interesting that similar defects are seen at this stage in both Bves- and VAMP3-depleted embryos, as this is when integrin-mediated adhesion is very important for migration across the blastocoel roof, which results in blastopore closure (Marsden et al., 2001). It should be noted that delayed blastopore closure can result from a range of defects, however, it generally is an indicator of impaired cell movement. Knockdown of VAMP3 in X. laevis embryos resulted in a range of phenotypes in tadpoles. The most severe embryos (Panel C; 4) displayed substantial edema, a curved body, and a drastically shortened anterior-posterior (AP) axis (16% of embryos; panel E, 4), while roughly half of the embryos (panel C; 2, 3) had a less extensive manifestation of this phenotype (49.4% of embryos, phenotypes 2 & 3; panel E, 2, 3). Approximately one third of embryos (34.7%; panel E, 1) were indistinguishable from COMO injected controls (panel C; 1).

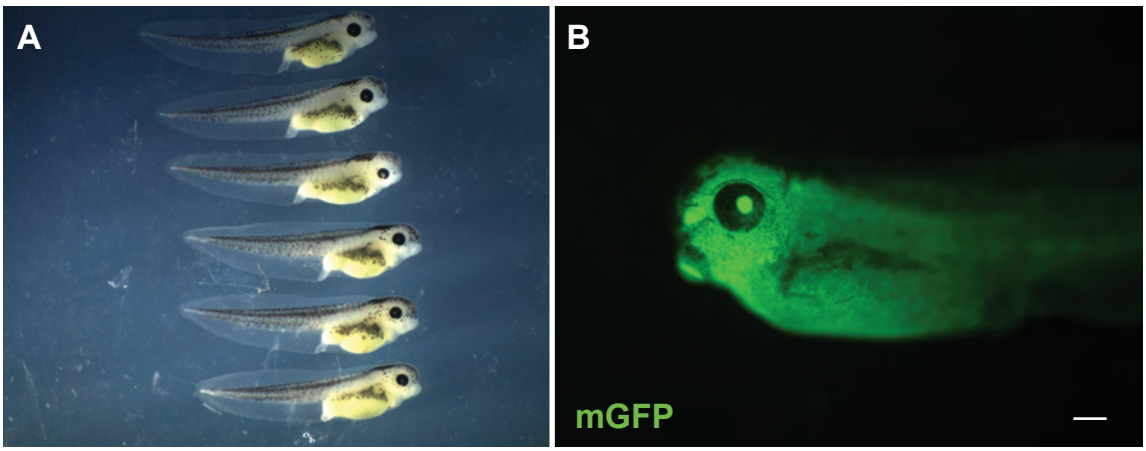


Figure 4.15 Bves Morpholino (MO) specifically knocks down Bves function. Embryos were injected into 2/2 cells with mGFP, 20ng of Bves MO, and 100pg of Xbves Rescue RNA that is mutated in the MO binding site. Stage 41 embryos (A) developed normally, with no gross defects in development, thus demonstrating full rescue. Embryos had membrane GFP labeling in the majority of cells (B), indicating the injected constructs diffused to each cell. This rescue data confirms Bves MO is specific, and off target effects are minimal.

when compared to controls (Table 4). SEM of Bves-depleted embryos determined that the anterior population of HM was severely disorganized (Figure 4.13H, J) with fewer overlaps, large spaces between cells, and no detectable polarity of cell orientation (Figure 4.13H, J; Table 4). In contrast, control embryos displayed a 'shingle-like' pattern of overlapping cells that are all situated in a similar direction relative to the leading edge of the involuting mesoderm (Figure 4.13G, I) (Winklbauer & Nagel, 1991). Taken together, these data suggest that gastrulation movements have been disrupted in both Bves MO and VAMP3 MO treated embryos, and that Bves function is necessary for proper orientation, cell contact, and morphology of HM during involution. Previous studies show that inhibition of integrin function results in overt defects in cellular movement, similar to those seen in Bves-depleted embryos (Figure 4.13) (Marsden & DeSimone, 2001; Na et al, 2003; Ramos & DeSimone, 1996).

Bves-depletion results in decreased *X. laevis* cell spreading on FN

Integrins are required for migration of the involuting HM over a FN gradient during gastrulation of *X. laevis* (Marsden & DeSimone, 2001). As integrins are recycled by VAMP3, we next determined if this was potentially an integrin-dependent phenotype (Luftman et al, 2009; Proux-Gillardeaux et al, 2005a; Skalski & Coppolino, 2005; Tayeb et al, 2005). By plating primary disassociated HM cells on FN, we found that COMO cells had defined lamellipodia and displayed spread morphology *in vitro* (Figure 4.16A), as defined by previous published studies (Ramos & DeSimone, 1996). Conversely, Bves-

Table 4

Figure 4.13

SEM Quantification

Cell Overlap

	Total Cells	Overlaps/Cell
COMO	67	1.66±0.42
Bves MO	64	0.641±0.41

Cell Polarity

	COMO	Bves MO
σ Avg	12.5±3.7	50.2±9.0

depleted cells exhibited distinctly decreased cellular spreading on FN (Figure 4.16B), with smaller cell protrusions. Previous reports have demonstrated disruption of integrin function results in round or spherical cells, phenocopying Bves depletion (Ramos & DeSimone, 1996). This decrease in spread morphology was not due to a decrease in integrin expression levels, as Bves MO injected embryos expressed the same level of integrin protein as COMO treated embryos (Figure 4.13F). The majority of Bves-depleted cells remain rounded (79.2±6%), with few filopodia anchoring them to FN (Figure 4.16B, arrows; Table 5a). Conversely, 73.6±4% of control cells were spread in morphology. This result was significant: $p < 0.0002$.

We next used live cell imaging to determine if Bves-depleted cells displayed impaired cell spreading, morphology, or movement over time; additionally, we extended this study to examine the effect of VAMP3 MO on HM cell morphology. HM cells injected with COMO, Bves MO, or VAMP3 MO displaying spread morphologies were chosen at the onset of image capture and were visualized by previously co-injected membrane GFP (mGFP, experimentals) or RFP (mRFP, control) (Wallingford et al, 2000). The behavior of individual cells was recorded over time and subjected to kymographic analysis for cell spreading and morphology. Both control and experimental cells display a heavily yolk-laden appearance (Figure 4.16C, E, G) and no cell in any group exhibited “directional” migration over the course of study. Time-lapse analysis demonstrated COMO injected cells (Figure 4.16C) displayed on average 4.45±2.3 lamellipodia per cell, while Bves or VAMP3 MO treated cells had only

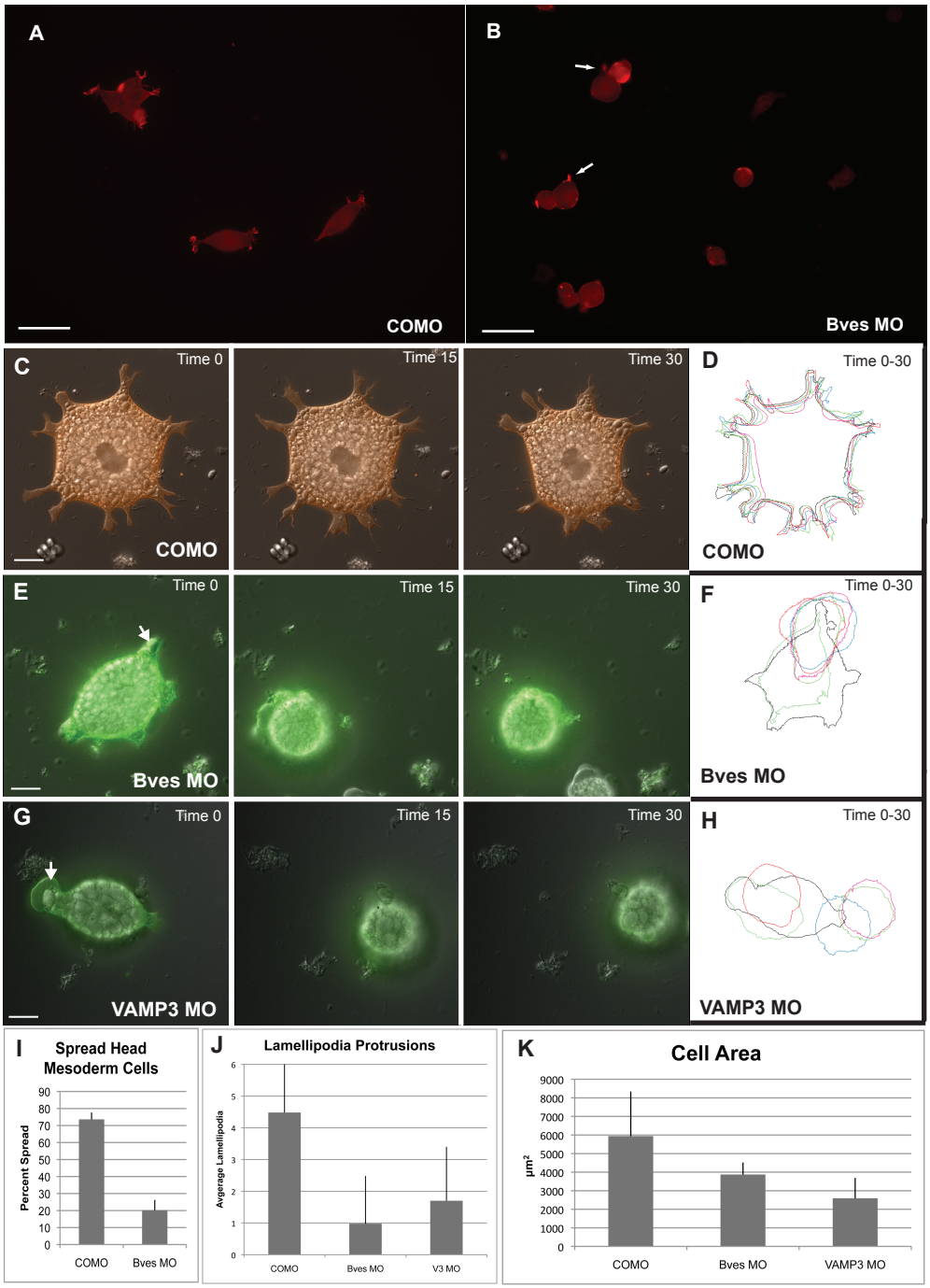


Figure 4.16 Bves- and VAMP3-depleted cells display decreased cell adhesion on FN. HM cells stained with Phalloidin-568 from COMO injected embryos (A) display distinctly spread morphologies on FN, while Bves-depleted cells (B) are round. Analysis over time (in minutes) indicates that as an mRFP labeled COMO treated cell moves (C, Time 0-30), it maintains a spread phenotype, extending several lamellipodia. Conversely, mGFP labeled Bves- (E, Time 0-30) or VAMP3- (G, Time 0-30) depleted cells are unable to maintain substrate adhesion and become rounded. Kymographs (D, F, H) depict cell morphology over time, underlining the distinct differences in cell shape, lamellipodia number, and cell area, which are quantified in graphs I, J, and K. Scale bars are 100µm (A, B) and 20µm (C, E, and G).

0.98±1.5 and 1.7±1.7 lamellipodia/cell (Figure 4.16J, Table 5b). As previously reported (Ramos & DeSimone, 1996), when integrins are non-functional, cultured HM cells remain round in appearance. This was clearly observed in both Bves- and VAMP3-depleted cells (Figure 4.16E and G). In a controlled side-by-side comparison of cells plated on the same FN coated dish, Bves-depleted cells (labeled with mGFP) became rounded over time while COMO treated cells (labeled with mRFP), remained spread. Additionally, both Bves- and VAMP3-depleted cells often exhibited large and very transient bleb-like protrusions that harbored yolk granules (Figure 4.16E, G, arrows); in control cells (Figure 4.16C), yolk granules indicate the stable boundary between the cell body and cell protrusion (Selchow & Winklbauer, 1997). These membrane blebs, known as circus movements in early development (Johnson, 1976) were short-lived, and are generally thought to be associated with decreased adhesion or breakdown of the actin-cytoskeleton network (Fackler & Grosse, 2008; Shook & Keller, 2003) Kymographs of the area of attachment reveal Bves- and VAMP3-depleted cells had a significantly smaller area of interaction with the substrate (Figure 4.16D, F, H, K; see Figure 4.17 for individual frames), when compared to the COMO injected cells. These statistically significant results (Table 5c) demonstrate that cell adhesion and process extension, processes regulated by integrins (Caswell & Norman, 2006; Holly et al, 2000b), are impaired in Bves- and VAMP3-depleted cells. These data, which are corroborated by our current findings with MDCK cells, further support a role for Bves in cell movement through VAMP3-mediated recycling of integrins.

Table 5

Figure 4.16

Head Mesoderm Cell Adhesion Quantification

(a) Cell Spreading

	COMO	Bves MO
% Spread	73.6±4	20.2±6
% Round	26.4±4	79.2±6
Total Cells	557	414
P Value <	0.0002	

(b) Lamellipodia Formation

	COMO	Bves MO	VAMP3 MO
Lamellipodia/cell	4.45±2.3	0.98±1.5	1.7±1.7
P Value <		0.0001	0.0001

(c) Cell Area (μm2)

COMO	Bves MO	VAMP3 MO
5941±2401	3,872±644	2,592±1097
P Value <	0.046	0.001

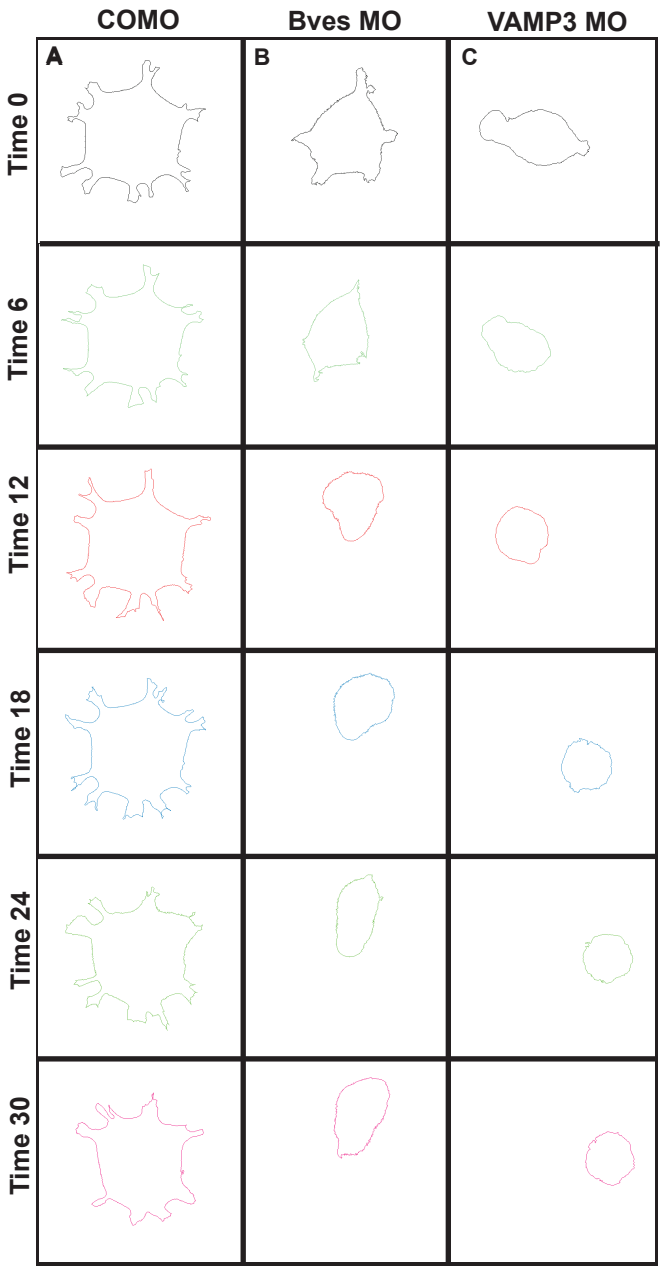


Figure 4.17: Kymographs of HM cells. Figure 4.16 demonstrates that cell adhesion to a FN substrate in Bves- and VAMP3-depleted X. laevis primary head mesoderm cells is disrupted over time. Individual cell tracings displayed here from COMO (panel A), Bves MO (panel B), and VAMP3 MO (panel C) demonstrate that lamellipodia protrusions and cell area are decreased in Bves- and VAMP3-depleted cells over time (Time 0-60; quantified in Table V). Overall, Bves or VAMP3 MO treated cells have distinctly different cell morphology, characterized by rounded or spherical cells which indicates decreased integrin-mediated cell adhesion, while COMO treated cells maintain cellular protrusions, as well as spread morphology.

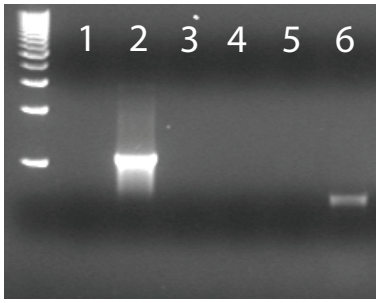


Figure 4.18 Stable Bves118 expression in MDCK cells is confirmed by RT-PCR. After selection in G418, stably transfected lines were assayed for RNA transcripts. Briefly, MDCK cell lysate was harvested using Trizol, and cDNA was made using Superscript II RT. PCR was carried out with the following primers: the forward primer began at pCMV-3Tag-4 vector nucleotide 584 (AAC CGT CAG ATC CGC TA) and the reverse primer began at nucleotide 943 (CTG GCA ACT AGA AGG CAC), thus spanning the multiple cloning site. Bves118 is 354 nucleotides, yielding a PCR product of 713 nucleotides as seen in lane 6. Lanes 1, 3-5 are negative controls, Lane 2 is a positive control.

Discussion

In this study we present data that link Bves to the fundamental cellular process of vesicular transport. Bves has been previously reported to regulate cell movement and cell-cell adhesion, although these functions were unexplained at the molecular level. Here we describe a mechanism that may elucidate the role Bves plays in these processes through its interaction with the vesicular transport protein, VAMP3. Our findings demonstrate that the intracellular domain of Bves interacts directly with VAMP3 and that these proteins co-localize in a variety of cell types. Furthermore, stable expression of a mutant form of Bves or elimination of Bves protein function results in a remarkably similar disruption in transport of two independent molecules, transferrin and β-1 integrin, both of which are trafficked by VAMP3 (Galli et al, 1994; Proux-Gillardeaux et al, 2005a). These findings are corroborated in *X. laevis* embryos, where Bves depletion (as well as depletion of VAMP3) results in impaired transferrin recycling in animal caps and morphological defects consistent with the disruption of integrins. Furthermore, in both model systems, cells with inhibited Bves function have disrupted cell adhesion or spreading, consistent with VAMP3-dependent trafficking of integrins. Based on these data, we propose that Bves is essential for VAMP3 function in vesicular transport, and is specifically required for the recycling of VAMP3-mediated receptors. Thus, we propose that Bves functions in broad cellular processes regulated by vesicular transport, explaining previously reported phenotypes unresolved at the molecular level.

Bves as a novel regulator of vesicular transport

Bves is essential for proper cell movement, although the exact role Bves played in this process was previously unknown (Ripley et al, 2006; Smith et al, 2008). We propose that, through interaction with VAMP3, Bves is necessary for the vesicular transport of the adhesion molecule, β-1 integrin, as attenuated integrin recycling is observed during cell migration when Bves is disrupted. This retardation in integrin recycling exactly phenocopies VAMP3 disruption and directly links Bves disruption to impaired integrin recycling. These data are corroborated by attenuated cell spreading or adhesion (processes dependent upon integrin integrity) observed with Bves inhibition. Furthermore, impaired recycling of integrins is a potential mechanism explaining the gastrulation phenotype observed in the embryo.

VAMP3 transports transferrin, hence, the rate of transferrin uptake is a general indicator of the integrity of VAMP3 trafficking (Galli et al, 1994). With Bves disruption, either by expression of a mutated protein or protein depletion, endocytosis of transferrin decreases over time and phenocopies VAMP3 inhibition. These data, along with decreased recycling of β-1 integrin, demonstrate that Bves is important for VAMP3-mediated vesicular transport of receptors.

Bves is important for the generation and maintenance of epithelial junction integrity (Osler et al, 2005). In the process of epithelial biogenesis, cell-cell adhesion is initiated as specific proteins are trafficked to the forming adherens

junctions (Bryant & Stow, 2004; Yap et al, 2007). This process is disrupted upon Bves depletion, as the canonical adherens junction molecule, E-cadherin, is not localized to points of cell-cell contact (Osler et al, 2005). Cadherin-based cell adhesion is a dynamic process with E-cadherin constantly being replenished at the cell surface via vesicular transport (Bryant & Stow, 2004; Yap et al, 2007). Defects in the vesicular transport of E-cadherin could lead to disrupted cell adhesion or signaling, which results in pathogenic states such as metastatic cancer (Hirohashi, 1998). Recently, it has been shown that efficient delivery of E-cadherin in both polarized and unpolarized cells is dependent upon functional Rab11 positive REs (Desclozeaux et al, 2008). Disruption of either Rab11 (a Rab GTPase and known player in vesicular transport) or the RE results in apical delivery of proteins. VAMP3 is a well-known member of the RE (Fields et al, 2007; Skalski & Coppolino, 2005) and thus it is plausible to hypothesize that VAMP3-mediated vesicle fusion may be important for the recycling of E-cadherin, and mislocalization of E-cadherin upon Bves depletion may be explained by disrupted VAMP3-mediated vesicular transport. Thus, the current data and previous results suggest that Bves may influence vesicular transport in a broad array of cell functions.

Bves in cell adhesion, spreading, and movement

We provide conclusive evidence that Bves-depleted embryos have disrupted cell movements during gastrulation. *X. laevis* gastrulation is a well-studied system where integrin-dependent cell adhesion and movement are

critical for development (Davidson et al, 2006; Marsden & DeSimone, 2003). We report that HM cells in Bves-depleted embryos fail to orient properly and, when isolated, display rounded morphology and impaired process extension when plated on FN. Additionally, these cells display transient membrane blebs, which have been associated with decreased cell adhesion during development (Shook & Keller, 2003), and indicate localized breakdown of the actin-cytoskeletal network (Fackler & Grosse, 2008). Evidence of this breakdown is seen in Bves-depleted cells as yolk granules, which are usually confined to the cell body, extend into lamellipodia (Selchow & Winklbauer, 1997). The decreased integrin-mediated cell-substrate adhesion, coupled with actin-cytoskeletal network breakdown found in these cells is not surprising. Bves depletion influences Rac and Cdc42 activity (Smith et al, 2008), both of which are molecules that are important for actin polymerization and communicate with integrins during cell movement (DeMali et al, 2003). Additionally, actin polymerization has a well documented role in the endocytic pathway (Lanzetti, 2007). Overall, these gastrulation-stage phenotypes are consistent with disrupted integrin function, as integrins are responsible for cell adhesion and spreading on FN (Marsden & DeSimone, 2001). As VAMP3 is known to recycle integrins, our data suggest VAMP3 interaction with Bves is necessary for proper integrin-dependent movements during early *X. laevis* development.

In turn, several previous reports have linked Bves to the regulation of cell movement. For example, germ cell migration in the developing *Drosophila* embryo is impaired with mutation of *Dmbves* (Lin et al, 2007), while

Bves/Popdc1-null mice exhibit impaired skeletal muscle regeneration due to the inhibition of myoblast movement (Andree et al, 2002). Previous work from our own group has demonstrated impaired movement and regulation of cell shape *in vitro* and in developing organisms (Ripley et al, 2004; Ripley et al, 2006; Smith et al, 2008). Still, until the identification of Bves-VAMP3 interaction, a molecular mechanism underlying these phenotypes was unresolved. Interestingly, the *Bves* gene is hypermethylated in specific cancer types (Feng et al, 2008), suggesting silencing of this gene may coincide with down-regulation of cell-cell adhesion. Thus, the role of Bves in the modulation of SNARE function may have broad impact on development and disease.

Bves as a moderator of diverse cellular pathways

In addition to interacting with VAMP3, we show that Bves interacts with VAMP2. It has been reported that VAMP2 and VAMP3 are promiscuous during development and *in vitro*, substituting for each other when one molecule is absent (Bhattacharya et al, 2002; Deak et al, 2006). This comes as no surprise, as rat VAMP2 and VAMP3 are highly homologous, differing by only one amino acid in their SNARE binding domain (McMahon et al, 1993). Bves interaction with VAMP2, and the potential overlapping function between different VAMP homologues, may explain the milder phenotype observed in VAMP3-depleted *X. laevis* embryos, and may suggest a broader role for Bves in influencing VAMP-mediated vesicular transport. Additionally, VAMP2 is expressed in muscle satellite cells and is up-regulated during skeletal muscle regeneration (Tajika et

al, 2007); interestingly, this process is delayed in Bves knockout mice (Andree et al, 2002). Finally, Bves is highly expressed in the brain, tissue where VAMP1 and 2 are also enriched, although Bves has never been studied in this context (Hager & Bader, 2009). It would be interesting to determine if Bves interacts with VAMP1, and if interaction with VAMP1 or 2 had any functional significance in the nervous system. Overall, Bves interaction with VAMP2 may suggest a broader role for Bves in a variety of tissue types that utilize SNARE machinery.

Current and recent studies have shown that Bves interacts with two proteins, VAMP3 and GEFT, that function through downstream targets to regulate convergent cellular processes (Smith et al, 2008). In light of the present data, it is intriguing to consider the overlapping cell operations in which GEFT and VAMP3 are involved. Although the direct functions of VAMP3 and GEFT within the cell are very different, they are nonetheless involved in a common pathway. GEFT activates Rho GTPases, which in turn regulate cell adhesion, cell motility, polarity, gene expression, and membrane trafficking (Etienne-Manneville & Hall, 2002). VAMP3, through regulation of protein trafficking, modulates cell motility, polarity, and gene expression (Schwartz & Shattil, 2000a). Indeed, several studies have even implicated Rho GTPase activity in the regulation of vesicular transport (Ridley, 2001b; Symons & Rusk, 2003), and integrins have been shown to recruit Rho GTPases necessary for modulation of the actin network (Caswell & Norman, 2008; Holly et al, 2000b). However, the interplay between integrin signaling and Rho GTPase function is not entirely understood. We hypothesize that through interaction with VAMP3 and GEFT, Bves may

provide crosstalk to achieve cellular synchrony in these essential cell processes. Taken together, our data suggest that Bves may play an unexpected role in a broad spectrum of cellular functions regulated by vesicular transport.

CHAPTER V

IDENTIFICATION OF A NOVEL INTRACELLULAR INTERACTION DOMAIN ESSENTIAL FOR BVES

This Chapter was published under the same title in *PLoS ONE*, May 21, 2008 (Kawaguchi et. al.) Dr. Michiya Kawaguchi completed the bulk of this work, however, I completed revisions for publication, contributing a figure and manuscript edits. I was second author on this study.

Abstract

While Blood vessel epicardial substance (Bves) confers adhesive properties, the molecular mechanism of regulating this activity is unknown. No predicted functional motifs in this highly conserved integral membrane protein, other than the transmembrane domain, have been identified. Here, we report for the first time that Bves interacts with itself through an intracellular interaction domain that is essential for its intercellular adhesion activity. Glutathion-S-transferase (GST) pull-down and SPOTs analyses mapped this domain to amino acids 268-274 in the intracellular C-terminus. Site-directed mutagenesis revealed that lysines 272 and 273 are essential for homodimerization and cell adhesion. Human corneal cells transfected with wild-type Bves trafficked the protein to the cell surface, assembled junction complexes and formed epithelial sheets. In contrast, cells expressing Bves mutated at these positions did not form

continuous epithelial sheets or maintain junctional proteins such as ZO-1 and E-cadherin at the membrane. A dramatic reduction in transepithelial electrical resistance was also observed indicating a functional loss of tight junctions. Importantly, expression of mutated Bves in epithelial cells promoted the transformation of cells from an epithelial to a mesenchymal phenotype. This study is the first to demonstrate the essential nature of any domain within Bves for maintenance of epithelial phenotype and function.

Introduction

Bves was discovered independently by Reese et al. (Reese et al, 1999) and Andree et al. (Andree et al, 2000) and is the prototypical member of the Popeye domain containing (*Popdc*) gene family (Andree et al, 2000). It is highly conserved and has been identified in a wide variety of vertebrate and invertebrates (Andree et al, 2000; Hitz et al, 2002; Lin et al, 2007; Reese & Bader, 1999; Reese et al, 1999). Both mRNA and protein of Bves are highly expressed in striated and smooth muscle and in various forms of epithelial cell types in the embryo and adult (Andree et al, 2000; Breher et al, 2004; DiAngelo et al, 2001; Osler & Bader, 2004; Osler et al, 2005; Osler et al, 2006; Reese & Bader, 1999; Reese et al, 1999; Vasavada et al, 2004). Biochemical analyses have determined that Bves is an integral membrane protein (Knight et al, 2003; Wada et al, 2001), while localization studies have found Bves at the lateral cell membrane and within vesicles of the Golgi apparatus (Knight et al, 2003; Wada

et al, 2001). Still, no molecular understanding of protein function is currently available.

Bves has the canonical structure of all predominant *Popdc* gene products. This includes a short extracellular N-terminus with two invariant glycosylation sites, three transmembrane domains with two intervening loops and a long intracellular C-terminus (Andree et al, 2000; Knight et al, 2003; Osler et al, 2005). While Bves has a highly conserved primary amino acid sequence among different species, there are no studies identifying any protein domain linked to any molecular or cellular function.

Phenotypic analyses of this gene family are only now emerging. Due to its subcellular localization and trafficking to points of cell-cell contact during epithelial sheet formation (Osler et al, 2005), we proposed that Bves might play a role in cell-cell adhesion. Transfection of Bves into normally non-adherent L-cells conferred adhesive activity (Wada et al, 2001) much like E-cadherin indicating that the transfected molecule confers adhesive properties (Nakada et al, 2000; Thoreson et al, 2000). Additionally, morpholino knockdown of Bves protein inhibited epithelial sheet formation and stability, and disrupted transepithelial electrical resistance (TER) (Osler et al, 2005). While *popdc1*-null mice do not show an overt embryonic phenotype, presumably due to redundancy of expression with *popdc2* and *popdc3* genes, regeneration of skeletal muscle is delayed due to an inhibition of cell-cell adhesion/interaction (Andree et al, 2002). Early inhibition of Bves function in *Drosophila* development results in disruption of pole cell migration (Lin et al, 2007), while gastrulation in *X. laevis* is severely

restricted due to failure in epithelial morphogenesis (Ripley et al, 2006). Still, no reports have identified any functional domains within Bves or described the molecular basis of Bves function for adhesion or any other possible activities in tissue or organ morphogenesis.

Here for the first time, we report a Bves-Bves molecular interaction through its intracellular C-terminus that is essential for molecular regulation of cell-cell adhesion. This domain lies within the highly conserved Popeye region of the molecule, which heretofore has no ascribed function. Further we identify two amino acids in this sequence (K^{272} and K^{273}) that are critical for homophilic binding. While transfection of wild type Bves promotes cell aggregation in L-cell assays, mutation or deletion of K^{272} and K^{273} abolishes this activity. Expression of these mutated transcripts dominantly interferes with normal Bves function in human corneal epithelial cells (HCE) resulting in loss of cell-cell adhesion, junction formation, TER and epithelial sheet integrity. Importantly, expression of mutated Bves leads to a change of cells from an epithelial to mesenchymal phenotype. This study is the first to identify a specific molecular mechanism by which Bves regulates cell-cell adhesion and to demonstrate that mutation of these sequences inhibits cellular functions attributed to this molecule.

Materials and Methods

Bves Constructs

Specific regions of the Bves molecule were cloned using Polymerase Chain Reaction (PCR) strategies for biochemical and cellular analysis. N-terminal

(amino acid (aa) 1-36) or C-terminal (aa 115-357) regions of Bves were cloned into the EcoRI and XhoI site of pGEX-5X-1 (Amersham, Piscateway, NJ) for bacterial expression and the Sal I and Not I site of pCIneo (Promega, Madison, WI) for eukaryotic expression. Wild type (WT) Bves and a C-terminal deletion series [Del-5 Bves (aa 1-309), Del-4 Bves (aa 1-284) and Del-3 Bves (aa 1-251)] were Flag-tagged (YYKDDDDK) on their C-termini and inserted into the Sal I and Not I site of pCIneo. Alanine substitution and deletion of K^{272} and K^{273} of Flag-tagged Bves were produced by a sequential PCR method and cloned into pCIneo. Sequences of all constructs were confirmed in the DNA sequencing core at Vanderbilt University.

Cells, Transfection and Production of Stable Cell Lines

COS-7 cells (ATCC, Manassas, VA) and L-cell (CLL 1.3, ATCC) were cultured in Dulbecco's Modification of Eagle's Medium (DMEM, Mediatech, Herndon, VA) with 4mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose and 1.0 mM sodium pyruvate supplemented with 10% fetal bovine serum (FBS, Atlanta biological, Lawrenceville, GA) and 10 mg/ml Penicillin-Streptomycin solution (Mediatech) Human corneal epithelial cells (HCE) were originally obtained from Dr. K. Araki-Sasaki (Osaka, Japan), maintained as previously described (Araki-Sasaki et al, 1995), and were grown in Defined Keratinocyte-SFM with growth supplement (Invitrogen, Grand Island, NY). L-cells were transfected with WT Bves, C-terminal Bves, N-terminal Bves, KK-Mut Bves and KK-Del Bves. Two mg of each construct were used for transfection with the

FuGENE6 transfection reagent (Roche, Indianapolis, IN). After 72 hours, positive clones were selected in growth medium containing G418 (0.4 mg/ml) and resistant cells were maintained in medium with G418 (0.2 mg/ml). HCE cells were also transfected with WT Bves, KK-Mut Bves and KK-Del Bves as described above. Stably transfected cell lines were obtained by using medium with G418 (0.02 mg/ml). Production of Bves protein was confirmed by immunochemical staining with anti-Bves (B846, (Wada et al, 2001)) and anti-Flag (M2, Sigma, St. Louis MO) antibodies.

GST Pull-down Assay

GST N-terminal, GST C-terminal Bves or GST proteins were prepared using standard methods (Osler et al, 2005) and mixed with WT Bves produced in COS-7 cells and incubated overnight at 4˚C. Replicate WT Bves transfected COS-7 cells were incubated with tunicamycin (2 mg/ml) to produce WT Bves protein devoid of glycosylation products. Reactions were then applied to glutathione beads and rocked at 4˚C for four hours. Beads were washed three times with Phosphate Buffered Saline (PBS) and the protein was eluted with SDS sample buffer. Eluted proteins were analyzed by SDS-PAGE and Western blot using anti-Flag antibody (Reese et al, 1999). The same experiments were repeated with a series of C-terminal deletion constructs to identify the precise region required for Bves-Bves homophilic interaction. Dilutions of antibodies were: primary antibodies (anti-Flag, M2, Sigma 1:1000), secondary antibody

(anti-mouse IgG alkaline phosphatase (AP) -conjugated, Sigma 1:10,000). Blots were developed in NBT/BCIP (Roche, Indianapolis, IN) in AP buffer.

SPOTs Protein Mapping

To more precisely identify the Bves-Bves interaction region in the C-terminus, a SPOTs blot membrane was generated by SIGMA-GENOSIS (Woodland, TX). Peptides were designed from the sequence of the C-terminus of Bves after the third hydrophobic membrane-spanning region. Synthesized 13-mer peptides (starting from aa 232) were covalently fixed to a cellulose membrane with ten overlapping amino acids between neighboring SPOTs (Figure 5.2A). The 20 total SPOTs contain the putative intracellular interaction domain between Del-4 Bves and Del- 3 Bves (aa 252-284). These SPOTs were assayed for binding with WT Bves and Del-3 Bves. Both proteins were prepared from COS-7 cell transfection and were incubated with anti-Flag antibody at 4˚C overnight to form a protein/antibody complex. The membrane was incubated in blocking solution (2% skim milk in TBST) for two hours at room temperature and then incubated with the protein/antibody complex for three hours at room temperature. After extensive washing in TBST, the membrane was incubated with secondary antibody (anti-mouse IgG horseradish peroxidase (HRP) conjugated, Sigma). Interactions were detected by chemiluminescence (ECL plus, Amersham UK Ltd., Buckinghamshire, England). The membrane was regenerated using manufacturer's recommendation.

Alanine Substitution Analysis

Identification of amino acid(s) critical for Bves-Bves interaction was performed on a second SPOTs membrane. On this SPOTs blot, each single amino acid of Peptide 12: TLNDKASKKIDRQ was individually substituted with L-alanine beginning with N-terminal amino acid and progressing to the C-terminal of the peptide; synthesized 13-mer peptides were fixed on a cellulose membrane. Blocking reaction, washing and development with WT Bves were identical to the protocol described above.

L-cell Aggregation Assay

Cellular adhesion activity of non-transfected control and stable L-cell lines transfected with Bves constructs (WT Bves, KK-Mut Bves, and KK-Del Bves) was compared in standard hanging drop suspension cultures (Nakada et al, 2000; Thoreson et al, 2000). Images were acquired using an inverted image microscope (Olympus IX70), an object lens (LCPlan FI 20X/0.40 Ph1), a camera (OPTRONICS MagnaFire-Model S60800) and software (MagnaFire 2.1A) at room temperature.

Immunofluorescence assay

Immunofluorescent analysis of protein expression and distribution was similar to previously published studies (Osler et al, 2005; Ripley et al, 2004; Wada et al, 2001). HCE cells transfected with WT Bves, KK-Mut Bves and KK-Del Bves or non-transfected parental cells were seeded on a four well-chamber

slide (Lab-Tek II, Nalge Nunc, Naperville, IL) and immunostained using following antibodies. Primary antibodies were: anti-Bves (B846, (Wada et al, 2001), 1:200), anti-Flag (M2, Sigma, 1:150), anti-ZO-1 (Zymed, South San Francisco CA, 1:200), anti-E-cadherin (Sigma, 1:200), anti-cytokeratin (DAKO, Carpinteria CA, 1:200), and anti-vimentin (AMF17b, Developmental Hybridoma Bank, Iowa City IA, 1:200). Anti-rabbit IgG conjugated with Alexa 488 (Molecular Probe, Eugene OR, 1:3000) and anti-mouse IgG conjugated with Cy3 (Jackson ImmunoResearch Lab, West Grove PA, 1:3000) were used as secondary antibodies. DAPI (Molecular Probes) was used at 1:3000 to stain nuclei. Images were acquired using a fluorescence microscope (Olympus AX70 TRF), an object lens (Olympus UPlan APO 40X/0.85), a camera (OPTRONICS MagnaFire-Model S60800) and software (MagnaFire 2.1A) at room temperature.

Transepithelial electrical resistance

To examine tight junction activity in confluent cultures, the TER was measured. Non-transfected and transfected HCE cells were used in this study. Cells (2×10^4 cells/cm^2) were seeded on a six well transwell chamber (cell culture insert, 0.4 mm pore, Falcon/BD lab ware, Franklin Lakes, NJ) and cultured for two weeks. TER was measured using an Epithelial volt-ohm-meter (EVOM) (World Precision Instruments, Sarasota, FL). TER was calculated as follows: TER = (Reading of EVOM - reading of blank) x surface area of the membrane (4.2 cm^2). The data are log-transformed to stabilize variances. Analysis of variance was applied to test for overall difference, followed by

Dunnett's method to compare the three experimental groups to the parental group while controlling the family-wise type I error rate at 5%. Simultaneous 95% confidence intervals of the group mean ratios were obtained using Dunnett's method. The reported p-values and confidence intervals are adjusted for multiple comparisons (Hsu, 1996).

Results

Bves intermolecular interaction through the intracellular C-terminus

The molecular basis of Bves adhesive function is unknown (Wada et al, 2001). To determine the molecular mechanisms that underlie this function, we explored whether Bves-Bves intermolecular interactions could be detected. We generated an array of wild type and truncated Bves constructs to identify possible Bves-Bves interaction domains (Figure 5.1A). In a first set of experiments, Flag-tagged Wild Type (WT) Bves harvested from COS-7 cells was reacted with GST N- or C-terminal Bves produced in E. coli. As seen in Figure 5.1B, GST C-terminal Bves readily precipitated WT Bves, while GST N-terminal Bves and GST alone did not. Elimination of the two N-terminal glycosylation sites had no effect on C-terminal interaction (Figure 5.1B, lane g). These results do not exclude the possibility of N-terminal interactions but demonstrate direct association between molecules through the C-terminus of Bves. To further define sequences in the C-terminus responsible for this activity, a series of C-terminal truncations (shown in Figure 5.1A) were reacted in similar manner. Deletion of C-terminal up to aa 284

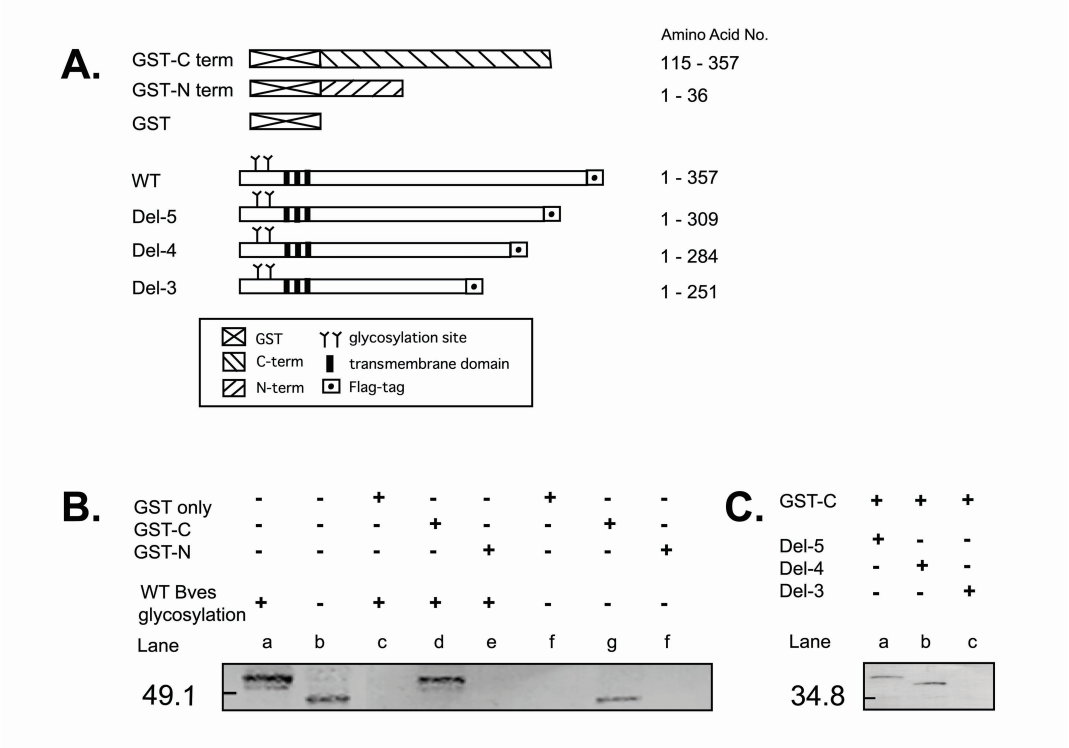


Figure 5.1 Bves constructs and GST pull-down assay. A, Diagram of GST-fusion proteins and deletion constructs. GST C- and N-terminal Bves proteins and serially deleted Flag-tagged Bves proteins. B, GST pull-down assay with C- or N-terminal Bves. Bacterial lysates of GST-Bves fusion proteins were reacted with COS-7 cell lysates transfected with Flag-tagged WT Bves and analyzed with Western blots using an anti-Flag antibody. The potential effects of glycosylation were also tested. Control band (WT Bves protein with or without glycosylation) are shown on lane a and b. Only GST C-terminal Bves reacted in this assay regardless of glycosylation state (lanes d and g) C, Serial deletion analysis of C-terminal interaction. GST C-terminal Bves was reacted with Del-5 Bves, Del-4 Bves and Del-3 Bves and processed for Western blotting to detect interactions. Del-5 Bves and Del-4 Bves (lanes a and b) interact with the GST C-terminal Bves while Del-3 Bves does not.

(Del-4 Bves) had no effect on precipitation efficiency (Figure 5.1C) but elimination of the next 33 amino acids (Del-3 Bves) completely abolished C-terminal interactions. This construct, Del-3 Bves, was used further as a non-interacting control in subsequent studies. These data are the first to identify Bves-Bves homodimerization.

Amino acids K^{272} and K^{273} are critical for Bves-Bves interaction

To further define the domain responsible for Bves-Bves intracellular interaction, a solid phase SPOTs methodology was employed. 13-mer peptides were synthesized from position 232 to 301 from the intracellular tail of Bves encompassing the putative interaction domain (neighboring peptides have 10 amino acid overlap, Figure 5.2A). These peptides were incubated with either WT Bves or Del-3 Bves that is missing the putative interaction domain. As seen in Figure 5.2B, WT Bves binds peptides 11-13 as predicted from the liquid phase precipitation analysis as these peptides lie within the 33 aa interaction domain. The apparent reactivity around, not within SPOTs 2 and 10 are spurious and do not appear in other reactions with the membrane. An additional, unpredicted interaction is detected in peptide 18. When the same blot is reacted with Del-3 Bves (Figure 5.2C), no reaction with peptides 11-13 was observed while reactivity with peptide 18 remained. WT Bves binding with peptide 18 may be a non-specific reaction or represent additional Bves-Bves interaction independent of aa 268-274.

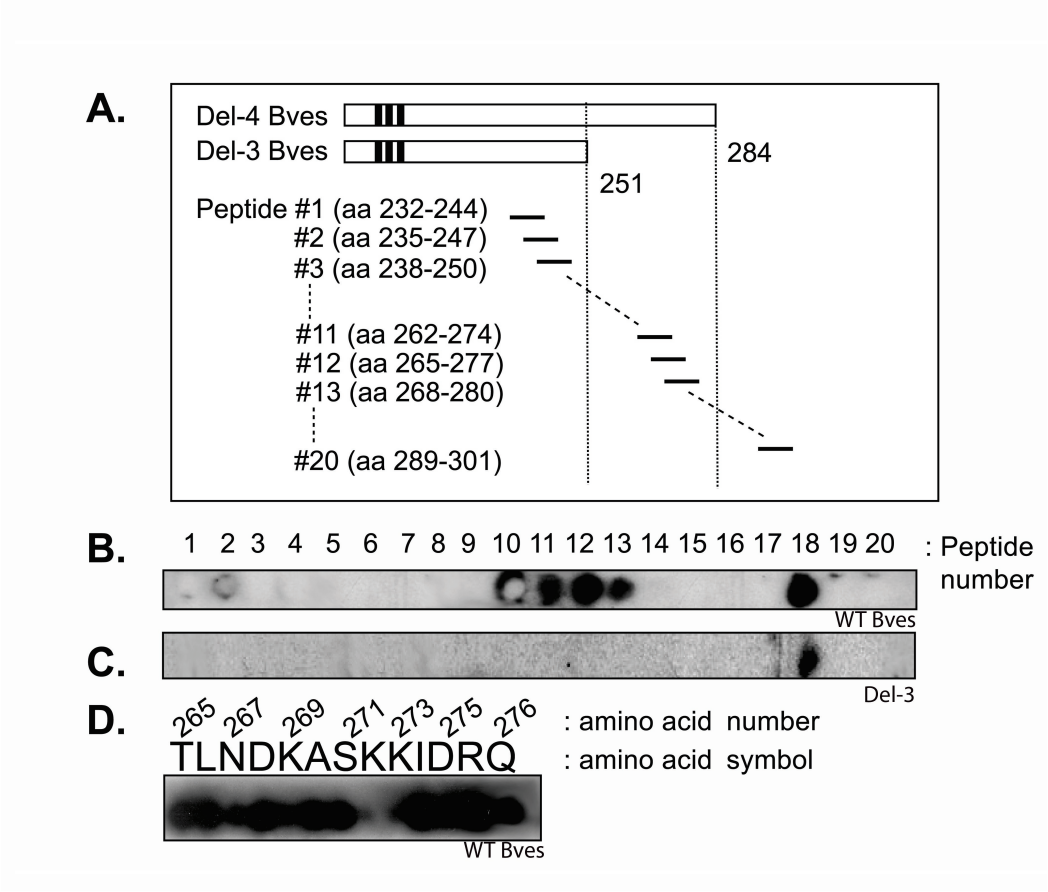


Figure 5.2 SPOTs protein mapping. A, Diagram of SPOTs blot (synthesized peptides). Twenty 13-mer peptides (SPOTs) were synthesized from the sequence of C-terminal Bves (amino acids 232-301) and fixed on a cellulose membrane for reaction with WT Bves or Del-3 Bves. B, Spots binding assay with WT Bves. A binding with WT Bves is clearly detected with peptides 11-13. Peptide 10 is likely a false positive, since the signal appears circumferential to the spot of protein. An additional unpredicted reaction is detected with peptide 18. C, Spots binding assay with Del-3 Bves. No binding of Del-3 Bves is detected with peptides 11-13 while peptide 18 is reactive suggesting a non-specific or interaction domain-independent association. D, Alanine substitution analysis. Numbers over the amino acid symbols are the amino acid number of WT Bves. Peptide 12, that showed positive binding at panel B was substituted at each amino acid position with alanine and incubated with WT Bves cell lysate. Substitution of lysines at positions aa 272 and aa 273 with alanine abolished binding with WT Bves protein.

Next, to determine whether specific amino acids are required for binding activity, alanine substitution of individual amino acids across this core element was conducted and the resulting peptides were reacted with WT Bves. Figure 5.2D shows that lysine to alanine substitution at positions 272 and 273 eliminates Bves binding to the core element peptide. Interestingly, peptides 11 to 13, which are reactive in SPOTs analysis, are the only targets that contain both K^{272} and K^{273}. These data indicate that aa 268-274 are required for at least one Bves-Bves interaction and that K^{272} and K^{273} are critical for this function.

The intracellular interaction domain is essential in Bves-mediated cell-cell adhesion

L-cells have been used to demonstrate adherent properties of transfected gene products (Thorson et al, 2000; Wada et al, 2001). As seen in Figure 5.3, non-transfected cells (3A) are non-adherent while transfection of WT Bves (3B) induces cell clustering as previously seen in Wada et al. We next explored whether the specific amino acids determined to be essential for C-terminal molecular interaction were critical for Bves adhesive function at the cellular level. L-cells were transfected with the two mutated forms of Bves, KK-Mutant (KK-Mut) Bves (Figure 5.3C) and KK-Deletion (KK-Del) Bves (Figure 5.3D). Both of these transfected cell lines failed to aggregate above levels seen in non-transfected parental cells (compare 3A, C and D). Taken together, these data demonstrate that mutation of the newly-identified Bves-Bves intracellular interaction domain abolishes the adhesive function.

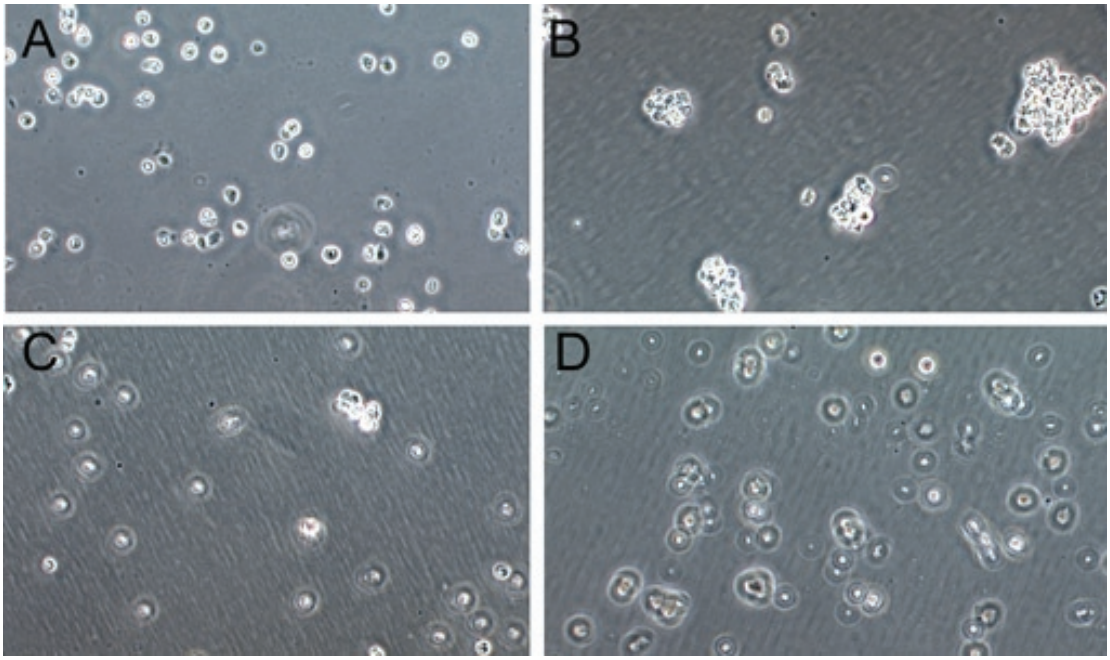


Figure 5.3 L-cell aggregation assay. Aggregation assays with various cell lines were performed using a standard hanging-drop method. A, Parental L-cells. B, L-cells transfected with WT Bves, C, KK-Mut Bves and D, KK-Del Bves. (Scale Bar 50 mm)

Expression of mutated Bves inhibits formation and stability of epithelial sheets

We next determined whether expression of mutated Bves molecules would disrupt native adhesive properties in epithelia using a human corneal epithelial cell line (HCE). Multiple stable HCE cell lines expressing WT Bves, KK-Mut Bves and KK-Del Bves were isolated and produced consistent results. As seen in Figure 5.4A in phase microscopy, parental HCE lines form confluent epithelial monolayers when seeded at a high density. HCE cells transfected with WT Bves (Figure 5.4B) also develop confluent monolayers, however, areas exhibiting very densely packed epithelium are observed (arrowhead). Cells expressing a mutated form of Bves, KK-Mut Bves, do not form completely confluent monolayers, even when seeded at high densities. Irregular gaps in the epithelium are observed (arrow Figure 5.4C), and cells appear rounded. A more pronounced phenotype is seen in cells expressing KK-Del Bves (Figure 5.4D). This cell line never forms a confluent epithelium. Several gaps between cells are observed (arrows) as these cells are unable to form a monolayer.

Using HCE cells as a model, we have shown that transfected WT Bves is properly trafficked to and maintained at the cell membrane (merge in Figure 5.5). It should be noted a minor but consistent population of endogenous Bves molecules, identified positive staining with anti-Bves antibodies (Figure 5A) and negative staining with anti-Flag (arrows, Figure 5.5B).

To detect transfected protein and endogenous Bves, cells were stained with anti-Flag and anti-Bves antibodies. Transfected WT Bves is detected at the cell membrane in these lines, and in areas of confluence, staining of both

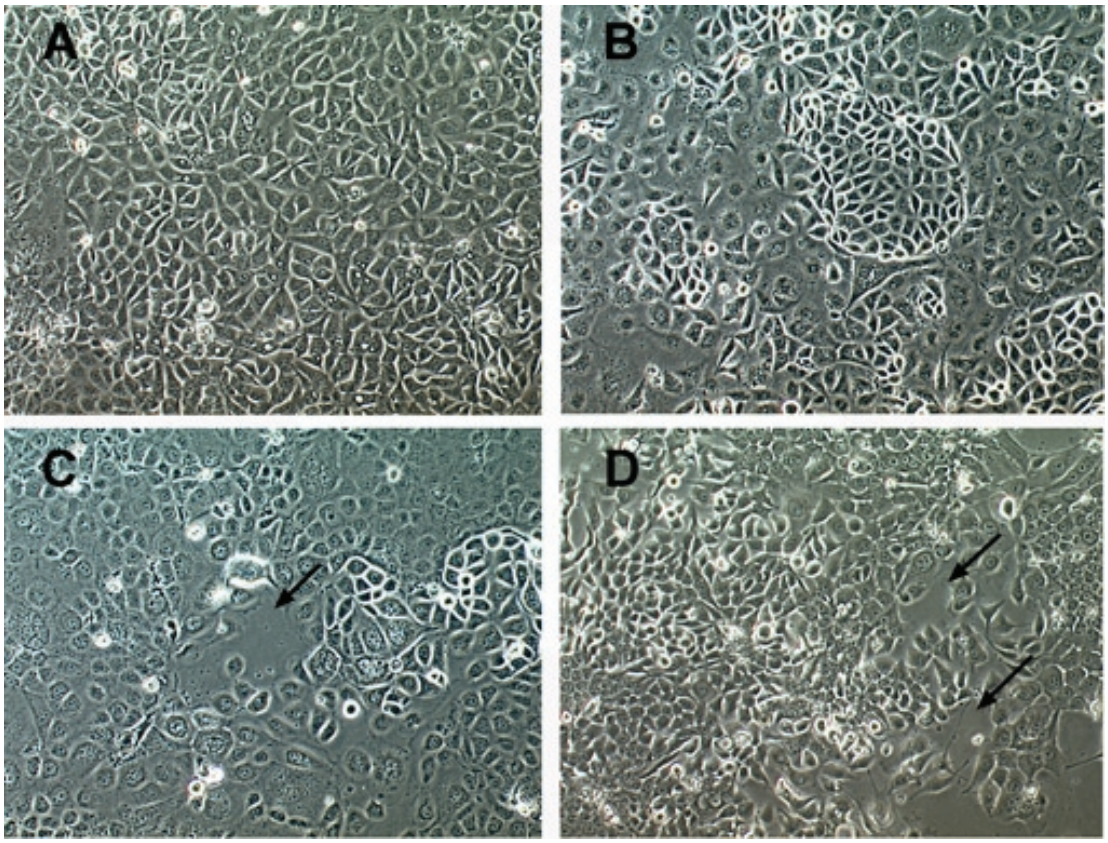


Figure 5.4 Phase contrast of transfected human corneal epithelial (HCE) cells. Non-transfected parental cells (panel A) form confluent epithelial sheets, as do HCE cells transfected with WT Bves (panel B). However, in WT Bves transfected cells, areas of tight adherence are readily observed (arrow head). In HCE cells transfected with mutant Bves, a contiguous monolayer is rarely formed. KK-Mut Bves transfected cells are more rounded and moderate gaps are viewed (panel C, arrows), while KK-Del transfected cells display distinct gaps.

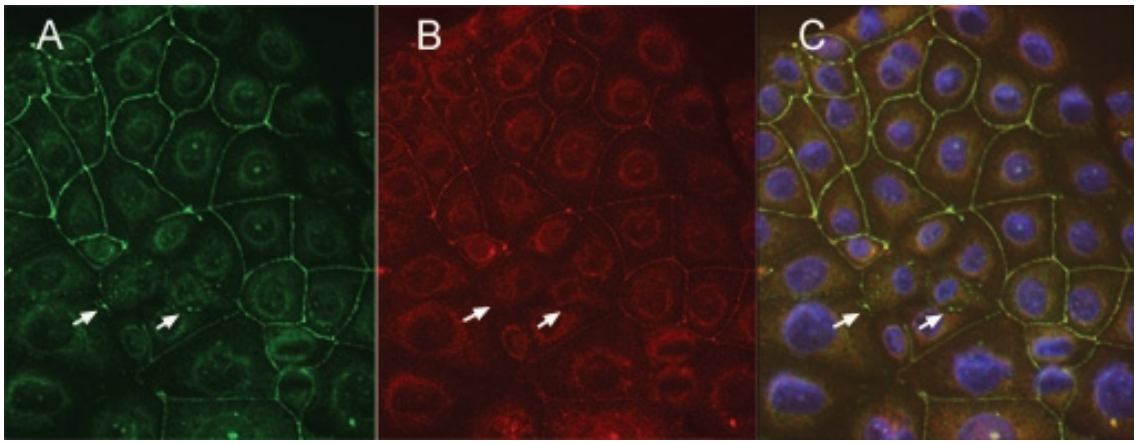


Figure 5.5 Distribution of transfected WT Bves in HCE cells. A stable cell line expressing WT Bves was grown to confluence and examined for Bves expression and distribution. Panel A demonstrates distribution of anti-Bves staining while B displays anti-Flag staining. Note that most staining overlaps (Panel C, merge) but small areas are observed that are positive for anti-Bves and negative for anti-Flag (arrows in A-C).

antibodies is observed around the entirety of cells. Intracellular staining of transfected and endogenous protein were also observed (Figures 5.5 and 5.6, panel J). Expression of either KK-Mut Bves or KK-Del Bves disrupts the pattern of anti-Bves staining in cells at high density. Flag-tagged protein is rarely observed at the cell surface and accumulates intracellularly (Figure 5.6G, H). It should be noted that all images were exposed and electronically processed identically. Thus, the reduced intensity of staining in Figure 5.6F and J can be compared to the greater signal observed in Figures 5.6G and H. In addition, the expression of a KK-Mut Bves inhibits trafficking and/or accumulation of endogenous Bves at the cell surface (Figure 5.6K). Still, in isolated areas, anti-Bves staining without overlapping anti-Flag staining was observed at the cell surface between adjacent cells indicating the presence of endogenous protein in the absence of transfected Bves. Anti-Flag immunoreactivity around the entire cell was rarely observed. In KK-Del Bves transfected cells distinct circumferential anti-Bves staining was also difficult to detect. These cells appeared smaller and had a significant intracellular accumulation of transfected proteins.

To determine whether mutations of Bves at positions 272 and 273 disrupt junctional adhesive complexes, cultures were assayed for the distribution of known junctional proteins. As seen in Figure 5.7, ZO-1 and E-cadherin, components of the tight and adherens junctions, respectively, are drastically redistributed in cell lines expressing exogenously mutated forms of Bves. ZO-1 staining is seen in small patches but not with that of the altered forms of the

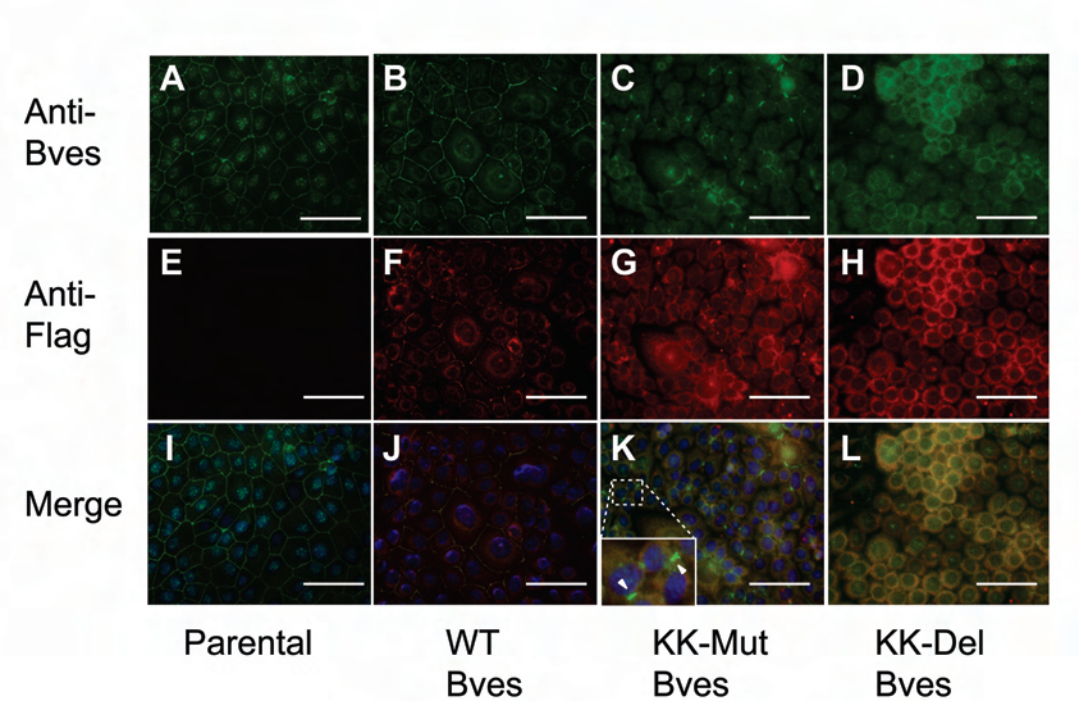


Figure 5.6 Detection of endogenous and transfected Bves in human corneal epithelial cells (HCE). Anti-Bves staining (top panels, A-D), anti-Flag staining (middle panels, E-H) and merge (bottom panels, I-L) are shown. A, Endogenous Bves is detected at the parental cell membrane in confluent monolayers. E, Parental cells do not react with anti-Flag. B, Endogenous Bves is expressed in WT Bves transfected cells. F, Transfected WT Flag-tagged Bves traffics to the cell membrane J, where it co-localizes with anti-Bves staining, which detects endogenous protein. C, G, K, Transfection of KK-Mut Bves and D, H, L, KK-Del Bves show a general loss of membrane staining for both endogenous and transfected protein. Inset K, importantly, sporadic green staining at the membrane is seen in merged images with transfected KK-Mut Bves suggesting localization of endogenous but not transfected protein. (Scale Bar 100 mm)

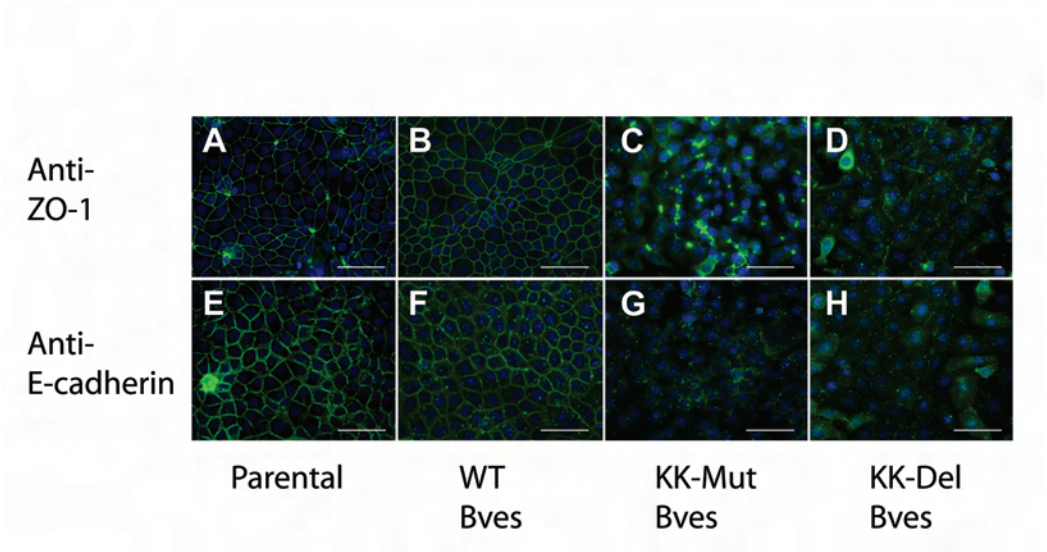


Figure 5.7 Expression of ZO-1 and E-cadherin in HCE cells. A, E, Parental HCE cells B, F, HCE cell lines transfected with WT Bves, C, G, KK-Mut Bves, and D, H, KK-Del Bves were stained with ZO-1 in panels A through D and E-cadherin in panels E through H. Note the loss of peripheral ZO-1 and E-cadherin staining in KK-Mut Bves and KK-Del Bves cells. (Scale Bar 100 mm)

protein. Circumferential staining of ZO-1 and E-cadherin were rarely if ever detected in cells expressing KK-Mut Bves or KK-Del Bves.

TER is a standard measurement of epithelial function and tight junction integrity. In order to determine the effect of mutation or substitution of K^{272} and K^{273} on tight junction activity, HCE cell lines stably transfected with WT Bves, KK-Mut Bves and KK-Del Bves were grown to confluence and assayed for TER (Figure 5.8). Transfected cultures were compared to the non-transfected parental cell line. A resistance of ~350W・cm^2 was observed with non-transfected controls while transfection with WT Bves produced elevated resistance. When we measured TER in HCE cell lines expressing KK-Mut Bves or KK-Del Bves, values were dramatically reduced to near background levels. TER for KK-Mut Bves [p-value < 0.0001, CI (0.07, 0.14)] and KK-Del Bves [p-value < 0.0001, CI (0.07, 0.15)] are significantly reduced compared to the control parental group, while TER for WT Bves is significantly greater than those of the control parental group [p-value = 0.006, CI (1.08, 2.28)]. Taken together, these data suggest the expression of Bves with mutation in the putative intracellular interaction domain produced marked disruption of epithelial structure and function.

Expression of mutated Bves leads to changes in epithelial cell phenotype

Alteration of cell-cell adhesion/interaction can lead to changes in cell phenotypes, most notably through processes involving epithelial-mesenchymal transition (Reese et al, 2002). Control parental and WT Bves-transfected cells expressed cytokeratin (Figure 5.9) and other markers of the epithelial phenotype

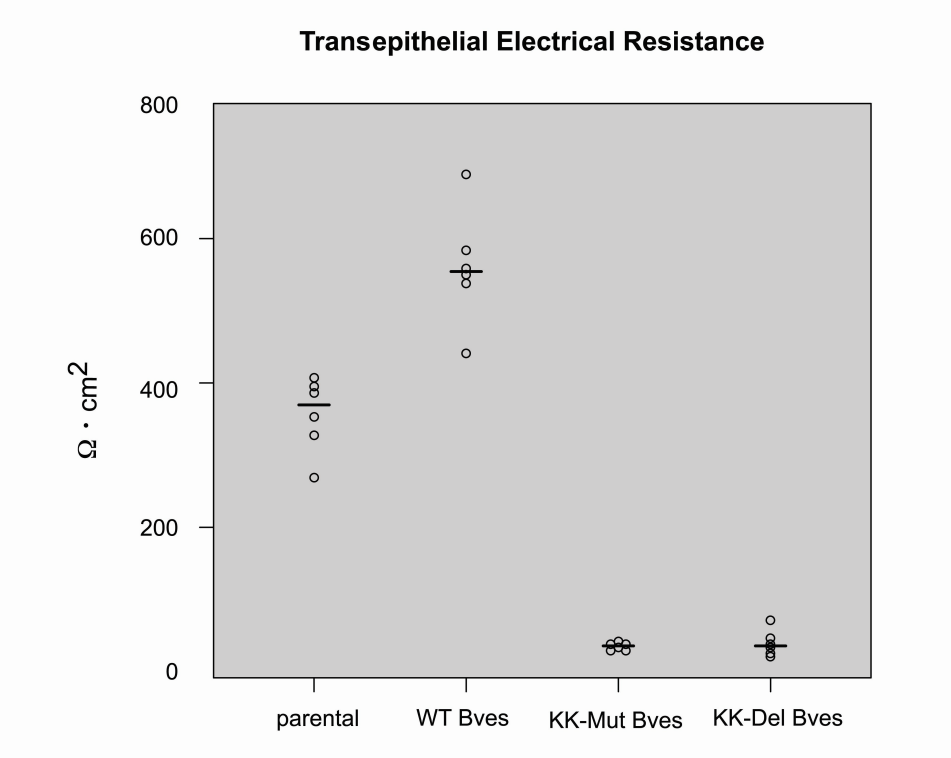


Figure 5.8 Measuring of transepithelial electrical resistance in HCE cells. Parental cells and cells expressing transfected WT Bves, KK-Mut Bves and KK-Del Bves were grown to confluence and assayed for transepithelial electrical resistance (TER) using EVOM volt-ohm-meter. Expression of transfected WT Bves increases TER. Conversely, cells expressing KK-Mut Bves or KK-Del Bves have TER values approaching background. The horizontal line indicates the median.

with no expression of mesenchymal phenotype. In contrast, cells stably transfected with KK-Mut Bves expressed and accumulated vimentin, a marker of mesenchymal phenotype (Hay, 2005). The number of cells with this phenotype was constant but low (Figure 5.9G). Conversely, a high percentage of KK-Del Bves expressing cells exhibited a mesenchymal phenotype even under culture conditions that favor maintenance of the HCE epithelial phenotype (Figure 5.9H). These data suggest that mutation of the Bves-Bves intracellular interaction domain leads to changes in cells consistent with a mesenchymal phenotype.

Discussion

Bves is a highly conserved transmembrane protein with cell-cell adhesion function that is expressed in a variety of epithelia and muscle from flies to humans. Previous studies suggest that the function of the *Popdc* gene family of which Bves is a prototypical member has great implications for development and disease. Still, the molecular mechanisms underlying this function are completely unknown. Additionally, no protein motif or domain has been identified to account for any activity of this gene family. Here for the first time, we demonstrate Bves-Bves interaction and identify a functional domain in the Bves molecule that regulates this process. Further, we show that mutation or deletion of specific amino acids within this domain abolishes the cell-cell adhesion mediated by the molecule and leads to predictable changes in cell phenotype. These novel studies identifying Bves-Bves interaction and its regulation have larger implications for the understanding of Bves function in development and disease.

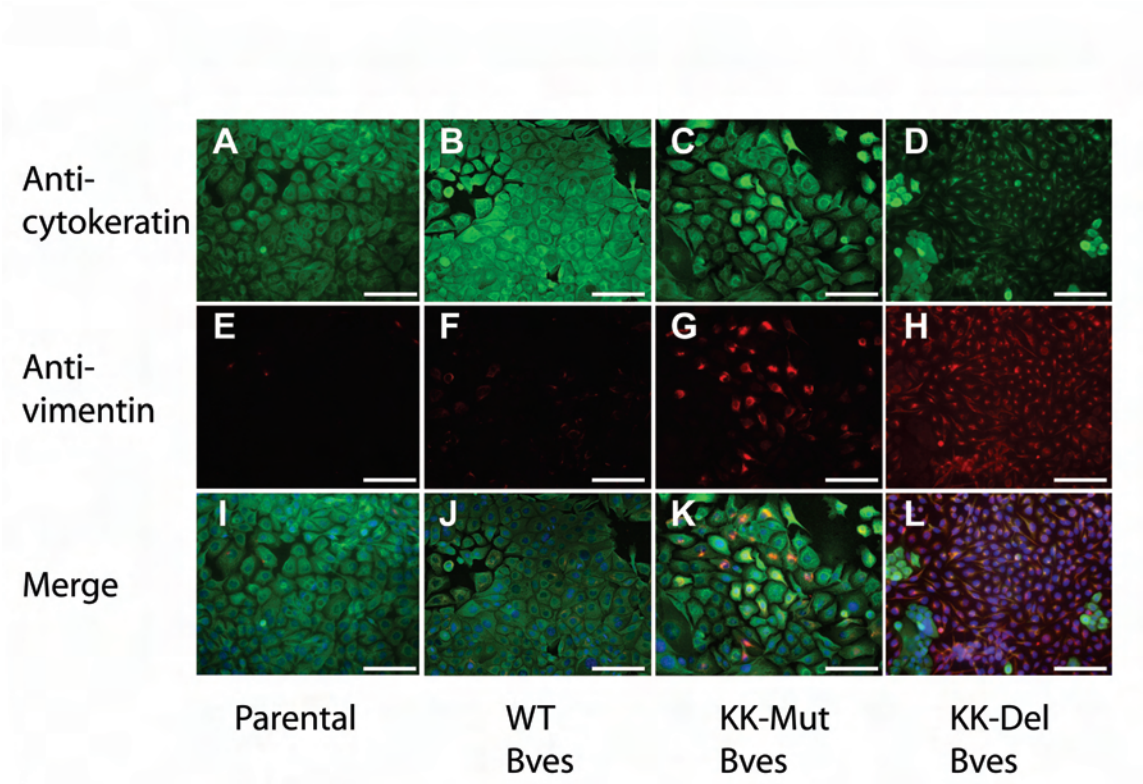


Figure 5.9 Immunostaining with anti-cytokeratin and anti-vimentin. Immunostaining with anti-cytokeratin (panels A through D), anti-vimentin (panels E through H) and merge (panels I through L) are given. A, E, I, Parental cells show high levels of cytokeratin and little vimentin staining. Transfection with WT Bves (B, F, J) shows similar staining patterns as parental cells. In contrast, cell lines expressing KK-Mut Bves (C, G, K) have a subpopulation of vimentin-positive cells and KK-Del Bves expressing cells (D, H, L) show nearly complete conversion to a vimentin-positive phenotype. (Scale Bar 100 mm)

Identification of an intracellular interaction domain in Bves

While members of the *Popdc* family are highly conserved, neither Bves nor any other family member harbors a predicted protein motif that would account for its cell-cell adhesive function (Andree et al, 2000; Reese & Bader, 1999; Reese et al, 1999). However, the presence of an intracellular Bves-Bves interaction domain responsible for membrane clustering of the molecule had been previously suggested by Professor Thomas Brand (Brand, 2005). Here, our truncation analysis of the C-terminus demarcates a region (aa 252-284) that mediates Bves-Bves intermolecular interaction and is sufficient for at least one homophilic binding event (Figures 5.1 and 5.2). It should be noted that the present study does not preclude the presence of additional Bves-Bves C-terminal interactions. Within this newly identified domain, lysines at positions 272 and 273 are critical for molecular interaction and are conserved in Bves protein sequences in mouse, human and chicken (NCBI). The identification of an intracellular domain that is essential for intercellular adhesive activity is not unprecedented, as an intracellular juxtamembrane domain in E-cadherin is essential for its intercellular adhesion function (Nagafuchi & Takeichi, 1988; Ozawa & Kemler, 1998a; Ozawa & Kemler, 1998b). As no previous studies have ascribed function to any motif within Bves, identification of this intracellular domain is critical for an understanding of the function of this molecule.

Mutation of Bves disrupts cell-cell adhesion

Expression of mutated Bves does not promote cell clustering in L cells in contrast to expression of WT Bves controls (Figure 5.3). Also, expression of mutant constructs greatly inhibits adhesion properties in normally adherent HCE cells (Figure 5.4-5.8). These assay systems provide strong corroborating data that suggest a functional significance for this intracellular interaction domain in cell-cell adhesion. We propose that over-expression of KK-Mut Bves or KK-Del Bves acts in a dominant-negative or interfering fashion in adherent cells that normally express Bves.

Additionally, immunofluorescence analysis of HCE cells expressing transfected WT Bves detects the molecule at the cell surface along with components of the adherens and tight junctions (Figure 5.7) and expression of WT Bves increases TER in transfected cells (Figure 5.8). In contrast, over-expression of either mutant form of Bves inhibits a function of the endogenous Bves protein as epithelial sheet integrity is compromised. It is interesting to note that only small puncta of endogenous Bves are observed at the cell surface (Figure 5.6K). Additionally, surface staining of adhesion molecules such as ZO-1 is punctate and also greatly diminished in cells expressing mutated Bves (Figure 5.7). These results indicate that: 1) the KK-containing domain is important for proper trafficking and/or stability of Bves since KK-mutated Bves disturbs membrane localization of endogenous Bves, and/or 2) the KK-containing domain is involved in regulating the membrane localization of other adhesion molecules. Therefore it is plausible that inhibition of Bves-Bves intracellular interaction in

turn disrupts the generation and/or maintenance of cell-cell adhesion. Bves is one of the first molecules to traffic to points of cell-cell contact and it interacts with ZO-1 a known mediator of cell-cell adhesion (Osler et al, 2005; Wada et al, 2001). Disruption of either one or both of these functions could inhibit the assembly or stability of forming junctions. This may lead to the loss of adhesion protein localization at the membrane and the observed drop in TER in epithelial cells expressing mutated Bves. These data support the hypothesis that Bves is a critical regulator of cell-cell interaction and clearly delineates essential intra-cellular interaction in the process. From these new data, we postulate that the intracellular interaction domain is critical for clustering of Bves molecules and that this aggregation is important for intercellular interaction functions of Bves and/or association with other protein components of cell-cell junctions.

Inhibition of Bves function leads to changes in cell phenotypes

A key finding in the present study is that cells expressing mutated Bves exhibit an altered phenotype. Many, but not all, epithelial cells expressing this molecule take on varying morphologies and express vimentin, a marker of the mesenchymal phenotype (Hay, 2005). These HCE cells, which normally do not exhibit mesenchymal behavior, take on this fate with altered Bves function and the lack of membrane localization and/or stability of the protein. This finding mimics the situation observed for Bves during coronary artery differentiation. There we have observed that, adherent epithelial cells of the developing epicardium express Bves at the lateral cell surface (Wada et al, 2001). When

individual cells undergo epithelial-mesenchymal transition, Bves is removed from the cell surface along with other adhesion molecules and cells assume a mesenchymal phenotype. It is possible that expression of mutated Bves inhibits cell-cell adhesion resulting in the production of mesenchyme not unlike that observed during those processes observed in vivo. Thus, it is possible that the regulation of cell-cell adhesion is controlled in part through inter-molecular association governed by the Bves intracellular interaction domain.

CHAPTER VI

XENOPUS LAEVIS EMBRYOGENESIS: BVES DISRUPTION

This study is ongoing and with future research will be compiled into a manuscript for publication.

Abstract

Epithelial sheet movement and integrity are critical for proper embryonic development and maintenance. Due to the role Bves plays in regulating cell motility and adhesion, the function of Bves was examined in the development of *Xenopus laevis*, where meticulous regulation of epithelial tissue movement and differential adhesion is imperative. Using a Morpholino knockdown and rescue strategy, we demonstrate that disruption of Bves results in defects at the single cell, organ, tissue, and organism levels. Specifically, Bves-depletion leads to severe membrane blebbing of mesodermal cells during movement; disruption of epithelia, characterized by a hypomorphic eye and a remarkably thickened epidermis; and severe morphological defects such as a shorter Anterior-Posterior axis and a curved body. Additionally, we demonstrate that the most conserved region of Bves protein, the Popeye domain, and the extracellular N-terminus are necessary for Bves function. Taken together, these data demonstrate that Bves is important for the development of *X. laevis*, and the phenotypes observed upon Bves depletion in the embryo confirm and extend previous *in vitro* reports of Bves

function. This ongoing study will next examine the effects of Bves depletion on epithelial components of the heart, as Xbves is highly enriched in this organ.

Introduction

For a number of years, *Xenopus laevis* (*X. laevis*) has been the standard amphibian model system in embryogenesis. Many classic studies of developmental biology have utilized this model organism to understand embryonic patterning, cell movement, organogenesis, and regeneration (Slack, 2006). *X. laevis* is cost-effective to maintain, lives for several years, can be induced to spawn at any time of the year, and produces massive quantities of eggs. These oocytes can be fertilized *in vitro* and exploited with a variety of surgical manipulations (Sive et al, 1998). *X. laevis* cells are densely packed with yolk granules and can thus survive outside the embryo in simple buffers, making them ideal for studying specific groups of primary cells (Slack, 2006). Most recently, with the introduction of *Xenopus tropicalis* and the adaptation of techniques in *X. laevis*, these model systems are being used for genetic studies that were previously too complex in *X. laevis* due to its allotetraploid genome (Amaya et al, 1998; Khokha et al, 2009; Kroll & Amaya, 1996).

One of the biggest advances in studying *X. laevis* gene disruption was the discovery of synthetic antisense oligonucleotides, called Morpholinos (MO) (Harland & Weintraub, 1985; Melton, 1985). These molecules specifically inhibit the translation of mRNA, thus allowing the study of development with inhibition of a particular gene of interest. MO disruption in an embryo must be tightly

controlled using standard assays to determine off-target effects that might alter development (Eisen & Smith, 2008). The gold standard control is expression of mRNA that is unrecognizable by the MO and thus restores the previously disrupted function to the embryo. This 'rescue' experiment must be conducted to ensure the specificity of a particular MO in recognizing the target mRNA.

Blood vessel/epicardial substance (Bves) is a transmembrane protein that is a member of the Popdc family of proteins (Hager & Bader, 2009). There are three family members (Bves, Popdc2 and Popdc3) and all share the highly conserved Popeye domain. Although conserved across all species, this domain shares no significant homology with any other characterized protein motif, thus making it difficult to predict function from structure (Hager & Bader, 2009). It has been shown that Bves interacts with itself via two conserved lysines found in mouse, human, and chick, and that this homophilic interaction is important for Bves function (Kawaguchi et al, 2008). Conversely, although Bves has been shown to interact with different proteins (outlined below and in Chapters III and IV), and these studies have convincingly demonstrated Bves function, it is not known which domains of Bves protein are crucial for interaction and subsequent functions in regard to these interacting proteins.

Bves has an established role in maintaining epithelial cell junctions, and when Bves is disrupted, the trans-epithelial resistance (which is a general indicator of epithelial integrity) is severely attenuated. Additionally, Bves is important for the localization of junctional proteins, such as E-cadherin, at the membrane (Osler et al, 2005). Recently, it has been shown that Bves interacts

with GEFT, a Rho GTPase exchange factor that catalyzes the exchange of GDP for GTP to activate Rho GTPases Rac1 and Cdc42. Disruption of Bves-GEFT results in decreased Rac1 and Cdc42 activity and corresponds to decreased actin-based filopodial and lamellipodial protrusions during cell movement (Guo et al, 2003; Smith et al, 2008).

In the early development of *X. laevis*, Xbves is the only homologue expressed and can be specifically knocked down using MO and then rescued with Xbves mRNA (Ripley et al, 2006). Disruption of Bves in the frog embryo results in rogue cell migration that is indicative of a general impairment of the gastrulation movements epiboly and involution. In epiboly, an epithelial sheet migrates towards the yolk plug, enveloping the embryo and eventually closing the blastopore (Keller, 1980). As Bves is important for epithelial junction stability, it is not surprising these movements are impaired when Bves is knocked down (Osler et al, 2005). During involution, migrating cells dive into the embryo at the dorsal blastopore lip and begin moving anteriorly across the blastocoel roof (BCR) (Keller, 1975). Further analysis of Bves depletion during involution demonstrates that head mesoderm (HM) cells are severely disorganized as they migrate across the BCR using integrin-mediated adhesion (Chapter IV). When plated *in vitro*, these same cells remain rounded and have difficulty adhering to a fibronectin (FN) substrate, which is indicative of impaired integrin function. Bves has also been shown to interact with VAMP3, a SNARE protein that mediates the recycling of β-1 integrins (Chapter IV). Thus, when this Bves-VAMP3 interaction

is disrupted in *X. laevis*, integrin-dependent cell movements during involution are impaired.

In this ongoing study we extend previous reports of Bves function to examine more closely the role Bves plays during the development of *X. laevis*. We demonstrate that disruption of Bves in HM cells results in severe membrane blebbing as seen previously *in vitro*; this phenotype is consistent with breakdown of Rho GTPase-modulated actin dynamics (Fackler & Grosse, 2008). We investigate the vast epithelial defects observed in Bves-depleted tadpoles and reveal that disruption of epithelial integrity is readily observed *in vivo*. This finding is in accordance with previous reports (Osler et al, 2005). Finally, we demonstrate through MO knockdown and rescue that the highly conserved Popeye domain (Hager & Bader, 2009) is necessary but not sufficient for Bves function. Additionally, the extracellular N-terminus, which is highly heterogeneous across species, is required for Bves function during development of *X. laevis*.

Materials and Methods

Antibodies and Morpholino

All antibodies and stains were obtained commercially as follows: E-cadherin (BD Laboratories, 610181); phalloidin (Molecular Probes, A12379, A12380); and Phospho-Histone H3 (PH3, Upstate Biotech, 06570). Membrane GFP (mGFP) is described in (Wallingford et al, 2000). Bves MO (Gene Tools,

LLC) and full-length rescue mRNA were described previously (Ripley et al, 2006).

X. laevis Embryos and Microinjections

Female *X. laevis* were obtained from Nasco and induced to ovulate by standard methods (Ripley et al, 2006). Embryos were fertilized *in vitro*, dejellied in 1% thioglycolic acid, and maintained in 1X – 0.1X Steinberg's Solution (SS); embryos were staged according to Nieuwkoop and Faber (Nieuwkoop & Faber, 1994). Embryos were microinjected with 5nL into both cells at stage two for membrane bleb analysis or one of two cells at stage two for all other assays; embryos were injected in 5% Ficoll in 1X SS, then switched to 0.1X SS before gastrulation. Bves MO or control MO (COMO) were injected into sister embryos along with mGFP (100pg) as a tracer at a concentration of 20ng (stage 35-42 analysis only) or 40ng/embryo (stage 10-12 analysis; Gene Tools, LLC) (Ripley et al, 2006; Wallingford et al, 2000).

Tissue Processing, Image Acquisition, and Data Analysis

Embryos were fixed in Mempha (Cold Spring Harbor Protocols) and whole mount pictures were captured with Magnafire (Olympus America Diagnostics). Histological analysis was carried out with standard hematoxylin and eosin staining (Ripley et al, 2006). Unless otherwise noted, all data was analyzed with Microsoft Excel.

Scanning Electron Microscopy (SEM)

Embryos were fixed in 2.5% glutaraldehyde in PBS and the involuting head mesoderm was dissected out (the overlying ectoderm or BCR was peeled away from this region to expose the surface of the cells that are attached to FN) with eyebrow knives. Specimens were post-fixed in 1% OsO_4, dehydrated in graded ethanol washes, and critically point dried. Specimens were then sputter coated with gold/palladium and imaged. SEM images were quantified as follows: membrane blebs (defined as spherical protrusions with a 2-dimensional diameter greater than one μm) from 11 different cells of multiple embryos were counted and measured for both control and experimental groups and then averaged to determine membrane blebs/cell and membrane bleb size.

Microdissections and Plating on Fibronectin

Microdissections of *X. laevis* embryos were carried out according to Ren et al., 2006 (Ren et al, 2006a). Briefly, the involuting HM was dissected at stage 10.25 in modified Barth's solution (MBS) using eyebrow knives. Explants were disassociated in Ca^{2+} Mg^{2+} free MBS and single cells from several embryos (control or experimental) were pooled and plated in MBS on MatTek dishes coated with 200μg/mL of FN (Sigma F4759). All FN-coated plates, dissection tools, and apparatuses were blocked with 5% BSA. Cells were cultured for two hours, washed three times in MBS to remove non-adhered cells, fixed in PFA overnight at 4° C, labeled with phalloidin, and imaged with a Zeiss Inverted LSM 510 Confocal Microscope using a 40X objective.

Cell Death and Proliferation

To assay for cell death, we used Terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) on whole mount embryos according to (Hensey & Gautier, 1998). Detection of proliferating cells was conducted using PH3 antibody. Briefly, embryos were microinjected with Bves MO or COMO into one of two cells as described above, flash frozen at stage 35-41, cryosectioned, and serial sections were labeled with PH3 antibody. Proliferating cells were counted on both the injected (the injected side was confirmed with mGFP labeling) and the non-injected control side in both Bves MO and COMO treated embryos; injected vs. non-injected sides were compared.

Rescue of Bves MO phenotype using truncation constructs

Truncations were prepared using PCR to propagate Xbves sequences of varying lengths from a *X. laevis* cDNA library. Six PCR products were created, each with a mutated MO binding site (the region directly upstream of the ATG site) at the 5' end, and a flag tag at the 3' end. Construct #6 (Figure 6.7) was missing the N-terminal region (the extracellular portion of the protein), which contains the ATG site, thus an artificial ATG site was engineered in frame. PCR products were cloned in frame into the multiple cloning site of PCR-SCRIPT (Stratagene). mRNA was produced from DNA constructs using mMESSAGE mMACHINE (Ambion), and concentrations were determined using a spectrophotometer. For rescue experiments, 100pg of mRNA was injected along

with 20ng of Bves MO into one of two cells. Phenotypes were classified and quantified according to the chart in Figure 6.2B. Rescue classifications are defined as follows: (observed X%) of embryos were indistinguishable from controls, with the remaining (observed X%) having a less severe phenotype. Full Rescue: > (80%), (20%); Almost Full Rescue: > (60%), (25%); Partial Rescue: > (35%), (40%); Little to Partial Rescue: > (5%), (50%).

Results

Membrane blebs are observed in involuting head mesoderm cells both *in vivo* and *in vitro*

As detailed in Chapter IV, Bves-depleted HM cells are severely disorganized *in vivo* and are impaired in their ability to adhere and spread on a FN substrate. In further examination of these cells using SEM, it was observed that HM cells displayed an increased number of membrane blebs when compared to controls (Figure 6.1B, Bves-depleted vs Figure 6.1A, COMO control). Bves MO treated embryos exhibited 5.2 blebs/cell, while COMO treated embryos displayed only 0.27 blebs/cell (Figure 6.1E). Membrane blebs in Bves-depleted cells were also +0.6 μm greater in size than COMO treated cells (Figure 6.1F). Membrane bleb formation upon disruption of Bves was also observed *in vitro*. When plated on FN, blebs (visualized with phalloidin) in Bves-depleted HM cells were readily observed (Figure 6.1D), but were not seen in COMO treated cells (Figure 6.1C). Membrane blebs (Figure 6.1B, D, arrows) are associated with localized breakdown of the actin-cytoskeletal network (Fackler & Grosse, 2008),

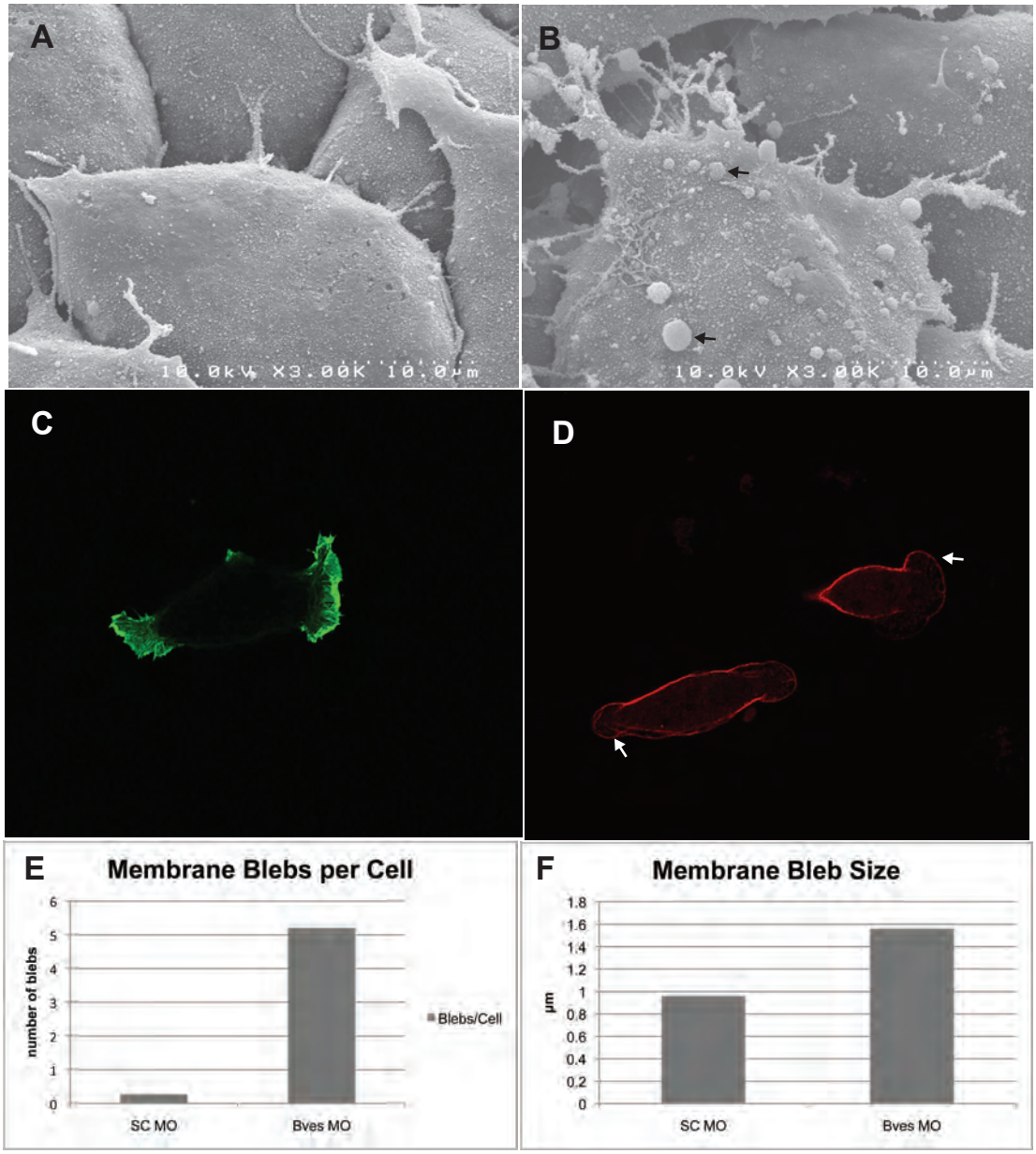
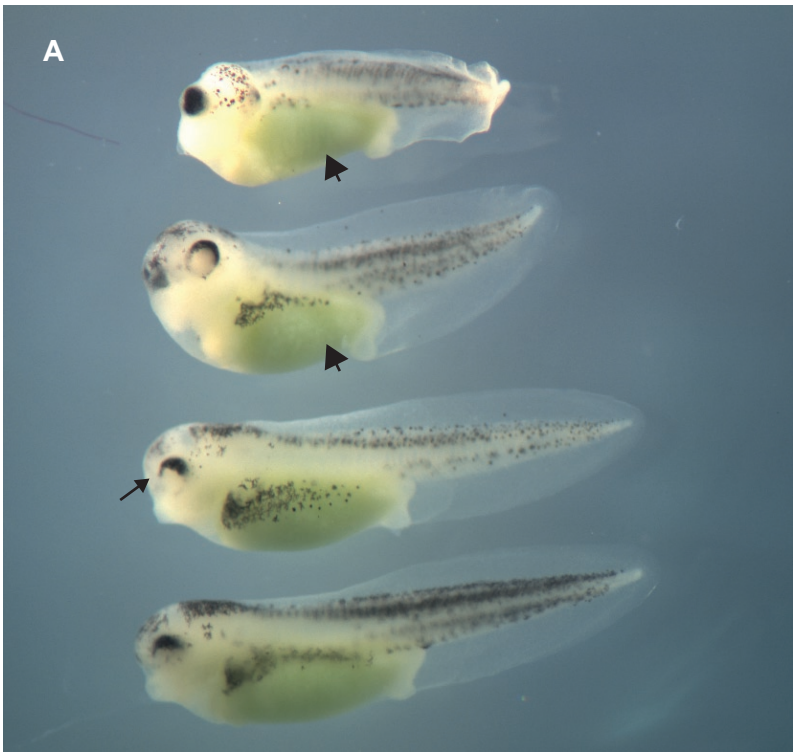


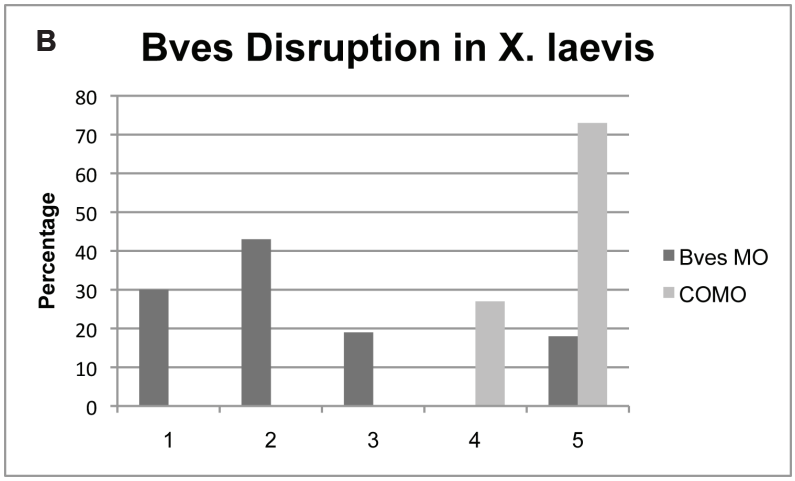
Figure 6.1 Bves depletion results in increased membrane bleb formation in *X. laevis* head mesoderm cells. SEM analysis of the anterior head mesoderm reveals increased membrane blebbing in Bves-depleted cells (B) as compared to COMO treated cells (A). When this population of cells is isolated and cultured on fibronectin, this phenotype is also observed by phalloidin staining in individual Bves MO treated cells (D). In contrast, COMO injected cells have normal lammelipodia protrusion on either side of the cell, with no apparent bleb formation (C). The number and size of membrane blebs observed *in vivo* via SEM analysis is quantified in panels E and F.

and have also been linked to decreased cell adhesion during development (Shook & Keller, 2003). Taken together, these data suggest the actin-cytoskeletal network may be destabilized upon Bves depletion.

Bves depletion results in gross morphological defects and disrupted epidermis

As reported by Ripley et al. (Ripley et al, 2006), global disruption of Bves in *X. laevis* results in developmental defects that are first observed during gastrulation. These defects involve the disruption of such critical cell movements as epiboly and involution and eventually lead to complete inhibition of gastrulation. Additionally, as described in Chapter IV, gastrulation-staged HM cells are impaired in cell adhesion *in vitro* and are severely unorganized *in vivo* as they migrate along the BCR. Having characterized Bves depletion in early *X. laevis* embryos, we wanted to understand the progression of these defects and the role Bves plays in later development. Using a similar MO knockdown strategy as previously reported (Ripley et al, 2006), we injected Bves MO (20ng) into one of two cells at the two-cell stage. This experimental design allows the non-injected side to serve as an internal control, as the *X. laevis* body plan is set at this stage and little crossover occurs between left and right sides. As seen in Figure 6.2A, severe morphological defects are observed on the injected side, and approximately 30% of embryos died before this stage (Figure 6.2B). In contrast, the general body plan is preserved in the non-injected control side of the embryo. Morphological defects are characterized by disrupted eye development (43% of embryos displayed some level of eye malformation, Figure 6.2B), shorter





C	Phenotype
1	Died
2	Disrupted eye development
3	Shorter AP axis, curved body
4	Slightly disrupted tissue morphogenesis
5	No gross morphological changes

Figure 6.2 MO knockdown of Bves results in severly impaired morphogenesis of *X laevis*. Depletion of Bves results in disrupted eye development (small arrow), an abbreviated AP axis, and curvature of the body (large arrows) in stage 41 embryos (A). This phenotype is quantified in panel B, with defining characteristics of the phenotype in panel C.

Anterior-Posterior (AP) axis, curved bodies, and ectodermal outgrowths. Disruption in eye formation was primarily visualized by malformed retinal pigmented epithelium (RPE), which is a tight epithelial layer that nourishes the retinal cells (Chung et al, 1975). Several embryos also displayed moderate edema of the abdominal cavity. It should be noted that the defects in anterior features are not surprising, given the impaired migration of precursor cells observed in younger embryos. Head structures are the progeny of involuting HM cells, the same cells that were previously observed to have adhesion and motility defects (Chapter IV).

Bves has been reported to play a role in the maintenance of epithelial cell junctions (Osler et al, 2005). This data, along with observation of superficial ectodermal epithelia defects, prompted us to determine the extent of epithelial disruption in tadpole-staged embryos. Using H&E staining, we observed a severely underdeveloped eye and a thickened outer epidermis on the Bves MO injected side of the embryo (Figure 6.3a, b). In contrast, the control non-injected side of the embryo displayed a thinner outer epithelium, and a well-formed eye with a visible RPE (Figure 6.3c). This section was taken from the anterior-dorsal portion of a stage 41 embryo (inset, line denotes area of section), and indicates a general defect in epithelial biogenesis and/or maintenance.

Disruption of Bves results in decreased localization of junctional proteins at the membrane, such as E-cadherin (Osler et al, 2005). This decreased localization was also observed upon Bves depletion in *X. laevis.* As described above, embryos were injected with Bves MO and mGFP as a tracer and then

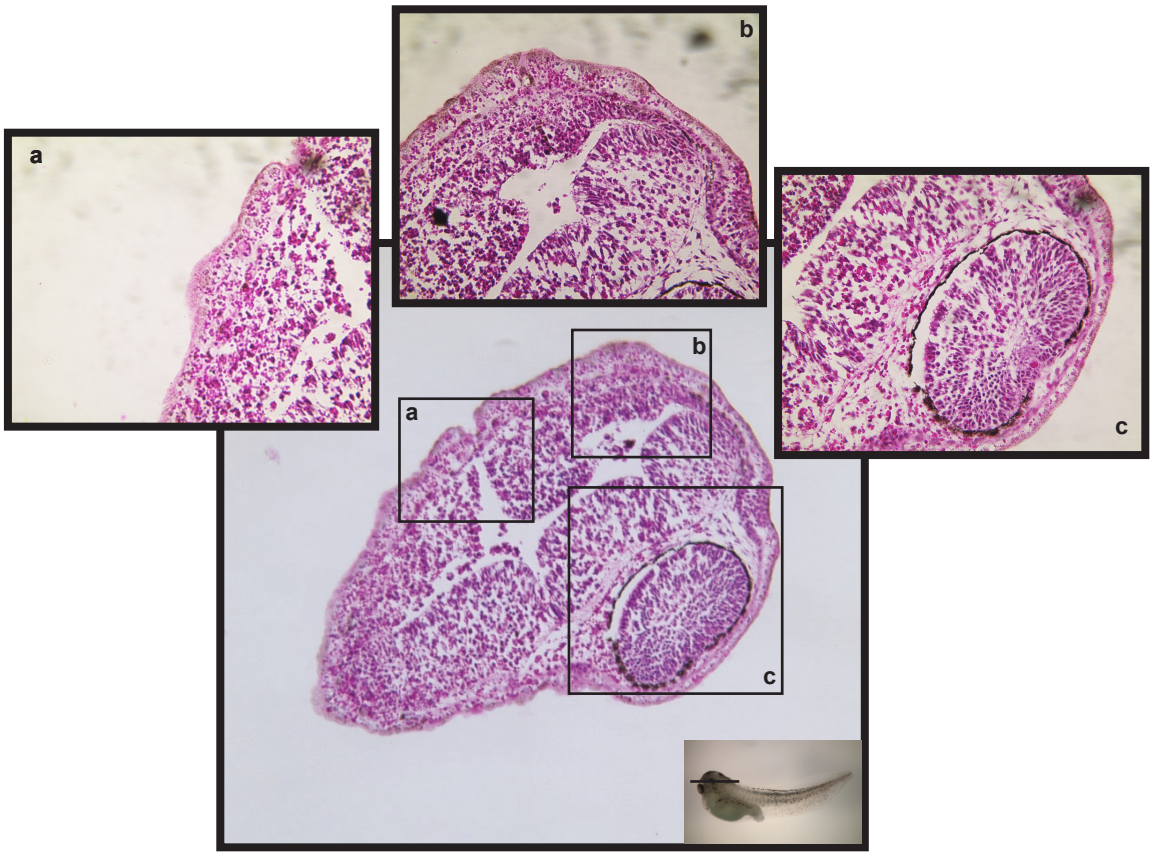


Figure 6.3 Bves depletion results in a severely thickened epidermis and disrupted eye formation. *X. laevis* embryos were injected with Bves MO into one cell at stage two. Histological analysis reveals disrupted eye formation on the injected side (a) in contrast to normal morphogensis on the non-injected control side (c). Additionally, the outer epidermis is observed to be much thicker when Bves is depleted (a, b). This section was taken from a stage 41 embryo (inset, line).

labeled with E-cadherin antibody. In Figure 6.4B, distribution of E-cadherin is observed on both sides of the embryo. However, there is decreased expression of E-cadherin where Bves is depleted (Figure 6.4B, inset: mGFP expression demonstrates the region shown in Figure 6.4A is indeed the Bves MO injected side of the embryo). On the non-injected side of the embryo (Figure 6.4C), E-cadherin localizes to the basolateral region of the epidermis, consistent with previously published distribution profiles (Levi et al, 1991). Contrastingly, when Bves is depleted, E-cadherin is localized to the membrane in some cells, but a consistent basolateral distribution is not observed. Disruption in E-cadherin expression, as well as defects in epithelia cytoarchitecture, suggest that Bves is necessary for the formation and maintenance of epithelial sheets during development.

Disruption of Bves results in cell death and decreased cell proliferation

As described above, knockdown of Bves in *X. laevis* results in disrupted epithelial architecture as seen with histological analysis (Figure 6.3). Additionally, decreased cell density is observed on the Bves-depleted side of the embryo, potentially causing the body axis curvature seen in a significant portion of tadpoles (Figure 6.5A, arrow). Fewer cells on the Bves MO injected side of the embryo could result from two different possibilities: increased cell death or decreased cell proliferation. To investigate the former possibility, we used TUNEL for *in situ* analysis on whole mount embryos (Hensey & Gautier, 1998). COMO labeled embryos demonstrated little to no apoptosis, consistent with

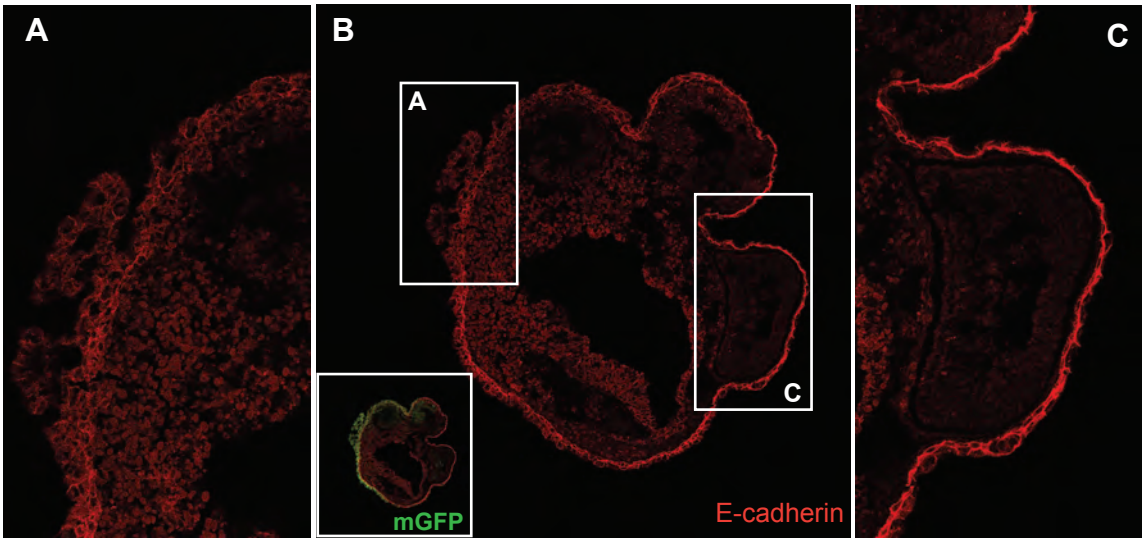


Figure 6.4 Bves depletion results in decreased E-cadherin expression. *X. laevis* embryos were injected with Bves MO and mGFP into one cell at stage two (B; mGFP expression shown in the inset confirms the injected side of the embryo). Disruption of E-cadherin expression is observed on the injected side of the embryo (A), while the control non-injected side displays basolateral labeling of the outer epithelia (C).

previous reports (Figure 6.5B). In contrast, the side of embryos injected with Bves MO displayed obvious cell death, as evidenced by the prominent punctate labeling in whole mount analysis (Figure 6.5C, magnified view in c), while the non-injected control side had little to no cell death (Figure 6.5D, magnified view in d), as seen with COMO injected controls (Figure 6.5B). Not surprisingly with Bves-depletion, cell death was readily observed in the anterior region where defects in epithelial structure and protein localization are found (Figure 6.2-4). This further suggests Bves function is important for the development of this region of the embryo and may play a crucial role in either the formation or the maintenance of head structures.

To investigate the possibility of decreased cell proliferation, we used PH3 antibody labeling to assay for dividing cells. As seen in Figure 6.6B, stage 35 tadpoles have fewer cells in M phase of mitosis on the Bves-depleted side of the embryo (the injected side is verified by mGFP labeling; proliferating cells are labeled with PH3 in red; DAPI mark nuclei in blue). In comparison, an embryo treated with COMO displays similar levels of proliferation on either side of the embryo (Figure 6.6A). Overall, disruption of Bves results in a 43% decrease in cell proliferation. Quantification is given for the Bves-depleted vs. non-injected control side in Figure 6.6D; regions from which the sections were taken is shown on the pre-sectioned embryo in Figure 6.6C. Taken together, these data suggest Bves is not only required for morphogenesis of *X. laevis* but also for maintenance of newly developed tissue.

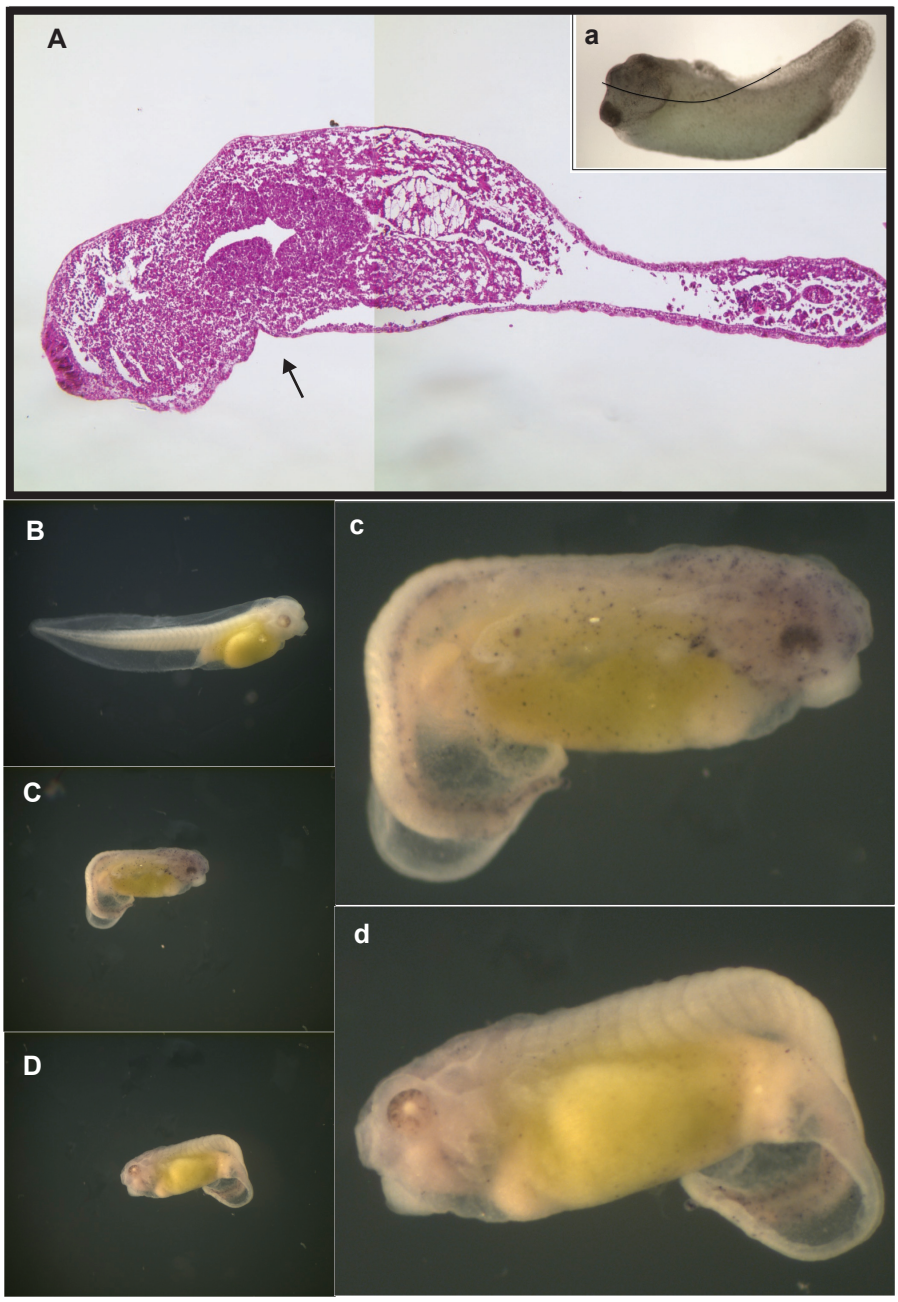
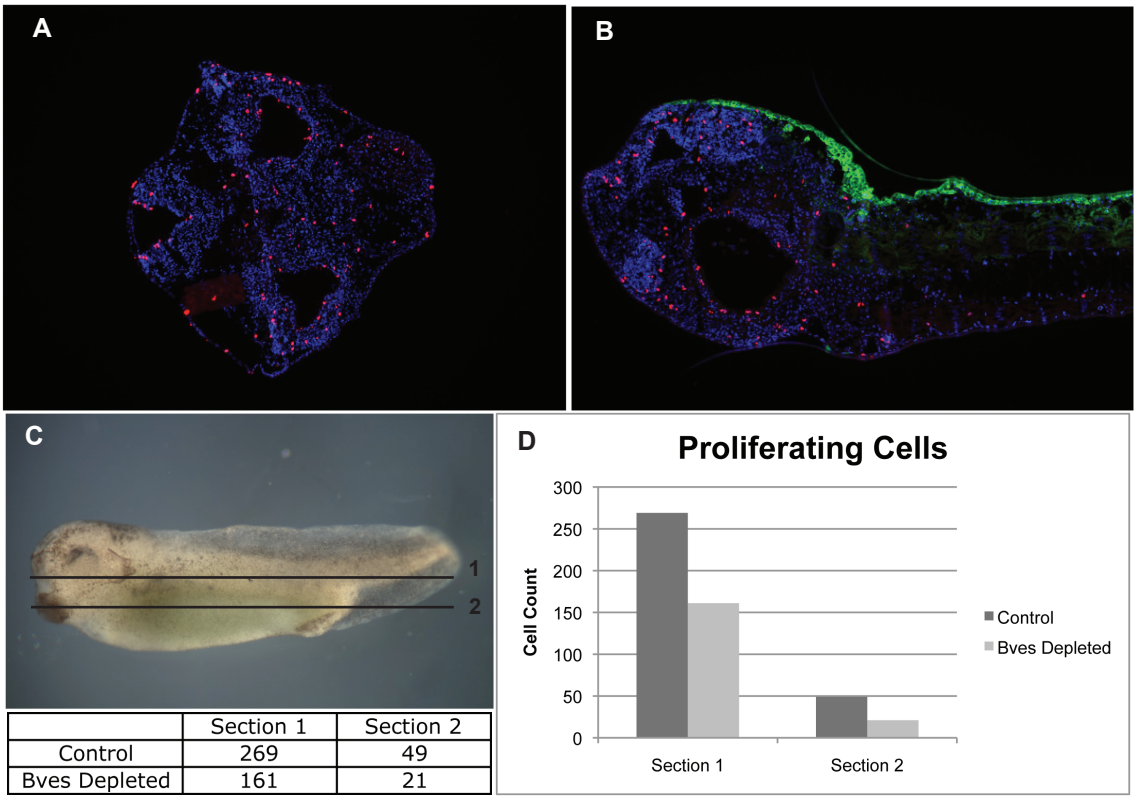


Figure 6.5 Bves depletion results in apoptosis throughout development. *X. laevis* embryos were injected with Bves MO in one of two cells. Histological analysis revealed that on the presumed injected side, Bves MO treated embryos had decreased cell density and were curved towards the side in which Bves was depleted (A, arrow; inset (a) marks the region of the section.) TUNEL staining demonstrates that Bves depletion resulted in increased apoptosis in whole mount (C; a magnified view is seen in c), however, the uninjected control side of the embryo undergoes little cell death (D, a magnified view is seen in d). Similarly, COMO injected embryos display little to no apoptosis (B).



	Section 1	Section 2
Control	269	49
Bves Depleted	161	21

Figure 6.6 Bves-depleted embryos exhibit decreased cell proliferation. One of two cells was injected with COMO (A), or Bves MO and mGFP (B). COMO treated embryos displayed similar cell proliferation on either side of the embryo as visualized by Phospho-Histone H3 (PH3) labeling (A, PH3 is red; DAPI is blue), while the Bves-depleted side of the embryo displayed decreased cell proliferation (B, the injected side is confirmed by the presence of mGFP). These data are quantfied in panel D, with the location of the sections shown with a solid black line in C (lines 1, 2).

MO knockdown of Bves is partially rescued by truncated Xbves constructs

An important control for Morpholino knockdown in *X. laevis* is to rescue the observed phenotype with mRNA that is unrecognizable by the MO (Eisen & Smith, 2008). This control assures that off target effects are minimal or undetectable and that knockdown of the protein of interest is responsible for the observed phenotype. Using these experiments, we see complete rescue of the Bves MO phenotype using Xbves mRNA that is mutated in the MO binding site, as detailed in Chapter IV and reported in (Ripley et al, 2006). This rescue was repeatable and convincing, thus we exploited this experimental design by creating truncated forms of our rescue construct to determine which region of the protein is important for Bves function. As explained above, Bves is a member of the Popdc family of proteins, and has no significant homology with any characterized protein motif (Hager & Bader, 2009). This family of proteins does have a Popeye domain, although no molecular function has yet been ascribed to this region and it has been defined only by its high conservation across species. Thus, from the structure of Bves, it is difficult to predict which domains of the protein are important for function. *X. laevis* is the most appropriate system in which to conduct assays of this type because Bves is the only Popdc family member expressed during frog early development (Hager & Bader, 2009), negating the effect of promiscuous homologues.

Our data demonstrate that ‘Full Rescue’ is only achieved with the full-length Xbves rescue construct (Figure 6.7, #1). ‘Almost Full Rescue’ (as defined in the methods) is seen with expression of a construct containing the

transmembrane and Popeye domains but is lacking the most distal C-terminus and critical KK region outlined in Chapter V (this region is essential for Bves-Bves homophilic interaction; Figure 6.7, #3). Surprisingly, a rescue construct that contains the entire Popeye domain and the KK region only results in 'Partial Rescue' of Bves MO treated embryos (Figure 6.7; #2). These unexpected but consistent results are further examined in the discussion. 'Little to Partial' rescue was seen with constructs that had truncated Popeye domains (Figure 6.7, #4, #5), suggesting this region is imperative for Bves function. Finally, expression of a rescue construct that is lacking the extracellular domain (Figure 6.7, #6) results in embryos indistinguishable from non-rescued tadpoles, suggesting that insertion of Bves in the membrane is essential for protein function (Wada et al, 2003). Overall, these results suggest that the Popeye domain, which is highly conserved across all species, is indispensible for Bves function.

Discussion

Bves has an established role in maintaining epithelial cell junctions and has recently been shown to be important for Rho GTPase activity and vesicular transport. Here, we report phenotypes in *X. laevis* development that further support a role for Bves in these diverse cellular processes and demonstrate domains of the protein that are critical for function.

We demonstrate that disruption of Bves function in *X. laevis* results in increased membrane bleb formation in HM cells. Membrane bleb formation occurs when the cell membrane becomes detached from the underlying actin-

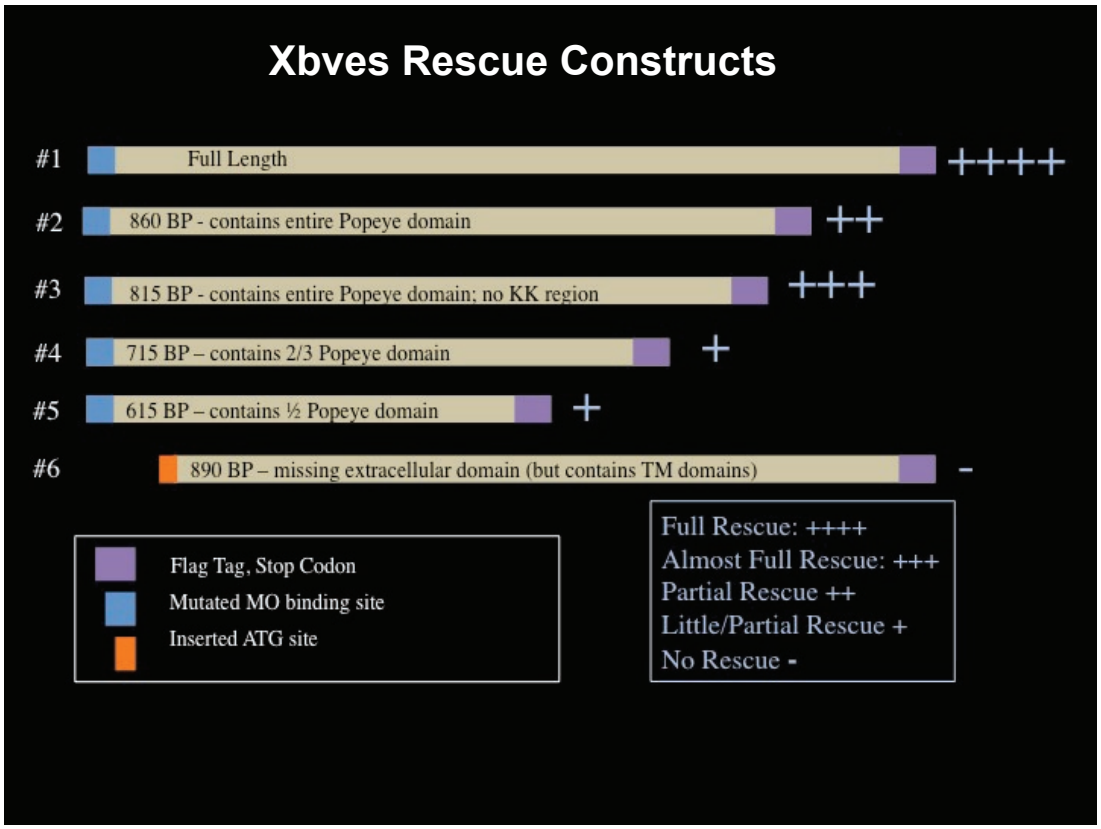


Figure 6.7 MO knockdown of Bves is partially rescued by truncated Xbves constructs. Full-length Xbves mRNA that is mutated in the MO-binding domain (#1) fully rescues the morphant phenotype observed in the frog, demonstrating there are minimal off-target effects of Bves MO. As Bves contains no previously characterized protein motifs, we have generated a series of Xbves truncation constructs to determine the functional domain of this protein. Rescue experiments show that the extracellular domain (contained in constructs #2-5) and the highly conserved Popeye domain (full length Popeye domain is in constructs # 2 and 3) are required for rescue. Additionally, the most distal portion of the intracellular C-terminus (amino acids 270-340) is important for full rescue of the embryo (only found in construct #1).

cytoskeleton, causing the membrane to transiently balloon outwards (Fackler & Grosse, 2008). Actin dynamics are in part modulated by Rho GTPases, specifically Rac1 and Cdc42 (Tapon & Hall, 1997). Thus, disruption of the function of these molecules could result in unregulated actin modulation and ultimately lead to increased membrane blebbing. Bves interacts with GEFT, a Rho-specific guanine nucleotide exchange factor for both Rac1 and Cdc42 (Guo et al, 2003; Smith et al, 2008). Upon disruption of the Bves-GEFT interaction, decreased Rac1 and Cdc42 activity is observed. Thus, the increased membrane bleb formation seen in gastrulation-staged HM cells may be indicative of localized breakdown of the actin network.

It has also been proposed that bleb formation occurs in response to decreased cell adhesion to a substrate (Shook & Keller, 2003). Bves-depleted HM cells clearly have trouble adhering to a FN substrate as frequent membrane blebs are observed with time-lapse imaging (Chapter IV). This phenotype has been linked to impaired recycling of integrins, molecules that are imperative for cell movement and have been shown to modulate Rho GTPase activity through signaling cascades (DeMali et al, 2003). Thus, we hypothesize that disrupted integrin-mediated adhesion and dysregulated actin dynamics underlie the membrane blebs observed in Bves-depleted HM cells.

Depletion of Bves protein in *X. laevis* results in a general disruption in the epithelial integrity of the outer epidermis. Interestingly, these defects are most apparent in the anterior portion of the embryo, which is the progeny of the previously described HM cells. At the gross morphological level, the most

obvious defect is disrupted eye development characterized by a malformed RPE. The RPE is a pigmented, single epithelial layer that nourishes retinal cells by selectively allowing the passage of ions (Hartzell & Qu, 2003). When Bves is depleted, this critical layer of cells is either completely missing or in rudimentary form. Additionally, histological analysis reveals the lens and retina are severely disrupted. Eye development consists of a series of induction events, with critical crosstalk occurring between epithelial layers (Chow & Lang, 2001). Bves depletion results in malformed eye structures, suggesting these complex tissue interactions do not occur in Bves-depleted embryos. Thus, the disruption of ocular epithelial demonstrates Bves is necessary for eye formation in *X. laevis* and suggests other epithelial layers may also be adversely affected.

Previously, knockdown of Bves has been shown to disrupt E-cadherin localization at the membrane and decrease the trans-epithelial resistance of a confluent epithelial sheet (Osler et al, 2005). Here, we show that defects in epithelial protein distribution and cytoarchitecture are also observed in Bves-depleted embryos. Consistent with previous reports (Osler et al, 2005), E-cadherin is mislocalized and overall expression levels are decreased upon Bves disruption in the frog. Thus, we demonstrate that the previously reported *in vitro* phenotype also holds true in the developing embryo. Based on our current understanding of Bves function in vesicular transport (Chapter IV), it is possible that E-cadherin is not trafficked properly or maintained at the membrane with the appropriate kinetics. This would result in the disrupted localization pattern observed both in *X. laevis* and *in vitro*. We also observe localized ectodermal

outgrowths (Figure 6.4A), which may indicate dysregulation of the cell cycle in this outer epithelial layer. As E-cadherin has an established role in regulation of cell cycle, this would be an interesting avenue to investigate (van Roy & Berx, 2008).

We observe both increased cell death and decreased cell proliferation in Bves-depleted tadpoles. As Bves is necessary for junctional integrity (Osler et al, 2005), loss of intercellular adhesion may undermine cell-signaling cascades in which Bves is involved (Rho GTPase and integrin signaling) and thus induce secondary defects in cell death or proliferation (Smith et al, 2008) (Chapter IV). Alternatively, specified cells may never localize to their appropriate developmental region, thus disrupting cell organization. Previously, Ripley et al. reported that depletion of Bves in 1 of 32 cells caused rogue cell movements, as cells did not migrate according to well-defined fate maps (Ripley et al, 2006). Taken together, we propose that increased cell death and decreased cell proliferation are secondary effects caused primarily by defects in cell adhesion, signaling, and migration.

We demonstrate that the highly conserved Popeye domain (Hager & Bader, 2009) is important for Bves function in *X. laevis*, but a full-length construct is required for complete rescue. This may be due to unknown protein folding or post-translational modifications that are inhibited without the entire amino acid sequence. Previously, it has been shown that two lysine residues (K^{272} and K^{273}, conserved in mouse, human and chick) are necessary for Bves-Bves homophilic interaction and subsequent function (Kawaguchi et al, 2008). Surprisingly, a

rescue construct that contained these lysines and the entire Popeye domain did not produce the most robust rescue of the Bves MO phenotype, suggesting they are not required for function in *X. laevis*. Although Xbves does contain two lysines in close proximity to those conserved in mouse, human and chick, they are in slightly different positions (Osler et al, 2006). Thus, it is possible that these lysines are not important for homophilic interaction or that homophilic interaction is not important for Xbves function. All engineered constructs displayed some rescue, aside from an Xbves truncation that lacked the extracellular N-terminal domain. Most likely, this protein product was unable to localize to the membrane without a signal sequence presumably contained in this extracellular domain. This finding is important, as it suggests Bves protein must be inserted into the membrane for proper function and further supports a role for Bves in regulating membrane dynamics. Taken together, these data demonstrate the most conserved region of Bves, the Popeye domain, is essential for function in *X. laevis*.

To date, this ongoing work demonstrates that Bves is important for *X. laevis* development and that Bves disruption in the embryo results in phenotypes consistent with previously reported functions.

CHAPTER VII

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Summary

The Bader laboratory discovered *Bves* in a screen to identify novel, heart enriched genes in 1997. The earliest characterization of this gene product focused on defining the expression pattern across species. After some debate in the literature, it was agreed that Bves was robustly expressed in muscle, brain, *and* epithelial tissue, thus eliminating a previously proposed muscle-specific function for Bves. It was soon shown that Bves was a highly conserved, integral membrane protein that was present in eukaryotes ranging from fruit flies to humans. Bves was first defined as a putative adhesion molecule, and subsequently, it was determined that Bves function was critical for epithelial integrity both *in vitro* and *in vivo*. However, the molecular basis of these early functions was unknown, and no clues could be gleaned from Bves structure. Thus, to understand Bves function at the molecular level, protein-protein interaction screens were initiated to identify Bves binding partners and potentially link Bves to a well-studied pathway. In this dissertation, I present work that identifies the molecular mechanisms underlying Bves function. As outlined in Chapter III, Bves interacts with GEFT and regulates cell motility and shape

through modulation of Rho GTPases Rac1 and Cdc42. In Chapter IV, Bves is shown to interact with VAMP3 and regulate VAMP3-mediated vesicular transport. Taken together, these studies explain the previously reported phenotypes upon Bves depletion at the molecular level and provide a basis to further examine the function of Bves. Thus, the significance of this work lies in the identification and characterization of the molecular mechanism underlying Bves function. Below, I discuss our current understanding of Bves function at the molecular level in regulating diverse signal cascades upstream of VAMP3 and GEFT, and present future experiments to further elucidate Bves function in these pathways.

Bves as a modulator of Rho Signaling Cascades

As outlined in Chapter III, Bves interacts directly with GEFT and regulates cell shape and movement through Rho GTPases Rac1 and Cdc42 (Smith et al, 2008). Yeast two-hybrid screens identified the highly conserved Popeye domain of Bves, and the Dbl Homology (DH) and Pleckstrin Homology (PH) domains of GEFT were important for this interaction. Previous reports have demonstrated that the PH domain in Dbl family proteins negatively regulates the DH domain, but interaction with phosphoinositides negates this inhibitory effect (Shimada et al, 2000). Thus, it is possible that Bves may bind the PH domain and similarly release the auto-inhibitory effect on the DH domain, allowing GEFT to activate Rac1 and Cdc42. Conversely, it has been proposed that Bves binds and sequesters GEFT, thus decreasing the amount of downstream Rho GTPase activity (Hager & Bader, 2009). Bves also interacts with Dbl family member Cool1

(which contains the DH domain), although this interaction was weaker or potentially transient when compared to Bves interaction with GEFT (Smith et al, 2008). Nonetheless, it is possible that Bves regulation of Rho GTPases through interaction with GEFT extends to other Dbl family members in different cellular contexts.

Bves and GEFT are both highly expressed in excitatory tissues and co-localization has been observed in skeletal, smooth, and cardiac muscle. GEFT has been shown to induce myogenesis and accelerate the rate of skeletal muscle regeneration (Bryan et al, 2005). In a similar vein, we demonstrate that expression of mutated Bves inhibits differentiation of C2C12 cells, suggesting Bves-GEFT interaction is important for skeletal myofiber formation (Smith et al, 2008). As both Bves and GEFT are highly expressed in the brain, and GEFT has a demonstrated role in neurite outgrowth and dendritic spine formation, it would be interesting to examine this interaction in the nervous system. Finally, Bves and GEFT are both highly expressed in the heart (Hager & Bader, 2009). Again, it would be interesting to examine the disruption of the Bves-GEFT interaction in the heart to determine the effect on this organ.

Rho GTPases have established roles in regulating cell migration through modulation of the actin-cytoskeletal network (Ladwein & Rottner, 2008). Expression of a mutated form of Bves in NIH 3T3 cells results in decreased activity of Rac1 and Cdc42. Additionally, these cells, which are expressing only the C-terminus of Bves, are 25% more round and have significantly decreased cell speed in comparison to controls (Smith et al, 2008). This report is consistent

with defects in actin-propelled lamellipodial and filopodial extensions resulting in decreased cell migration (Ladwein & Rottner, 2008). Previously, Bves has been shown to be important for epithelial wound healing (Ripley et al, 2004), gastrulation of *X. laevis* (Ripley et al, 2006), germband extension in *Drosophila* (Lin et al, 2007), and skeletal muscle regeneration in the mouse (Andree et al, 2002). All of these events require coordinated cell movements. Until identification of the Bves-GEFT interaction, the molecular mechanism underlying impaired migration in these broad systems was unknown. Now, it is clear that motility defects observed upon Bves inhibition are at least *in part* caused by disrupted Rho GTPase activity.

In addition to influencing cell migration, Rho GTPases also have an established role in mediating epithelial biogenesis. Disruption of either Rac1 or Cdc42 influences E-cadherin mediated cell-cell adhesion, although this phenotype can be dependent upon cell type and junction maturity (Fukata & Kaibuchi, 2001). Specifically, Rac1 and Cdc42 are necessary for E-cadherin based cell-cell adhesion (Hordijk et al, 1997) and expression of a dominant negative form of Rac1 results in decreased E-cadherin at the cell junction (Braga et al, 1997). Bves is also required for localization of E-cadherin at points of cell-cell contact. When Bves is disrupted, E-cadherin is not trafficked to cell junctions, and the integrity of the epithelial sheet is compromised *in vitro* (Osler et al, 2005). Similar disruption of E-cadherin distribution is observed *in vivo* in *X. laevis* along with general defects in epithelial architecture (Chapter VI). This suggests that disrupted Rho GTPase activity may underlie the defects in epithelial biogenesis

observed upon Bves depletion. It has been demonstrated that in addition to Rho GTPases effecting cadherin-based cell-cell adhesion, E-cadherin may also signal to Rac1 and Cdc42. Reports have shown that in response to E-cadherin mediated cell-cell adhesion, levels of active Rac1 and Cdc42 are increased (Kim et al, 2000; Nakagawa et al, 2001). This demonstrates that crosstalk or mutual regulation occurs between junctional molecules and Rho GTPases. This essential communication is most likely disrupted upon Bves inhibition. As stated above in relation to cell migration, the molecular mechanism of Bves function in maintaining epithelial integrity was previously unknown. In light of the current data, Bves interaction with GEFT may affect downstream targets that regulate epithelial biogenesis. Inhibition of this signal cascade may *in part* explain the epithelial phenotypes observed upon Bves depletion.

Bves as a novel regulator of vesicular transport

VAMP3 has an established role in transport of the transferrin receptor, and recently, several reports have demonstrated that VAMP3 transports integrins to and from the membrane during cell migration (Galli et al, 1994; Luftman et al, 2009; Proux-Gillardeaux et al, 2005a; Skalski & Coppolino, 2005; Tayeb et al, 2005). We demonstrate in Chapter IV that Bves interacts with VAMP3 and regulates VAMP3-mediated vesicular transport.

The intracellular C-terminus of Bves is important for interaction with VAMP3, and this portion of the protein has also been shown to interact with the VAMP homologue, VAMP2. VAMP3 and VAMP2 are highly homologous in their

SNARE domain and have overlapping tissue distribution *in vivo* (McMahon et al, 1993). These proteins have been shown to substitute for one another both in development and *in vitro* (Bhattacharya et al, 2002; Deak et al, 2006). However, unlike VAMP3, VAMP2 is also expressed in neural tissue (Kweon et al, 2003). VAMP1, the third VAMP homologue that is cleaved by Tetanus toxin, is only expressed in the nervous system (Trimble et al, 1988) and, along with VAMP2, is imperative for the release of neurotransmitters (Wang & Tang, 2006). As mentioned previously, examining the potential role of Bves in the brain may reveal a critical function for Bves in synapse biology, underscoring a broader role for Bves in behavior or cognition.

Bves and VAMP3 co-localize in several epithelial cell lines and also in skeletal and cardiac muscle (Chapter IV). Overlapping distribution is seen both at the membrane and in intracellular vesicles, suggesting a conserved function for Bves in VAMP3-mediated vesicle fusion at the external cell membrane and in intracellular endosomes. Although co-localization does not confirm interaction between two proteins, it can suggest subcellular locations where proteins may bind. Bves and VAMP3 are both integral membrane proteins, but the membrane dynamics of their interaction are not known, although several possibilities exist. For example, Bves and VAMP3 may interact when they are on apposing membranes with Bves on the target membrane (Figure 7.1A), or alternatively when Bves and VAMP3 are in close proximity on the same membrane (Figure 7.1B). In the former possibility, Bves may act as a vesicle docking protein on the target membrane, priming VAMP3 (which is localized to the incoming vesicle) for

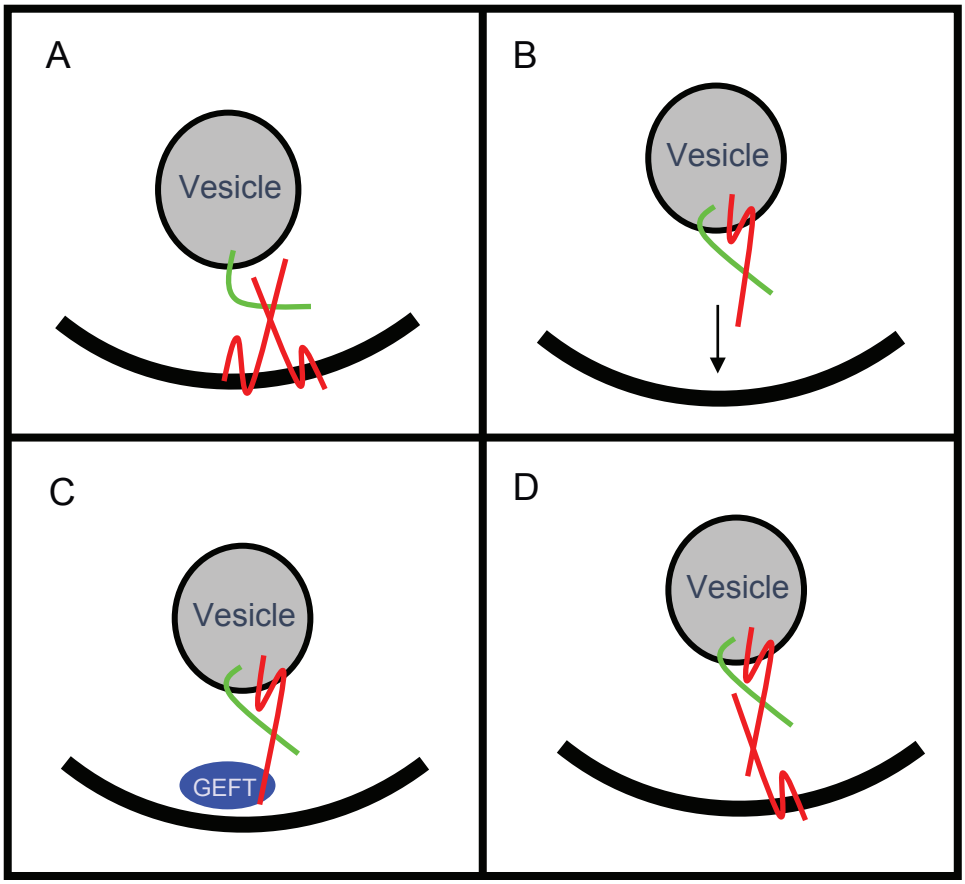


Figure 7.1 Bves interaction with VAMP3. Bves (red) may interact with VAMP3 (green) while proteins are on apposing membranes (A) or while both proteins are on the same membrane (B). Additionally, Bves and VAMP3 may complex with GEFT (C, blue). Finally, Bves may interact with itself while individual Bves molecules are on apposing membranes (D).

interaction with other SNARE proteins (Figure 7.1A). As VAMP3 has been shown to only interact with specific syntaxins (Hu et al, 2007) on the basolateral surface of epithelia, interaction with Bves could provide an additional level of specificity. At the present time, few regulators of VAMP3 trafficking are known, and no proteins have been observed to aid specifically in VAMP3 membrane docking through interaction (Leabu, 2006).

In the alternative possibility, Bves is transported to the external cell membrane in VAMP3-positive vesicles, and interaction of Bves-VAMP3 occurs while both proteins are localized to the same vesicle or membrane (Figure 7.1B). Evidence for this possibility is observed in cell lines without VAMP3 expression (Chapter IV). Bves is observed in intracellular vesicles in these cells, but Bves localization at the membrane is significantly attenuated. This suggests Bves is dependent upon VAMP3 for localization at the membrane. Additionally, Bves, VAMP3, and internalized β-1 integrins all localize to the same vesicle in wildtype cells, further suggesting Bves is trafficked via VAMP3 action (Chapter IV). In this situation, Bves may still function as a docking protein. Instead of Bves being integrated into the target membrane and tethering VAMP3 from this location, a Bves-VAMP3 complex may be part of the incoming vesicle and interact with proteins on the target membrane. Thus, Bves would form a protein complex with molecules on the target membrane, potentially with membrane localized GEFT (Figure 7.1C). As Bves has been shown to undergo homophilic Bves-Bves interaction, Bves may interact with other Bves molecules to essentially dock itself (and VAMP3) at the target membrane (Figure 7.1D).

A final and potentially more realistic alternative exists: all of the previously described situations may occur (Figure 7.1A-D). Bves may be trafficked to the membrane in VAMP3-positive vesicles where interaction within the same vesicle occurs; simultaneously, Bves and VAMP3 interact while on apposing membranes. In both situations, Bves could aid in VAMP3 docking as described above. Current data neither provide conclusive evidence for any of these three scenarios, nor do these data exclude any possibilities. Thus, further analysis of the subcellular location of Bves and VAMP3 interaction is necessary to fully understand this complex situation.

VAMP3 is important for the transport of transferrin receptors and β-1 integrins (Galli et al, 1998; Luftman et al, 2009; Proux-Gillardeaux et al, 2005b; Skalski & Coppolino, 2005; Tayeb et al, 2005). Our data conclusively demonstrate that the trafficking of these two molecules is impaired upon Bves disruption, and side-by-side analysis confirms that our data directly phenocopy inhibition of VAMP3 function. Additionally, integrin-dependent processes such as cell spreading and cell-substrate adhesion are impaired in two different model systems. Integrins are imperative for migration, as recycling of these adhesion molecules and subsequent interaction with the ECM provides the basis for coordinated cell movement (Caswell & Norman, 2008). Thus, the migration defects reported previously in various model systems (*X. laevis*, *Drosophila*, epithelia, and NIH 3T3 cells) may *in part* be a result of impaired integrin recycling.

For epithelial biogenesis to occur, junctional proteins must be trafficked to the external cell membrane in a coordinated manner. Generally, adherens junction proteins E-cadherin, β-catenin, and α-catenin are first localized to the membrane to initiate cell-cell contacts (Hartsock & Nelson, 2008). Next, tight junctions are established by the arrival of occludin and claudin proteins at the cell membrane, enabling selective passage of ions and small molecules (Hartsock & Nelson, 2008). Clearly, vesicular transport to the cell membrane and then tight regulation of endo- and exocytosis is necessary to maintain newly formed junctions. Disruption of endocytic pathway kinetics would lead to defects in epithelial biogenesis and/or maintenance. As discussed above, Bves is necessary for maintaining the integrity of epithelial tissue. When Bves is disrupted, adherens junction proteins such as E-cadherin and β-catenin are no longer localized at sites of cell-cell contact (Osler et al, 2005). It is possible that inhibition of vesicular transport, specifically VAMP3-mediated transport, may underlie the disruption in junctional protein localization. Recent evidence supports this hypothesis. E-cadherin has been shown to recycle through Rab11 positive recycling endosomes (Desclozeaux et al, 2008), and VAMP3 is a well-established member of the recycling endosome (Mallard et al, 2002). Thus, impaired VAMP3-mediated transport may underlie the disrupted localization of junctional proteins observed when Bves is inhibited. Taken together, disrupted vesicular transport may *in part* explain the previous epithelial phenotypes observed upon Bves inhibition.

Bves role in the development of *X. laevis*

Bves has been shown to play a role in regulating epithelial migration, adhesion, and cell morphology (Andree et al, 2002; Lin et al, 2007; Ripley et al, 2004; Ripley et al, 2006), thus we utilized gastrulation-staged embryos (where differential cell adhesion, movement and shape are critical for proper morphogenesis) to understand Bves function in development. In Bves-depleted embryos, disrupted development is first observed during gastrulation, which is consistent with previous reports of Bves regulation of cell migration and adhesion. The most overt developmental defect observed was delayed blastopore closure (Chapter IV). This phenotype is a general indicator of epithelial movement defects, but may result from disruption of several different molecules or pathways. Interestingly, this phenotype is observed when integrin function is disrupted (DeSimone & Hynes, 1988). Numerous studies by DeSimone and colleagues have shown that integrins are required for involution of mesoderm and subsequent migration of head and trunk mesoderm across the blastocoel roof (BCR) (Davidson et al, 2006; Marsden & DeSimone, 2001; Marsden & DeSimone, 2003; Ramos et al, 1996). These movements occur on a fibronectin (FN) matrix covering the BCR and position the future anterior features of the embryo. Isolation of these mesodermal cells reveals that without integrin function, these cells remain rounded in cell morphology and do not adhere to a FN substrate (Ramos & DeSimone, 1996). Similarly, isolated Bves-depleted cells also display this rounded cell morphology when plated on FN, suggesting the impaired integrin recycling observed *in vitro* also occurs *in* vivo (Chapter IV).

SEM analysis of Bves-depleted cells in the embryo reveal they are severely disorganized when compared to controls, suggesting impaired integrin-dependent migration across the BCR. Finally, time-lapse analysis demonstrates that cells with impaired Bves function are unable to adhere to a FN matrix *in vitro* as they display rounded cell morphology over time. Thus, as was demonstrated *in vitro*, impaired integrin recycling may *in part* underlie the migration and adhesion defects observed in the embryo upon Bves depletion.

Additionally, Bves-depleted cells exhibited many transient membrane blebs when observed over time *in vitro* and also when intact mesoderm was visualized with SEM (Chapter VI). Membrane blebs have been associated with decreased cell adhesion in development and also with localized breakdown of the actin cytoskeletal network (Fackler & Grosse, 2008). As described above, dysregulation of the actin network is intrinsically linked to defective modulation of Rho GTPases. Studies by Symes and colleagues have shown that disruption of Rac1 in *X. laevis* results in gastrulation defects that are distinctly pronounced in the head and trunk mesoderm (Ren et al, 2006a; Tahinci & Symes, 2003). Specifically, these studies demonstrate disrupted organization and migration of the involuting mesoderm, and impaired development of the head (which is the progeny of the involuting mesoderm) in tadpoles. Additionally, it has been shown that Cdc42 regulates convergent extension during *X. laevis* gastrulation (Choi & Han, 2002). Interestingly, Bves depletion also results in impaired gastrulation movements; specifically, mesodermal migration and polarity are impaired, and disrupted anterior features in tadpoles are observed (Chapter IV). Thus, the

gastrulation defects observed in Bves-depleted embryos may *in part* be due to disrupted Rho GTPase regulation.

Global function of Bves protein

Thus far, I have proposed that dysregulation of Rho GTPases *and* defects in the vesicular transport of integrins underlie the phenotypes observed upon Bves depletion. Bves interacts with VAMP3 and disruption of this interaction results in decreased VAMP3-mediated integrin recycling (Chapter IV). Bves also interacts with GEFT and inhibition of this interaction leads to decreased activation of Rac1 and Cdc42 (Smith et al, 2008). In both reports, disruption of Bves leads to phenotypes consistent with inhibition of *either* integrins or Rho GTPases, and therefore, these molecules contribute *in part* to Bves function. So, how does one delineate between such similar phenotypes to truly understand the molecular mechanism underlying Bves function?

Auspiciously, this is not just a problem for understanding the biology of Bves function, rather, it is also a conundrum for all biologists who study the intimately linked signaling pathways of integrins and Rho GTPases. Both integrins and Rho GTPases have been shown to be important for similar processes such as cell spreading and protrusion formation (Caswell & Norman, 2006; Holly et al, 2000a; Ridley, 2001a). Integrins depend on Rho GTPases for actin-propelled cell processes during migration, and Rho GTPases require integrin-based cell-substrate adhesion to maintain cellular extensions (Schwartz & Shattil, 2000b). Furthermore, it has been shown that integrin-mediated signals

are regulated by Rac1 and Cdc42 (Clark et al, 1998), and conversely, integrins activate Rac1 and Cdc42 upon interaction with the ECM (Price et al, 1998). Thus, crosstalk must exist between Rho GTPase and integrin signaling cascades, and disruption of one pathway would inevitably affect the other (Schwartz & Shattil, 2000a).

Integrins and Rho GTPases may influence each other in additional ways to directly regulate the physical propulsion of vesicles (Ridley 2006). In order for integrins to participate in cell adhesion, they must be trafficked to the leading edge of a cell during migration (Caswell & Norman, 2008). This directed localization of a vesicle may depend upon Rho GTPase-modulated actin polymerization, which drives the vesicle to the cell surface (Ridley, 2006). Once integrins are at the cell surface and interact with the ECM, they initiate cell-signaling cascades that have been shown to activate Rho GTPases as described above (Price et al, 1998). Thus, mutual regulation and crosstalk may be occurring on several levels between integrins and Rho GTPases to achieve cellular synchrony.

Although integrin and Rho GTPase signaling cascades are closely related, no direct connection has been established between these two pathways. Our data demonstrates that through interaction with VAMP3, Bves is important for the recycling of integrins (Chapter IV). Analogously, Bves interaction with GEFT is imperative for activation of Rho GTPases Rac1 and Cdc42 (Smith et al, 2008). Thus, as the reader must certainly understand, these two different pathways contribute *in part* to Bves function. Bves interacts with both VAMP3 and GEFT

and is necessary for the similar cellular processes regulated by these two proteins. Thus, we have established a link between these diverse but interrelated proteins and propose that Bves functions to coordinate their function in cell movement and adhesion by regulation of integrin and Rho GTPase signaling cascades (Figure 7.2). With this being said, we do not propose that Bves is the ultimate regulator of these two pathways. Given the diversity of integrin and Rho GTPase molecules across all tissue types, it is probable that several proteins function on many different levels to regulate and integrate these signaling cascades (Schwartz & Shattil, 2000a). However, based on our data, it is likely that Bves functions to regulate the downstream effector proteins of VAMP3 and GEFT. Thus, we hypothesize that Bves is imperative for tight regulation of membrane dynamics in cell adhesion and migration through modulation of multiple signaling pathways.

Future Directions

Bves function at the organ level

Bves is highly expressed in tissues that couple or form junctions: muscle, brain, and epithelia. However, most studies (as detailed in this dissertation) have focused on the function of Bves in epithelia. Based on what we have learned about Bves function in recent years, it would be interesting to expand these studies to either muscle or nerve tissue. Below, I discuss strategies and rationale for examining Bves function in heart and/or brain.

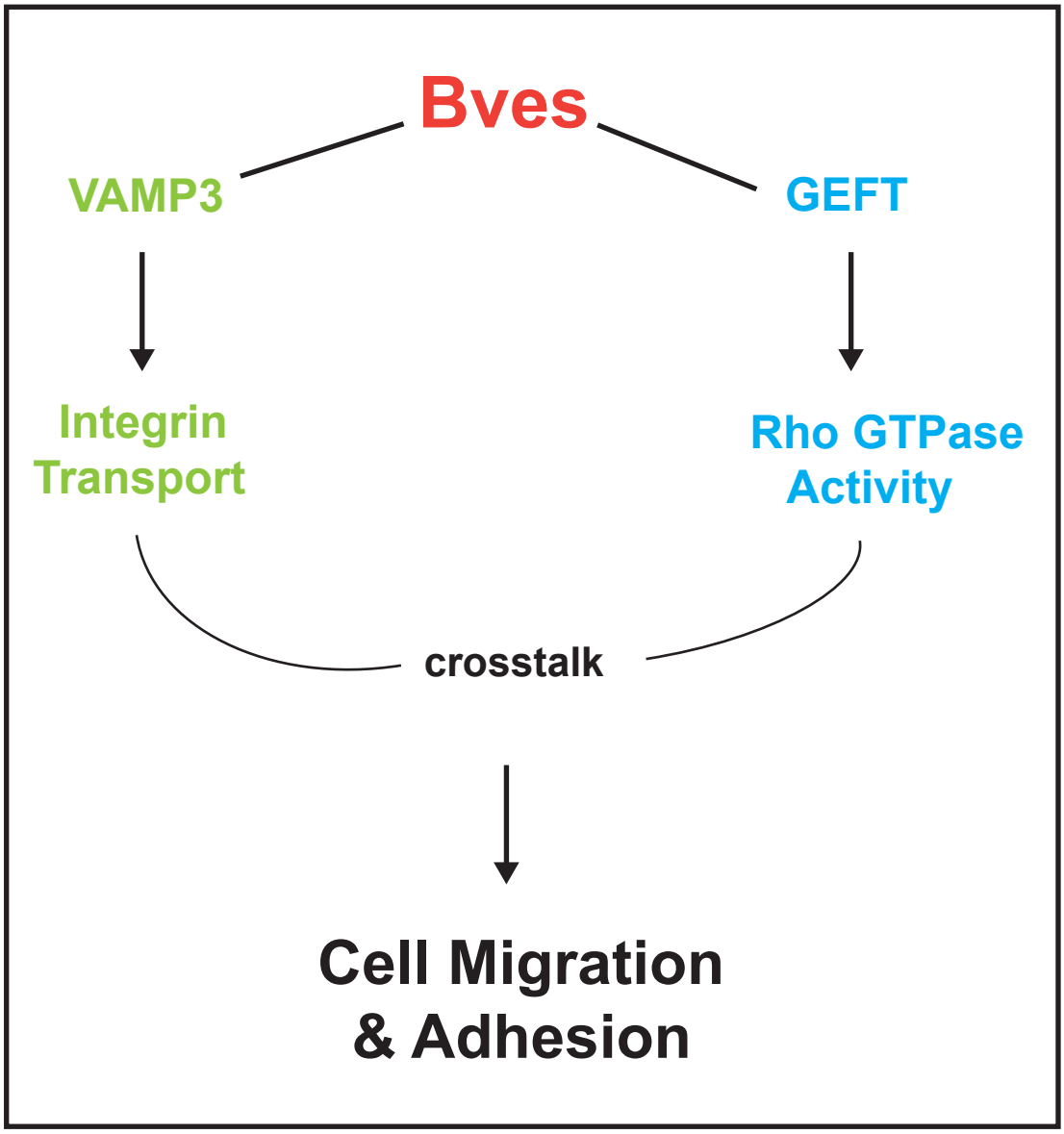


Figure 7.2 Global function of Bves protein. Bves interacts with VAMP3 and GEFT and is important for the cellular processes governed by these proteins: integrin transport and Rho GTPase activity. Through regulation of these two signal cascades, Bves is necessary for cell migration and adhesion *in vitro* and *in vivo*.

Cardiac development and adult heart function

Given the history of the Bader laboratory in the field of heart development (Bader et al, 1982), this would be the most appropriate avenue to study Bves function. Our data demonstrate Bves is important for the regulation of vesicular transport. Specifically, Bves mediates the VAMP3-dependent recycling of integrins in epithelial tissues. In the developing and adult heart, integrins are important signaling molecules and structural determinants. Although the precise regulation of integrins in the heart is poorly understood, it is known that tight spatial and temporal expression of these molecules is necessary for proper organ development and function (Ross & Borg, 2001). It has been shown that disruption of integrins in isolated cardiac myocytes results in disrupted cytoarchitecture and impaired myofibrillar patterning (Simpson et al, 1994). Additionally, reduction of β-1 integrins (the same integrin subunit that is recycled by Bves in epithelia) results in a severe decrease in postnatal heart function, ultimately resulting in cardiac failure (Shai et al, 2002). VAMP3 is also expressed in the heart (McMahon et al, 1993), but has never been studied in this context. We have shown that in the heart, Bves and VAMP3 interact via split ubiquitin (full-length mouse Bves was screened against an adult heart mouse library), and both proteins co-localize at the myocyte periphery (Chapter IV). Given the phenotypes observed with Bves disruption in the epithelia, it would be interesting to examine the Bves-VAMP3 interaction in the heart and determine its potential contribution to organ function.

As reported previously, disruption of Bves leads to decreased localization of adhesion proteins at cell junctions in epithelia, suggesting Bves is necessary for the transport of these molecules to the cell surface (Osler et al, 2005). In the heart, myocyte junctions are essential for proper cardiac development and function (Westfall et al, 1997). Distinct protein localization underlies the formation of these junctions, known as intercalated discs (ID), which mediate coupling between adjacent myocytes (Eppenberger & Zuppinger, 1999). However, the molecular mechanism underlying this highly specialized protein localization has not been resolved. Integrins, E-cadherin, and β-catenin are all resident members of the ID and are also proteins influenced by Bves disruption in epithelia (Carver et al, 1994; Hilenski et al, 1992; Kostin et al, 1999). Thus, it is likely that Bves modulates the localization of these molecules in cardiac tissue. In the developing heart, Bves undergoes dynamic redistribution during both myocardial trabeculation and epicardical EMT. During the early morphogenetic phases of cardiomyogenesis, Bves is expressed around the entire cardiac myocyte, but later in development and in the adult, Bves is also seen at the ID (Figure 7.3). Thus, given the disrupted junction phenotype found in epithelia when Bves is depleted, and Bves role in VAMP3-mediated vesicular transport, it would be interesting to examine disruption of Bves at the cellular level in coupled cardiac myocytes.

Finally, Bves has been shown to play a role in regulating Rho GTPase activity through interaction with GEFT (Smith et al, 2008). Although the studies of Rho GTPases in the heart are limited, Wei et al. discovered that down-regulation

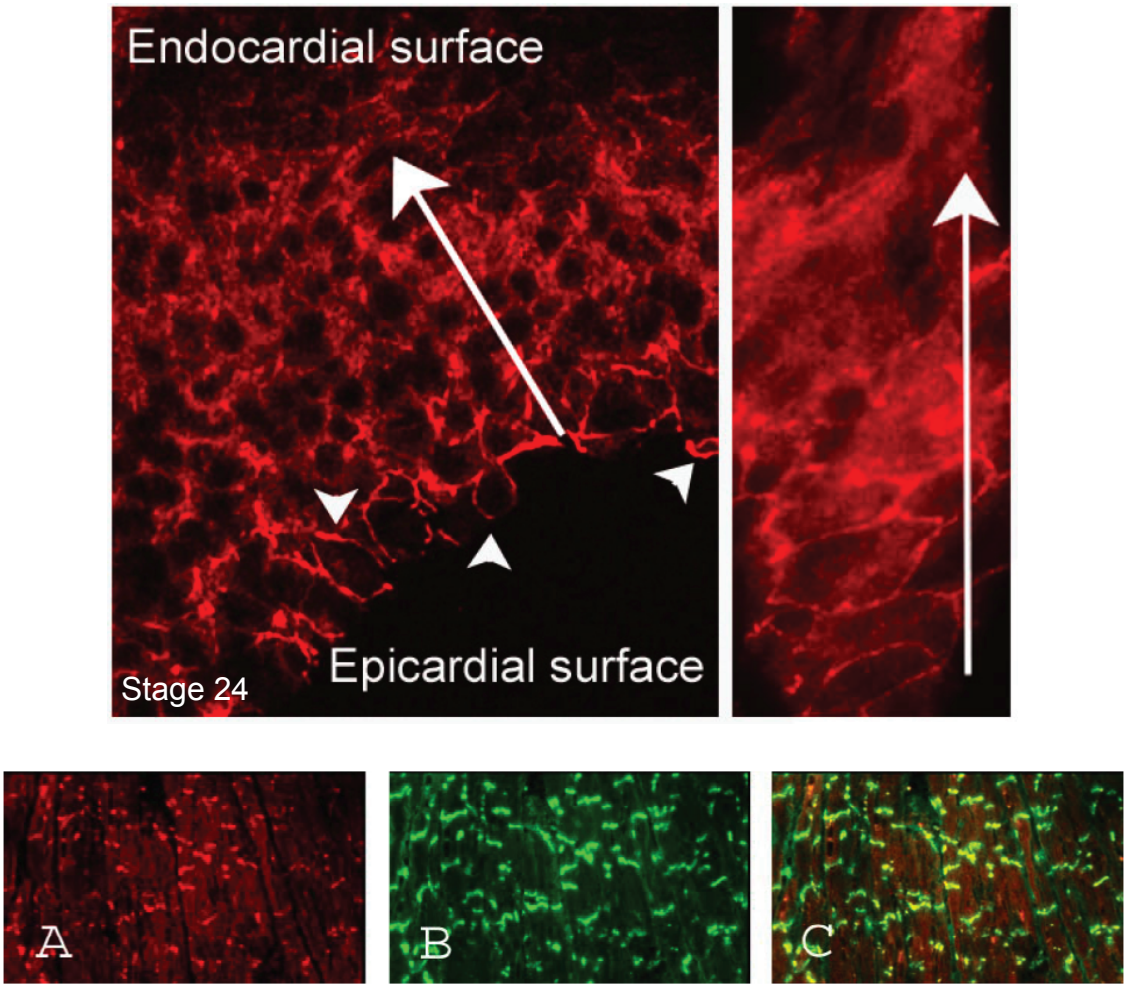


Figure 7.3 Dynamic Bves expression in cardiac tissue. Bves is expressed around the entire myocyte near the epicardium (top panel, arrow heads) in the developing heart. As myocytes undergo trabeculation (top panel, arrows), Bves is lost at the cell surface. Later, Bves (A, bottom panel) co-localizes with Connexin43 (B, bottom panel) and is present at the ID (C, merge; Connexin43 marks the ID). The images above are compliments of previous Bader laboratory members.

of these molecules specifically in the mouse heart results in embryonic lethality (Wei et al, 2002). Analysis of these embryos at the time of death revealed inhibition of cell proliferation and incomplete cardiac morphogenesis. Thus, through regulation of Rho GTPases, Bves may play a role in heart development.

Andree et al. reported minimal defects in mice that have a null mutation for Bves (Andree et al, 2002). This unexpected result may be due to redundancy of the Popdc family members. Thus, in order to study the function of Bves in the mouse, creation of a triple knockout mouse of all Popdc homologues is warranted. Alternatively, depletion of Bves in *X. laevis* results in a striking gross morphological phenotype (Ripley et al, 2006) (presumably because Bves is the only Popdc homologue expressed in early development); this phenotype has never been characterized in the heart. As *X. laevis* has been used to study heart development for years (Warkman & Krieg, 2007), this may be a potential model system in which to investigate Bves function in the heart.

Bves function in the nervous system

Bves is imperative for the transport of VAMP3-recycled receptors to and from the membrane. Our data demonstrate that Bves also interacts with VAMP2, a homologue expressed in neural tissue (Kweon et al, 2003). This is quite intriguing, as Bves is highly expressed in the brain and VAMP2 is necessary for neurotransmitter release in the brain. Thus, given the role Bves plays in VAMP3-mediated recycling in epithelia, it is possible that Bves may influence the release of neurotransmitters in the nerve terminal through interaction with VAMP2. Thus,

Bves may play an unexpected role in the biological basis of cognition or behavior.

Bves function at the molecular level

Although the function of Bves is now partially understood at the molecular level, many questions remain. For example, what specific domains are responsible for Bves interaction with VAMP3 and GEFT? Do these three proteins form a protein complex, or are these interactions competitive? Where do these interactions occur within the cell – at the external cell membranes or within the cell in vesicles? What are the kinetics of these interactions, i.e., are these interactions transient or static? These questions and many more must be examined before Bves function in the regulation of integrin and Rho GTPase signaling cascades is understood.

Bves has been demonstrated to interact with NDRG4, a little studied protein highly expressed in the heart and brain (Cross et al., in prep) (Zhou et al, 2001). With this in mind, what other proteins does Bves interact with? Are these interactions specific to particular organs, or is there a conserved mechanism of Bves function across all tissue types? The split-ubiquitin and yeast two-hybrid screens were only conducted against mouse heart libraries. Thus, if other tissues (brain or epithelia) were screened, would this reveal different Bves interacting partners or just homologues of the interacting partners already identified? Identifying additional proteins that Bves interacts with across all tissue types may reveal a global theme of Bves protein function.

Two different mutated forms of Bves protein have been used to perturb function *in vitro*: expression of only the intracellular C-terminus missing the transmembrane domains and expression of only the transmembrane domains minus the C-terminus. The former Bves disruption strategy was used to study Bves interaction with GEFT, while the latter was used to examine Bves interaction with VAMP3. Both modes of Bves disruption reveal complementary phenotypes, however, it would be interesting to study how expression of only the C-terminus of Bves affects VAMP3-mediated vesicular transport. Likewise, it would be insightful to know how expression of only the transmembrane domains of Bves affects GEFT-mediated activation of Rho GTPases. Conducting these experiments may reveal the dynamics of these interactions and subsequent functions, and provide insight to questions posed above.

Additionally, the definitive function of the Popeye domain has not been established. This domain is highly conserved throughout all species expressing Popdc homologues, thus it has been deduced that the Popeye domain must be important for protein function; recent data supports this (Chapter VI). However, the functional significance of this domain and how it exerts this function within Bves protein is unknown. Given the ascribed molecular functions of Bves, it is possible that the Popeye domain may simply act as a scaffolding protein, bringing several proteins in close proximity with the membrane. Alternatively, the Popeye domain may possess some novel enzymatic capacity (for example, a new protein kinase potential), effectively modifying proteins with which it comes

in close contact. Determining the function of the Popeye domain will illuminate our understanding of the Popdc family of proteins.

Finally, on a more general level, the biochemistry of Bves protein is almost entirely unstudied. Aside from N-glycosylation, other post-translational modifications have not been identified. Additionally, protein turnover kinetics, folding conformations, and enzymatic activities are largely uncharacterized for Bves protein. In the future, understanding the biochemical structure of Bves may provide insight into protein function.

Thus, although recent advances have been made in understanding the function of this highly conserved and widely expressed protein, there is still much to be learned about Bves and its function at both the cellular and organismal level.

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