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Review

Bves: Ten years after

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Summary. Bves was discovered in 1999 by two independent laboratories using screens to identify novel genes that were highly expressed in the developing heart (Reese et al., 1999; Andree et al., 2000). 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.

Key words: Cell adhesion, Cell motility, Epithelia, GEFT, Popdc

Popdc gene family

The *Popdc* family, of which Bves is the founding member, is comprised of three highly conserved, completely novel genes (Reese et al., 1999; Andree et al., 2000). 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 Popeye domain containing (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; Parnes et al., 2007; Froese and Brand, 2008). 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 (Parnes et al., 2007; Froese and Brand, 2008). 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 (Fig. 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 the function of these structurally unique genes and underscore their overall biological significance.

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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 (Fig. 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 and 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, 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 (Reese et al., 1999; Andree et al., 2000). Since its initial isolation, Bves expression has been identified in heart, smooth and skeletal muscle, brain, and various epithelia (Fig. 3) (Andree et al., 2000; Osler and Bader,

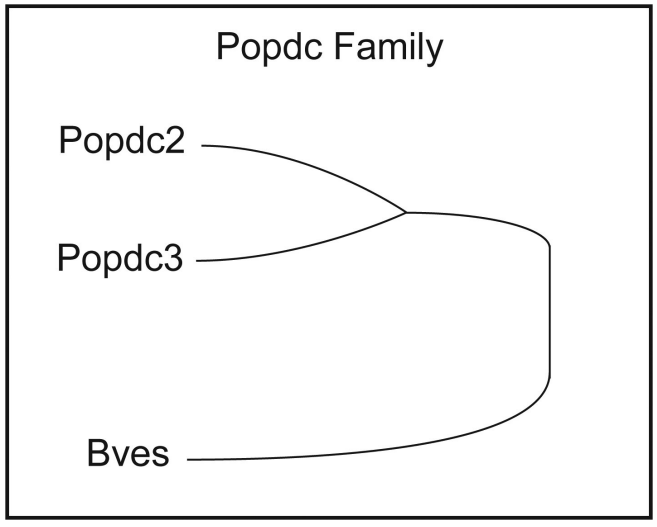

Fig. 1. The Popdc Family. Bves is only 25% conserved with either Popdc2 or Popdc3, suggesting it is the outlier of this gene family. The function of Bves is only now being uncovered, whereas the function of Popdc2 and Popdc3 are completely unknown.

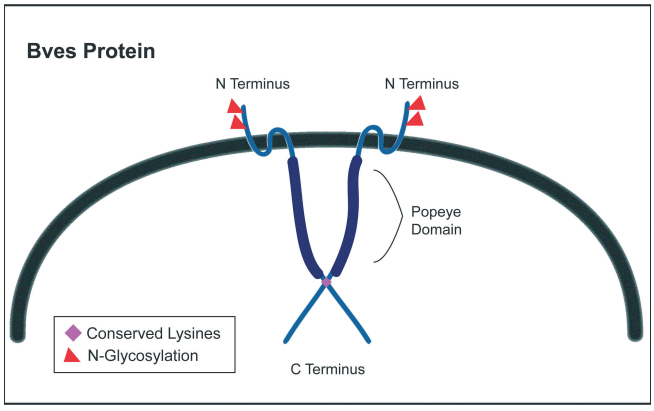

Fig. 2. Bves Protein. Bves is a transmembrane protein that exists in the plasma membrane as a multimer. There is a short, extracellular N-terminus with two N-glycosylation sites and an intracellular, self-associating C-terminus. Located within the C-terminus is the Popeye domain, named for its high conservation across species. To date, no function has been specifically linked to this motif.

2004; Ripley et al., 2004; Vasavada et al., 2004; McCarthy, 2006; Smith and Bader, 2006; Torlopp et al., 2006). 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 and Bader, 2004; Ripley et al., 2004; Smith and 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.

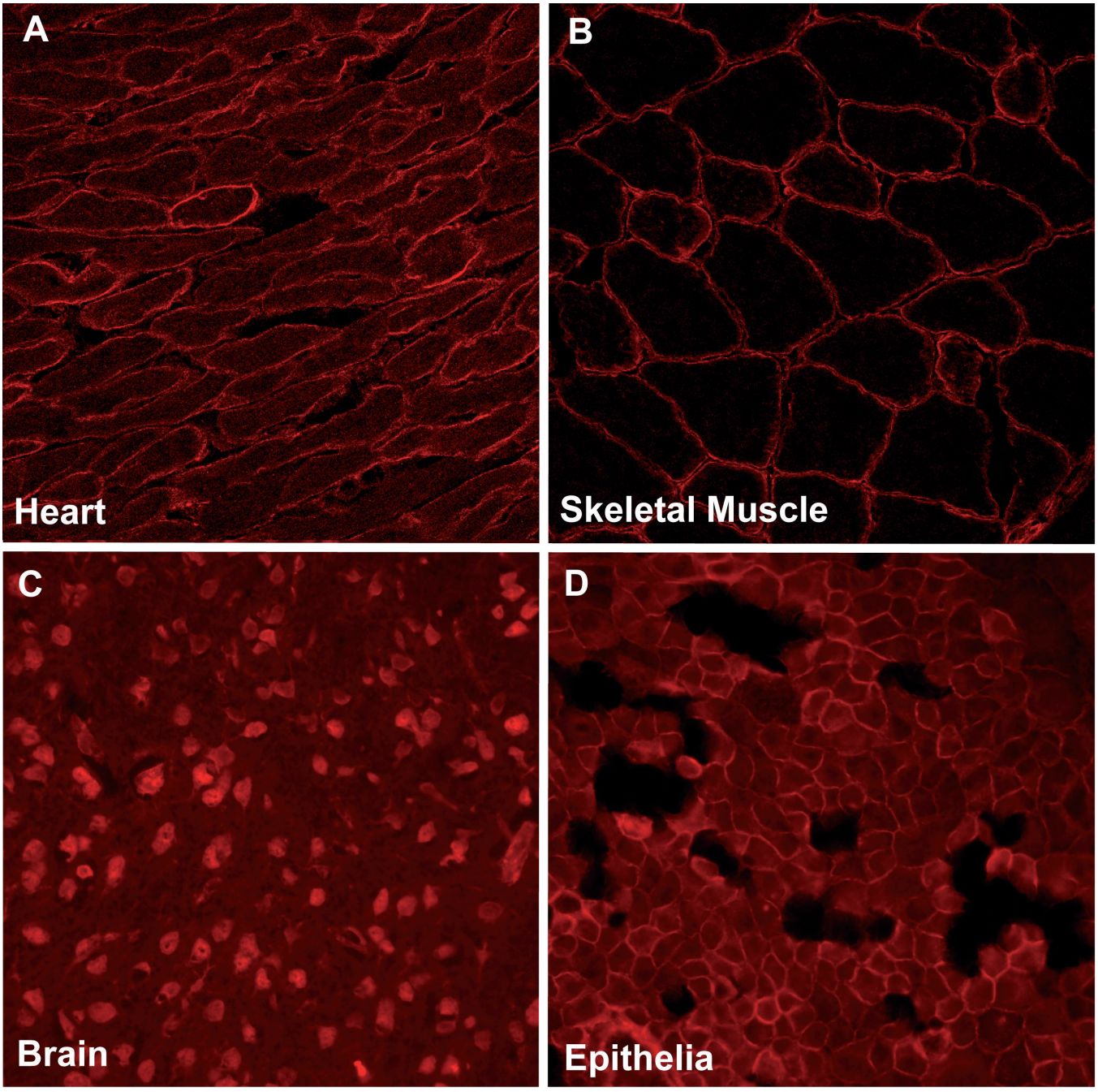

Fig. 3. Bves Expression. Bves is expressed in cells that associate or couple: Heart **(A)**, Skeletal Muscle **(B)**, Brain **(C)** and Epithelia **(D)**.

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 and 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 and 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 and 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 and 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 (Wada et al., 2001; Osler et al., 2005). 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 and 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 and 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 and 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; Ripley et al., 2006; Lin et al., 2007). 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 (Ripley et al., 2006; Lin et al., 2007).

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 and 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 and 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 and 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., 2000, 2002; Breher et al., 2004; Smith and Bader, 2006; Froese and Brand, 2008). 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 and 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 ooctye, regulating downstream effectors in this area (Gilbert et al., 2006). In *Drosphila*, 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 (Wada et al., 2001; Kawaguchi et al., 2008). 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 (Wada et al., 2001; Osler et al., 2005). 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 and 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 (Knight et al., 2003; Kawaguchi et al., 2008). 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 and 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 (Guo et al., 2003; Bryan et al., 2004, 2005, 2006). GEF distribution is spatially regulated and thus localization at the membrane can potentially be indicative of GEF activation (Schmidt and 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 (Fig. 4A). In general, GEFs are modulated in three distinct ways: 1) self-regulation through an inhibitory intramolecular association; 2) activation via interaction with another protein; 3) modulation through subcellular localization (Schmidt and Hall, 2002).

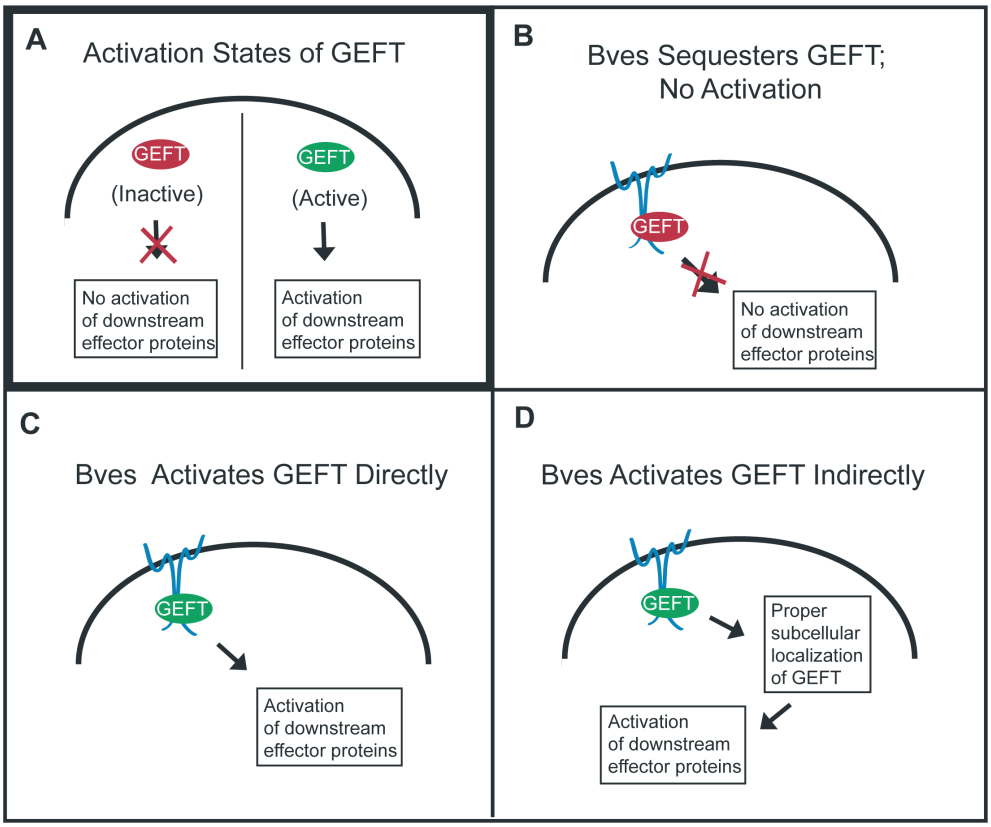

Fig. 4. Bves Regulation of GEFT. In order to induce downstream effectors, GEFT must be activated **(A)**; the mechanism by which this occurs is not specifically known. Bves interaction with GEFT may regulate the activation state of GEFT. Three mechanisms of modulation are outlined: 1) Bves may sequester 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)**.

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 (Fig. 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 (Fig. 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 (Fig. 4D). As mentioned earlier, GEFT may be regulated by its localization within the cell (Schmidt and Hall, 2002). If GEFT cannot be transported to or be retained at its activation site, this would cause a 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 (Malliri and Collard, 2003; Braga and Yap, 2005; 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 (Ripley et al., 2006; Lin et al., 2007). 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 and 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.

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