# ANALYSIS OF BVES FUNCTION THROUGH IDENTIFICATION OF INTERACTING PROTEINS

By

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To my parents, always supportive

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# CHAPTER I

# INTRODUCTION

Bves (blood vessel epicardial substance) was initially described in the literature eight years ago (Reese et al. 1999). Despite the existence of nearly 20 publications investigating this gene, very little has been learned about the functional significance of this protein. Several phenotypic abnormalities have been generated via alteration of *bves* expression levels, yet little evidence exists to substantiate a molecular mechanism underlying these phenotypes. Of course, determination of the function of a novel protein is a very attractive goal for those interested in basic science, and was the topic that I selected for my doctoral research.

This introductory chapter will provide a synopsis of the existing literature concerning Bves. Additionally, a brief review of Rho-family GTPase signaling is also provided, which will be helpful in understanding the potential functional implications of the work described in this doctoral thesis.

## Bves: Discovery and initial characterization

To identify novel genes expressed during heart development, we performed a subtractive hybridization screen in the HH stage 18 chicken heart (Reese et al. 1999). *bves (**b**lood **v**essel **e**picardial **s**ubstance)* is a novel

message identified through this screen, as reported by Reese et al. (1999). We assigned the name Bves to the protein product because our first antiserum recognized the protein in cells of the epicardium and developing coronary vascular system. Using a similar screen, Thomas Brand and colleagues independently identified an identical chick cDNA, which they termed "*pop1a*", and characterized a related family of genes in chick and several other species (Andree et al. 2000). As these transcripts were identified in heart and skeletal muscle, they assigned the name *popeye* to the gene family to reflect the robust expression pattern in muscle types. The Duncan laboratory also independently identified Bves in a screen for genes transcriptionally-regulated in eye development (M. Duncan, personal communication) and later reported the characterization of two monoclonal antibodies directed against the protein (DiAngelo et al. 2001). For clarification, the correct nomenclature in mouse and human for the *bves/pop1a* gene and protein is Bves. The accepted names for the protein products of family members *popdc2* and *popdc3* are Popdc2 and Popdc3, respectively (Mouse Genome Informatics, Jackson Labs; HUGO Gene Nomenclature Committee). The gene family is known as the *popdc* (popeye domain-containing) family.

Since the initial identification of *bves* in chicken, mouse and human, cDNAs representing this gene have been reported in all classes of vertebrates. Homologous Bves sequences have been identified in several invertebrates, including insects *Drosophila* and *Anopheles*, through a search for EST clones

with internet-based search engines (NCBI, Ensembl). Additionally, related transcripts have been reported in the cephalochordate *Amphioxus floridae* (Andree et al. 2000), as well as *Ciona intestinalis* (Davidson and Levine 2003) and the ascidian *Boltenia villosa* (Davidson et al. 2003). To date, *Bves* has not been reported to be in the *C. elegans* genome, in any single cell organism, or in plant species.

The genomic location and structure of *Bves* is known for several species (Table 1). Mouse *Bves* lies on chromosome 10, human *Bves* has been mapped to chromosome 6q21, and the chicken gene is located on chromosome 3. The cloning of *bves* in many organisms has allowed a cross-species comparison of gene structure, which varies considerably. The mouse gene consists of 11 exons, while human *BVES* has only five exons. The chicken and *Drosophila bves* genes have eight and seven exons, respectively. Chicken *bves* generates a message of approximately 1.7kb, while mouse *bves* is 1.8kb (Reese et al. 1999; Andree et al. 2000). A high sequence similarity exists at the nucleotide level (>70%). In addition, the presence of at least four individual transcripts from the chicken *bves* gene has been demonstrated, and originally reported as Pop1A-Pop1D (Andree et al. 2000). The splice variants appear to be generated principally in the extreme 5' and 3' ends of the coding region (Andree et al. 2000). To date, no insight concerning function has been derived from the genomic structure. Currently, none of the promoter or enhancer elements that drive cell-specific expression have been described for *bves* or any other *popdc* genes, although *bves* has been

Table 1: Popdc family gene characteristics

| Species | Gene | mRNAs | Accession no. | Gene position | Protein | Size (aa) | Isolated/reported by |
|---|---|---|---|---|---|---|---|
| | *Bves* | *Bves* | NM_007073 | 6q21 | Bves | 357 | Reese et al. (1999) |
| *H. sapiens* | *Popdc2* | *Popdc2* | NM_022135 | 3q13 | Popdc2 | 364 | Andree et al. (2000) |
| | *Popdc3* | *Popdc3* | NM_022361 | 6q21 | Popdc3 | 291 | " |
| *M. musculus* | *Bves* | *Bves* | AF204174 | 10 | Bves | 358 | Reese et al. (1999) |
| | *Popdc2* | *Popdc2* | AF204175 | 16 | Popdc2 | 367 | Andree et al. (2000) |
| | *Popdc3* | *Popdc3* | AF204176 | 10 | Popdc3 | 291 | " |
| | *Bves* | *Bves* | AF208398 | 3 | Bves | 360 | Reese et al. (1999) |
| | " | *Pop1b* | AF208399 | " | Popdc1b | 288 | Andree et al. (2000) |
| | " | *Pop1c* | AF208400 | " | Popdc1c | 305 | " |
| | " | *Pop1d* | AF208401 | " | Popdc1d | 695 | " |
| | *Popdc2* | *Popdc2a* | AY388621 | 1 | Popdc2a | 356 | " |
| *G. gallus* | " | *Popdc2b* | AY388622 | " | Popdc2b | 353 | " |
| | " | *Popdc2a/b* | AY427076 | " | Popdc2a/b | 378 | " |
| | " | *Popdc2c* | AY388624 | " | Popdc2c | 276 | " |
| | " | *Popdc2d* | AY388623 | " | Popdc2d | 276 | " |
| | *Popdc3* | *Popdc3* | AF204170 | 3 | Popdc3 | 305 | " |
| *D. melanogaster* | *Bves* | *Bves* | AF247183 | X | (dm)Bves | 415 | S. Lin (abstract) |
| *X. laevis* | *XBves* | *Bves* | AF527799 | ? | XBves | 338 | Ripley (2006) |
| *D. rerio* | *Popdc1* | *Popdc1* | AY293117 | 13 | Popdc1 | 316 | Brand (abstract) |
| | *Popdc3* | *Popdc3* | AY293116 | 25 | Popdc3 | 298 | " |

identified as a target of Pax3, a key developmental transcription factor in muscle (Barber et al. 2002).

This dissertation will focus primarily on the *bves* gene and its gene product, Bves. However, it is necessary and appropriate to point out similarities and differences between the other family members, *popdc2* and *popdc3,* which have been both been identified in mouse, human, and chicken (Andree et al. 2000). The gene structure and sizes of *popdc* family members have been determined (Table 1). The chromosomal location of the *popdc2* and *popdc3* genes has been determined in many species (Table 1). Mouse *popdc3* is located in close proximity to *bves* on chromosome 10 while *popdc2* is on chromosome 16. Similarly, human *popdc3* has been mapped to chromosome 6q21 (like *bves)*, while *popdc2* has been mapped to chromosome 3q13. Chicken *bves* and *popdc3* genes are both located on chromosome 3 and *popdc2* is on chromosome 1. However, not all species have multiple *popdc* genes. To date, only one transcript has identified in *Xenopus* (Hitz et al. 2002; Ripley et al. 2006), and in *Drosophila* through EST databases and cDNA cloning (NCBI).

In summary, *popdc* genes appear in a broad spectrum of invertebrate and vertebrate species. Due to potential redundancy of *popdc* gene family members, genetic analysis of function may proceed more rapidly in organisms that have only one gene, whereas studies in mice, which are essential for understanding mammalian gene function, will apparently require disruption of two or more of the genes (Andree et al. 2002).

## Protein Size and Structure

The full-length protein sequence of *bves* has been determined in chicken (357 a.a.), mouse (358 a.a.), and human (360 a.a.) (Table 1). Conservation of Bves protein sequence exists across a wide variety of vertebrate species (~80%; Figure 1). At present, groups studying Bves are in agreement that the protein possesses three transmembrane domains and has an extracellular glycosylated N-terminus and intracellular C-terminus (Figure 1) (Andree et al. 2002; Knight et al. 2003). A closer examination of sequence similarity reveals that certain regions of the protein exhibit higher degrees of homology (Figure 1). For example, while the amino acid sequence of chick and mouse are 75% homologous, the highest degree of homology (92%) lies within the C-terminus. Outside of the regions that encode glycosylation sites, the N terminal sequence is rather diverse across species (Andree et al. 2000). While I will focus on the gene product, Bves, from this point forward, I will mention that the mouse, chick, and human *popdc2* encodes a ~360 a.a. (41kD) and *popdc3* encodes a ~290 a.a. protein, which has a shorter C-terminus (37kD) (Table 1). Like Bves, the additional gene products in the Popdc family are highly conserved across species (~80%) (Table 1). However, within a single species Bves is ~25% homologous to Popdc2 and Popdc3. Interestingly, Popdc2 and Popdc3 are ~50% identical, indicating that these gene products are more closely related to each other than to


Figure 1: Alignment of vertebrate Bves sequences. Bves is highly conserved across species. Transmembrane/hydrophobic sequences are contained by red boxes. The Popeye domain is denoted (black line). Sequences to which antibodies were generated are indicated by blue (D033), green (XBves), and low (B846) lines. An additional monoclonal antibody generated by DiAngelo et al. (2001) was raised against amino acids 91-358 of the chick sequence. A pair of lysine residues within the C-terminus, denoted by a pink box, has been shown to be critical for epithelial integrity.

Bves, which appears to be the outlier of this family. This is surprising in light of the arrangement of the three family members in the genome (see Table 1).

Immunochemical detection of Bves protein yields bands ranging between a ~42-48kD under reducing and denaturing conditions (Reese et al. 1999; Andree et al. 2000; DiAngelo et al. 2001; Smith and Bader 2006). The size variation may result from posttranslational modification of the protein in the form of N-linked glycosylation (Knight et al. 2003). Early computer-based modeling predicted that Bves possesses three hydrophobic domains (Reese et al. 1999; Andree et al. 2000). Presence of these hydrophobic domains was confirmed and shown to be essential for membrane insertion/retention, as demonstrated by in vitro transcription/translation reactions in the presence of microsomes (Wada et al. 2001). Despite these findings, the structure of Bves was initially difficult to resolve and Wada et al. originally reported that the C-terminus of Bves was extracellular (2001). Subsequent immunocytochemical and biochemical data suggest that the short N-terminus (36-39a.a.) is extracellular, while the longer C-terminus is intracellular (Knight et al. 2003). Two N-glycosylation sites have been found within the N-terminus in all species studied thus far (Knight et al. 2003), although the physiological significance of these sites is unknown.

The C-terminal intracellular portion of the protein is a likely candidate region for interactions with other proteins and, thus, its dissection has been a primary focus. However, this region of Bves lacks known motifs, such as a PDZ, SH3, leucine zippers, or other protein-protein interaction domains that would

suggest function. However, a highly conserved domain (a.a. 172-266) in the C-terminal intracellular portion of Bves for which function(s) is unknown has brought about the renaming of the Popeye family to Popdc (popeye domain containing), and this newly identified domain may be a motif for protein-protein interaction unique to the Popdc family. The absence of any conserved or characterized domains in the Bves protein has greatly complicated early inquiries into the function of the protein, as the unique nature of Bves at the amino acid level precludes any sort of candidate approach in experimental design. In the absence of known functional domains, it has been necessary to identify interacting partners using immunoprecipitation, protein pull-downs, and yeast two-hybrid analyses to ascribe function. Later, I describe the isolation of two interacting proteins using genetic screens, and describe the characterization of one of these interactions.

## Expression of Bves during development

Bves expression has been analyzed at the RNA and protein level in the mouse, chick, and frog during various developmental stages (Reese et al. 1999; Andree et al. 2000; DiAngelo et al. 2001; Wada et al. 2001; Andree et al. 2002; Hitz et al. 2002; Osler and Bader 2004; Ripley et al. 2004; Vasavada et al. 2004; Ripley et al. 2006). Interestingly, expression analysis using in situ hybridization or antibody detection did not always lead to congruent results, and some of the Bves expression data appeared difficult to reconcile. One major point of

contention has been the question whether Bves is expressed in the epicardium of the heart, and more generally, in epithelial cells.

Published literature agrees that Bves is clearly expressed in muscle cell types. Reese et al. (1999) and Andree et al. (2000) originally identified the *Bves* transcript in a screen for gene expression in the developing chick heart. Without doubt, in situ hybridization, Northern blot, RT-PCR, and lacZ knock-in experiments have demonstrated that *Bves* is highly expressed in muscle cells of the embryonic heart in all vertebrates examined thus far (Reese et al. 1999; Andree et al. 2000; Hitz et al. 2002; Ripley et al. 2006). Immunocytochemical studies have confirmed this robust cardiac muscle expression. Using the B846 polyclonal antiserum (Wada et al. 2001; Osler and Bader 2004; Ripley et al. 2004), the XBves polyclonal antiserum (Ripley et al. 2006), the 3F11 monoclonal antiserum (DiAngelo et al. 2001; Vasavada et al. 2004), Bves has been detected in the developing myocardium of embryos and in the adult heart. Expanded analysis of both mRNA and protein expression have detected *Bves* in skeletal muscle of chick, mouse and frog (Andree et al. 2000; Andree et al. 2002; Ripley et al. 2006). It should be noted that Hitz et al. (2002) report no Bves expression in the skeletal muscle of *Xenopus* embryos by in situ hybridization. The reason for this conflicting result is presently not known. Interestingly, Swalla and colleagues found an ascidian *popdc* gene to be expressed in cells of the primordial heart and tail muscle lineages, suggesting a conserved function for Bves in striated muscle across species (Davidson et al. 2003). Taken together, these published studies

permit the conclusion that Bves is expressed in all striated muscle continuously from development through adulthood. Furthermore, primary and immortal myocyte cell lines have been employed to analyze Bves expression and localization in a cell culture system. Overall, the published work pertaining to Bves expression in muscle indicates that Bves mRNA and protein are clearly observed in cardiac, skeletal, and smooth muscle types (Table 2).

The tissue around which much of the dispute in the literature was centered was the epicardium. While a thorough discussion of the epicardium and its significance will not be included here, a brief introduction to the epicardium and development of the coronary vasculature will be presented. During the process of cardiac looping, the epithelial proepicardium migrates to the surface of the myocardium, proliferates rapidly, and covers the surface of the myocardium (Viragh and Challice 1981; Viragh et al. 1993; Mikawa and Gourdie 1996). This epithelium persists through development and adulthood, comprising the epicardium and pericardium. A subpopulation of cells of the epicardium undergo an epithelial-mesenchymal transition, delaminate from the epithelial epicardium, and infiltrate the developing myocardium (Mikawa and Gourdie 1996; Reese et al. 1999). These mesenchymal cells give rise to the smooth muscle, vascular endothelium, and cardiac fibroblasts of the coronary vasculature (Mikawa and Gourdie 1996). The presence or absence of Bves in the proepicardium and mature epicardium, as well as in the derivatives of these tissues, has been a central topic of debate in the literature

Table 2: Expression data are reported as interpreted by published literature. Sources are numbered in order of publication date. Corresponding numbers are used to indicate expression findings in chart form based on technique. A consensus of expression is presented as positive, negative or both, in the case where conflicting reports exist. Reference numbers with a strikethrough indicate that expression was analyzed in the noted report and not observed. A question mark indicates that the expression has not been tested and/or determined. Species are abbreviated as C (chick), M (mouse), H (human), X (frog), not applicable (n/a).

Expression of Bves in muscle

| **Muscle type** | Cardiac | Skeletal | Vascular smooth | Extracardiac smooth | Primary myocytes | **Species,** Reported by |
|---|---|---|---|---|---|---|
| **mRNA detection** | + | + | − | + | + | 1. **C,** Reese (1999) |
| in situ | 1–3,5,13 | 1–3,5,13,~~7~~ | ? | 5 | n/a | 2. **H,** Reese (1999b) |
| RT-PCR | 1-3,5,9 | 2,3,5,7,11,12 | ? | 3 | ? | 3. **C,M,** Andree (2000) |
| Northern blot | 1-3,11 | 1-3,11 | ? | 3 | ? | 4. **C,** Wada (2001) |
| lacZ embryos | 5,6 | 5,6 | ~~5,6~~ | 5,6 | 5,6 | 5. **M,** Andree (2002) |
| **Protein detection** | + | + | +/− | + | + | 6. **M,** Andree (2002b) |
| D033 pAb | 1 | 9 | 1,4 | ? | n/a | 7. **X,** Hitz (2002) |
| B846 pAb | 9,14 | 15 | 4,14 | 14 | 15 | 8. **C,** DiAngelo (2001) |
| XBves pAb | 13 | 13 | ? | 14 | n/a | 9. **C,** Osler (2004) |
| 3F11-D9-E8 mAb | 8,10 | 10,12 | ~~10~~ | ? | 10 | 10. **C,** Vasavada (2004) |
| | | | | | | 11. **M,** Breher (2004) |
| | | | | | | 12. **M,** Brand (2005) |
| | | | | | | 13. **X,** Ripley (2006) |
| | | | | | | 14. **C,M,H,X,** Osler (2006) |

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EST database searches suggested that the message also existed in non-striated muscle sources. Using Northern blot and RT-PCR analyses, the Bves transcript was identified in other organs such as the brain, kidney, stomach, lung, spleen, and the uterus of a pregnant mouse, (Andree et al. 2000). Based on these data and the results described above, Brand and co-workers hypothesized that Bves is expressed within the smooth muscle of these organs. However, Northern blot and RT-PCR analyses of organs or tissues do not allow the identification of the specific cell types expressing a certain transcript, presenting a disadvantage of these techniques for detailed expression studies at the cellular level. Using immunocytochemistry, Bves protein has been detected in the smooth muscle surrounding the coronary vessels and in gastric visceral smooth muscle (Reese and Bader 1999; Wada et al. 2001), but also in epithelia, as discussed below. Analysis of Bves lacZ knock-in mice further suggested Bves expression exists in the smooth muscle of the gut tube, notochord, and neural tube (Andree et al. 2002). While absolute resolution has not been reached concerning vascular smooth muscle expression, literature agrees that Bves is present in several smooth muscle cell types throughout the embryo.

Several lines of evidence indicate that Bves is expressed in epithelial cell types, in addition to expression in various striated and smooth muscle cells. Since *Bves* had originally been isolated from a screen for heart-specific genes (Reese et al. 1999; Andree et al. 2000), the expression pattern was consequently expected to be restricted to the cardiac muscle progenitors and the myocardium.

Thus, the initial investigations concentrated only on heart development, with a broadened focus on muscle development upon detection of the gene/protein in skeletal and smooth muscle. Consequently, studies of Bves expression were extended to embryogenesis as a whole. Following comprehensive mRNA and protein analysis in many species, Bves was found to be expressed in a large variety of epithelial tissues in addition to muscle (Wada et al. 2001; Osler and Bader 2004; Ripley et al. 2004; Vasavada et al. 2004; Osler et al. 2005; Ripley et al. 2006)(Table 3).

While existing data do not suggest whether Bves is restricted to a particular subset of epithelia, the protein is expressed in all three germ layers of the developing embryo, and in many epithelial structures during morphogenesis and in the adult (Osler and Bader 2004; Ripley et al. 2004; Osler et al. 2005; Ripley et al. 2006). The first clue that Bves could be a significant epithelial component arose with the identification of expression in the chick proepicardium and its derivative, the epicardium. (Figure 2A, Table 3). To this point, Bves expression in the epicardium has been detected using several monoclonal and polyclonal immunoreagents in chick and mouse embryos (Reese et al. 1999; Wada et al. 2001; Osler and Bader 2004; Vasavada et al. 2004). However, Bves expression in chick epicardium was reported to be of transient nature in one particular analysis using a monoclonal $\alpha$-Bves antibody (Vasavada et al. 2004). These data point out that the persistence/continuity of Bves expression in the epicardium is not resolved at present. Furthermore, all reports of epicardial

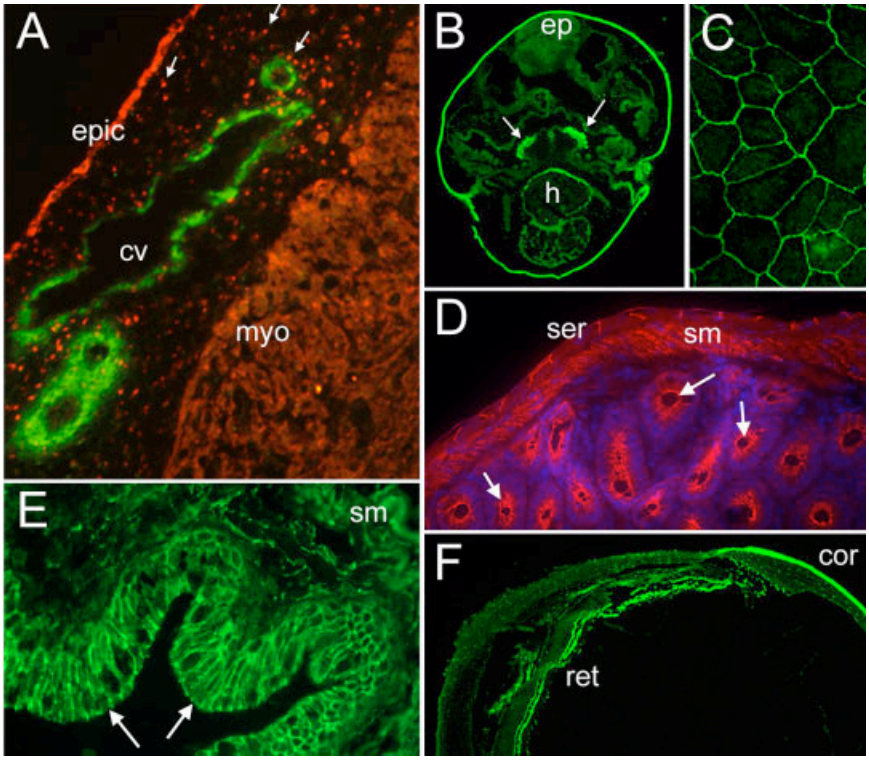



Figure 2: Bves expression in muscle and epithelia. A: B846 polyclonal immunoreagent detects Bves (red) in the epithelial epicardium (epic), delaminated migratory cells (arrows), and myocardium (myo) in a section through a developing chick heart. Smooth muscle actin (green) labels forming coronary vessels (cv) in the subepicardial space. B: Five-day frog embryo labeled with -XBves (green). Positive cells are found in the epidermis (ep), the developing heart (h), and the velar plate (arrows). C: Bves is distributed at the cell circumference in cultured human corneal epithelial cells, as labeled by B846 polyclonal antisera. D: Bves is detected by -Bves B846 (red) in the serosa (ser), the smooth muscle (sm), and the gastric epithelium (arrows) of the adult mouse intestine. The 4,6-diamidine-2-phenylidole-dihydrochloride (DAPI) labels the cell nuclei (blue). E: -XBves detects epithelium (arrows) and the smooth muscle (sm) cells of the adult Xenopus gut (green). F: Xenopus Bves is recognized by -XBves in the epithelial layers of the adult frog eye, including the cornea (cor) and retina (ret).

Table 3: Expression data are reported as interpreted by published literature. Sources are numbered in order of publication date. Corresponding numbers are used to indicate expression findings in chart form based on technique. A consensus of expression is presented as positive, negative, or both, in the case where conflicting reports exist. Reference numbers with a strikethrough indicate that expression was analyzed in the noted report and not observed. A question mark indicates that the expression has not been tested and/or determined. Species are abbreviated as C (chick), M (mouse), H (human), X (frog), not applicable (n/a).expression come from studies in the chick embryo and it remains unclear whether this observation could be species-specific.

| **Epithelial type** | Epicardium | Gut | Gastrula | Eye | Epidermis | Early somite | Cell lines | **Species**, Reported by: |
|---|---|---|---|---|---|---|---|---|
| **mRNA detection** | − | + | + | + | + | + | + | 1. **C,** Reese (1999) |
| in situ | ~~2~~ | ? | 13 | 13 | 13 | 13 | n/a | 2. **C,M,** Andree (2000) |
| RT-PCR | ~~10~~ | 8 | 8 | 7 | 8 | ? | 8 | 3. **C,** Wada (2001) |
| lacZ embryos | ~~4,5~~ | ? | ? | ? | ? | ? | n/a | 4. **M,** Andree (2002) |
| **Protein detection** | +/− | + | + | + | + | + | + | 5. **M,** Andree (2002b) |
| D033 pAb | 1,3,8 | ? | 8 | ? | 8 | 8 | ? | 6. **C,** DiAngelo (2001) |
| B846 pAb | 3,8,14 | 8 | 8 | 7 | 8 | 8 | 3,7,8,11 | 7. **C, H,** Ripley (2004) |
| XBves pAb | ? | 14 | 13 | 13 | 13 | ? | 13 | 8. **C,** Osler (2004) |
| 3F11-D9-E8 mAb | 6,9,~~12~~ | ? | ? | ? | ? | ? | ? | 9. **C,** Vasavada (2004) |
| | | | | | | | | 10. **M,** Breher (2004) |
| | | | | | | | | 11. **M,H,** Osler (2005) |
| | | | | | | | | 12. **M,** Brand (2005) |
| | | | | | | | | 13. **X,** Ripley (2006) |
| | | | | | | | | 14. **C,M,H,X,** Osler (2006) |

In addition to the epicardial epithelium, Bves is detected in a variety of other epithelia, including the gut epithelium and the serosa, epithelia of the respiratory system, the epidermis, the eye and the ependyma (Osler and Bader 2004; Ripley et al. 2004; Ripley et al. 2006) (Figure 2). Of these organs, the eye expression pattern is of particular interest since Duncan and colleagues identified the Bves transcript from a screen for genes essential for eye development (Duncan, personal communication). Importantly, proper eye development results from appropriate orchestration of signals between the primordial retina, lens and cornea, all of which are epithelial in nature. Unpublished work in *Xenopus* underscores the importance of Bves in epithelial morphogenesis of the eye, as development is impaired following XBves depletion (Osler, unpublished data). Notably, the Bves transcript has also been detected by RT-PCR in early embryonic stages in the chick and frog, prior to differentiation of the heart and skeletal muscle (Osler and Bader 2004; Ripley et al. 2004; Ripley et al. 2006). This supported the idea that Bves must be an epithelial component, since the cell layers of early, gastrulating embryos are epithelial in nature, and lack muscle gene expressing cells.

Epithelial cell lines have permitted the analysis of Bves expression in a controlled culture system. Our group demonstrated a conserved presence in clonal epithelial cell lines of endodermal, mesodermal and ectodermal origin, as predicted by expression in embryos (Wada et al. 2001; Osler and Bader 2004;

Ripley et al. 2004). Antibodies (B846, D033, XBves) recognize Bves in various epithelia and clearly show this protein is present in epithelia (Figure 2; Table 3).

Despite the agreement of investigators in the field concerning Bves expression in cardiac and skeletal muscle, numerous disagreements still exist about expression in other cell types. As stated above, expression in epithelial cells and some smooth muscle remains a topic of debate in the literature. While immunochemical analyses indicate that Bves is expressed in a variety of epithelial cell types and in cardiac smooth muscle; surveys of expression involving detection of *bves* message disagree with these findings. It is critical to resolve the discrepancies in this field regarding expression, as important inferences concerning protein function can be made from expression patterns (Kamberov et al. 2000; Roh et al. 2002; Hurd et al. 2003; Roh et al. 2003). For example, if Bves were found to be a protein expressed specifically in striated muscle, this would indicate that the function of Bves might be a "muscle-type" function, such as contraction, response to electrical stimulus, or adhesion between myocytes (Wang et al. 1999; Sinn et al. 2002). However, determination that Bves is not restricted to muscle would preclude many of these potential functions, and indicate that Bves may have a more "generic" function that would be applicable to a broader range of cell types. In a following chapter, I describe our efforts to remove these discrepancies from the field through generation of a panel of Bves-specific monoclonal antibodies, and our subsequent survey of Bves expression during murine embryogenesis

## Function

Of course, the most important scientific question about Bves is: *What is the function of the Bves protein?* The lack of identifiable protein motifs and a potential redundancy of function between members of the *Popdc* gene family in coelomates have made ascertainment of Bves function difficult. By necessity, initial experiments conducted to determine function were broad in nature. Thomas Brand and colleagues published the first report of a Bves-null mouse (Andree et al. 2000). These animals displayed no overt embryonic phenotype (Andree et al. 2002), presumably due to redundant functions of *Bves* with *Popdc2* and *3*. However, the adult mice showed a delay in skeletal muscle regeneration *in vivo* following cardiotoxin injection. Attempts to determine the developmental function using genetics in the mouse await generation of an animal where all *Popdc* genes are inactivated.

Genetic analyses in *Drosophila* and zebrafish are also attractive, as only one *Popdc* family gene is present in *Drosophila,* while the number is currently unresolved in zebrafish. Recently, Bader and colleagues addressed function in vivo by depleting the *X. laevis* Bves homolog in developing frog embryos (Ripley et al. 2006). In this study, Ripley and Osler et al. show that global depletion of XBves by $\alpha$-XBves morpholino oligonucleotides (Gene Tools, Inc.) causes a gastrulation block, while clonal depletion of XBves results in rogue movements by the progeny of the injected cell. These findings underscore the essential nature of this protein in large-scale epithelial rearrangements that occur during early

development (Ripley et al. 2006). However, these experimental findings do not indicate any sort of molecular mechanism for Bves, nor do they explain how alteration of Bves expression leads to the observed phenotypes.

In the absence of genetic systems, advances in examination of Bves function have developed from in vitro studies using cell culture models (Wada et al. 2001; Ripley et al. 2004; Osler et al. 2005). Early findings support a role in cell-cell adhesion/cell-cell interaction. Experiments using fibroblastic L-cell hanging-drop aggregation assays support an adhesive function for Bves. These standard adhesion assays demonstrated that transfection of chicken, *Xenopus*, or *Drosophila* Bves resulted in increases in cell-cell adhesion in these normally non-adherent cells (Wada et al. 2001; Ripley et al. 2006).

Published work from the Brand and Bader groups points to Bves-Bves homophillic interaction in both N- and C-termini (Wada et al. 2001; Andree et al. 2002) and supports the results of L-cell adhesion assays. Vasavada et al. have also demonstrated that Bves in its native conformation forms dimers (2004) (Figure 3). Whether Bves heterophillically interacts with related Popdc family members is currently unknown. Additional work from the Backstrom laboratory has indicated that intermolecular disulphide bonding plays a role in Bves-Bves interactions (Knight et al. 2003). Thus, it is plausible that oligomeric forms of Bves participate in cell-cell interaction at some level. Clearly, evidence exists from a variety of experimental methods that Bves is important for cell-cell interaction/adhesion.

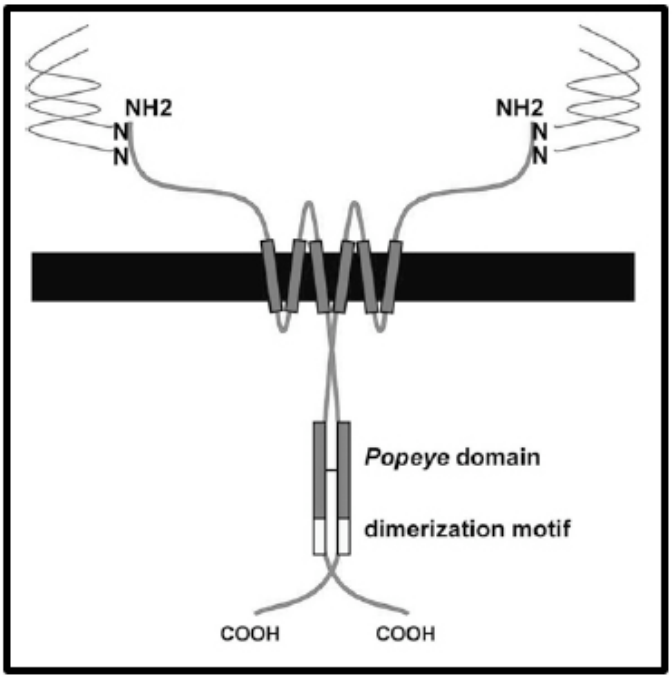



Figure 3: Predicted structure of Bves. Bves, a protein containing ~360 amino acids, is predicted to have 3 transmembrane domains. The extracellular amino terminus possesses two N-linked glycosylation sites. The intracellular C-terminal tail contains a domain responsible for oligomerization (Kawaguchi and Bader, unpublished results) and the Popeye domain, a common feature of all popdc family members.from a variety of experimental methods that Bves is important for cell-cell interaction/adhesion.

Epithelial cells express Bves at points of cell-cell contact, reminiscent of proteins involved in cell adhesion (Osler et al. 2005). In confluent epithelial cells, Bves surrounds the cell border and significantly colocalizes with TJ proteins Occludin and ZO-1 (Osler et al. 2005). As previously stated, investigation of Bves interaction with other proteins is a critical avenue of exploration for determination of Bves function as little indication of function is provided by analyses of domains contained in the Bves protein. GST pull-down analysis demonstrated an interaction between the intracellular C-terminus and a protein complex containing ZO-1 (Osler et al. 2005). ZO-1 is a scaffolding protein that interacts with a multitude of tight, adherens and gap junction proteins (Itoh et al. 1993; Fanning et al. 1998; Itoh et al. 1999; Barker et al. 2001).

Consequently, the localization and interaction at the TJ led to a functional assessment of TJ integrity in Bves-depleted human corneal epithelial cells. Knockdown of Bves in epithelial cells leads to a disruption of epithelial sheet integrity, a concomitant loss of transepithelial resistance, and displacement of ZO-1 from the TJ domain, further suggesting interaction between Bves and the TJ (Osler et al. 2005). While this finding indicates that Bves somehow affects TJ integrity in epithelia, perhaps through an interaction with ZO-1, neither the nature of this interaction nor the precise domain of Bves that interacts with ZO-1 have been determined. While this interaction and phenotype are interesting, a

mechanistic explanation for how the interaction of Bves with a protein complex containing ZO-1 may be potentiating this phenotype is lacking at this time.

In summary, the function of Bves has been largely undetermined. Tantalizing phenotypes, such as the epithelial wound assay, tight junction perturbation upon Bves depletion, defective cellular organization during *Xenopus* gastrulation upon Bves knockdown, and delays in skeletal muscle regeneration upon Bves global inactivation exist, yet no molecular mechanism has been determined to explain these phenotypes. I have determined that Bves interacts with GEFT, a protein that modulates small Rho-GTPase signaling. This interaction, and its effects on Rho-GTPase signaling, provides the first description of a protein that interacts with Bves directly and provides a molecular mechanism for the Bves protein that may explain some or all of the phenotypes listed above.

## Small GTPase signaling

Small GTPase signaling plays a variety of roles in the developing embryo as well as the mature organism. GTPases serve as the molecular switches for cellular processes such as migration and shape change via regulation of the actin cytoskeleton (Kaibuchi et al. 1999). Myriad cellular activities, including formation of stress fibers, cellular morphology, aggregation, motility, membrane ruffling, lamellipodia formation, filopodia formation, cytokinesis, and cellular adhesion have all been demonstrated to be controlled by the Rho-family GTPases

(Kaibuchi et al. 1999). This family consists of at least 10 members in mammals, among those are Rac1, Cdc42, and RhoA—the three best understood members of the Rho GTPase family.

Rho-family GTPases bind to both GTP and GDP, and have intrinsic GTPase activity. These proteins cycle between a GTP-bound active state and a GDP-bound inactive state. Several accessory proteins modulate this exchange activity, such as GEFs (guanine nucleotide exchange factors), GAPs (GTPase activating proteins), and GDIs (GDP dissociation inhibitor). GEFs stimulate the release of GDP from small GTPases, which in turn leads to increased GTP binding, thereby activating the small GTPase (Cerione and Zheng 1996). GDIs play the opposite role by repressing the dissociation of GDP from GTPases, thus favoring the inactive state of these proteins (Takai et al. 1995). GAPs stimulate the GTPase activity of GTPases, leading to their conversion to a GDP-bound inactive state (Takai et al. 1995). In summary, these proteins regulate GTPase activity along the following broad cyclical pathway: GDP-bound GTPases (inactive) in the cytoplasm are complexed with GDIs. When a signal for activation is received, GEFs stimulate dissociation of GTPases and GDIs. GDP dissociates from the GTPase, and the GTPase then binds free GTP in the cytoplasm (active). The active GTP-bound GTPase is then targeted to the membrane where it interacts with its targets. GAPs enhance the intrinsic GTPase activity of these GTP-bound proteins, catalyzing conversion to a GDP-

bound (inactive) state. At this point, GTPases complex with GDIs to begin the cycle again (Figure 4) (Kaibuchi et al. 1999).

Rho-family GTPases have numerous targets, some of which are shared between family members, and some of which are specific to a particular GTPase (Kaibuchi et al. 1999). Rho targets in mammalian cells include Rho-kinase, myosin phosphatase, PRK1, rhophilin, rhotekin, citron, and p140mDia (Van Aelst and D'Souza-Schorey 1997). These effectors elicit a variety of cellular activities, including smooth muscle contraction (Amano et al. 1996; Kimura et al. 1996), stress fiber formation (Leung et al. 1996; Amano et al. 1997), neurite retraction (Amano et al. 1998), cytokinesis (Yasui et al. 1998), and actin polymerization (Watanabe et al. 1997).

Rac1 and Cdc42 target p21 activated kinases (PAKs), WASP and N-WASP, IQGAP1, MRCK, Por1, p140Sra-1, and Posh . Rac1 and Cdc42 share several of these targets. Formation of lamellipodia and filopodia (Miki et al. 1998), degradation of stress fibers and focal adhesions (Manser et al. 1997; Sells et al. 1997), activation of JNK and p38 transcriptional cascades (Bagrodia et al. 1995; Zhang et al. 1995) have all been shown to be regulated by these Rac1/Cdc42 effectors. Interestingly, activation of some of these effectors functions as positive feedback mechanisms to propagate GTPase signaling (Manser et al. 1998).

These small GTPases are critical for control of a number of cellular processes, and commonly function as a “relay” between extracellular signals and

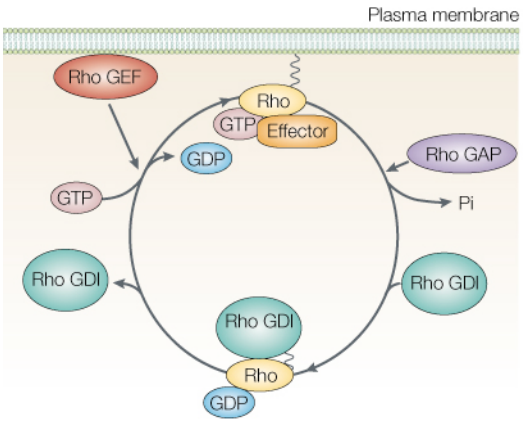



Figure 4: In resting cells, Rho-famliy GTPases exist mostly in the GDP-bound form and in complexes with GDIs (GDP dissociation inhibitors) in the cytosol. When cells are stimulated with the appropriate extracellular factors, Rho-family GTPases are probably dissociated from GDIs and targeted to specific membranes by its carboxy-terminal prenyl group. At the membrane, specific GEFs (guanine nucleotide exchange factors) for Rho-family GTPases are activated and GDP-GTPase is then converted to GTP-GTPase. GTP-GTPase interacts with specific effectors to exert its functions. GAPs (GTPase-activating proteins) enhance the GTPase activity of Rho-family GTPases and reconvert GTPases to their inactive GDP-bound form. GDIs can then form a complex with GDP-GTPases and extract it from the membrane back into the cytosol. (adapted from Fukata and Kaibuchi, 2001)

intracellular processes. In many cases, the intracellular process most directly affected by Rho-family GTPase signaling is the dynamic organization of the actin cytoskeleton. Via control of the cytoskeleton, Rho GTPases are able to exert influence upon are numerous cellular processes.

For example, control of the actin cytoskeleton during cell-cell and cell-matrix adhesion is exerted through the Rho GTPase family. Integrins, which link the cytoskeleton to the extracellular matrix, activate both Rac1 and Cdc42, and this activation is critical for normal cell spreading (Price et al. 1998; del Pozo et al. 2000). Similarly, cadherin signaling during formation of adhesion complexes also activates Rac1 and Cdc42, while repressing RhoA. Disruptions of these GTPase activations and repressions have been shown to block the formation of cell-cell adhesions (Braga et al. 1997; Hordijk et al. 1997; Kuroda et al. 1997; Zhong et al. 1997; Jou and Nelson 1998).

Perhaps the most commonly known function of the Rho GTPases is control of processes that govern cellular motility. In Swiss 3T3 fibroblasts, Rac1, Cdc42, and RhoA are all activated during cell motility. On the leading edge of movement, Rac1 and Cdc42 are active, producing filopodia and lamellipodia. The combination of this protrusive activity driven by Rac1 and Cdc42 coupled with acto-myosin based retraction on the trailing edge, catalyzed by RhoA activity, allows directed cellular movement in response to extracellular cues (Figure 5) (Etienne-Manneville and Hall 2002). Complex processes regulated by Rho GTPases, such as cell motility, are closely regulated by crosstalk between

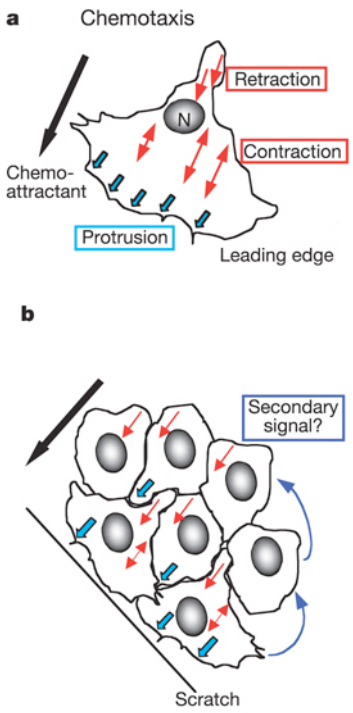



Figure 5: Independent movement controlled by Rho-family GTPase signals. Cells move through the polarized and dynamic re-organization of the actin cytoskeleton, involving a protruding force at the front (blue arrows), combined with a contractile force in the cell body (double-headed red arrows). This contractile activity leads to retraction of the rear of the cell as the adhesions are lost (single-headed red arrows). Rho-family GTPases act spatially and temporally to control all these aspects. Rac regulates actin polymerization at the front to promote protrusion. Cdc42 acts at the front to control direction in response to extracellular cues. Rho stimulates actin-myosin contraction in the cell body. Invading cancer cells are probably not directed by outside signals, but deregulated Rac is thought to have an important role. b, Coordinated movement. In in vitro scratch assays, cells sense the free space left by the scratch and migrate together as a sheet. Rac is essential for forward movement in fibroblasts. Migrating cells are oriented perpendicularly to the scratch. How cells behind the front row migrate coordinately with front row cells is unclear, but may involve secretion of soluble factors and/or mechanical tension. The Rac1/Cdc42-dependent secretion of a soluble TGFbeta-like factor (in blue) is required to promote migration of the entire monolayer. When cells approach each other, Cdc42- and Rac-dependent filopodia and lamellipodia protrude to execute wound closure. (Adapted from Etienne-Manneville and Hall, 2002)

members of the Rho GTPase family. These mechanisms are poorly understood at this time, and vary by cell type in which they exist, but are the topic of active investigation.

It is clear that the Rho-family GTPase signaling pathway is a key regulator of many cellular processes. Data presented in a later chapter demonstrate that Bves interacts with GEFT, a protein that directly controls the activity level of Rho GTPases (Guo et al. 2003). The interaction of Bves with a regulatory component of this signaling pathway not only has important implications for understanding the function of Bves, but also for developing a more complete understanding of Rho-family GTPase signaling and how it controls the variety of cellular behaviors previously described.

### The Dbl-family of guanine nucleotide exchange factors

As mentioned previously, GEFs stimulate GDP dissociation from GTPases. This dissociation catalyzes GTP binding to GTPases, as the cytosolic concentration of GTP is 5x higher than the concentration of GDP (Cerione and Zheng 1996). The activation of Rho-family GTPases through these proteins is often stimulated by extracellular signals via various cell-surface receptors. Receptors of the tyrosine kinase, adhesion, cytokine, and GPCR families all stimulate downstream Rho-family GTPase activity (Kjoller and Hall 1999; Sah et al. 2000). The intermediates between cell-surface receptors and GTPases activation are GEFs.

The prototypical mammalian Rho family GEF is Dbl. This GEF was isolated from diffuse B-cell-lymphoma cells (Eva et al. 1988; Hart et al. 1991). This protein was found to share a highly conserved domain with Cdc24, a *Saccharomyces cerevisiae* protein that interacts with Cdc42 during yeast budding and polarity. Later, Dbl was demonstrated to have GEF activity for human Cdc42. 69 distinct members of the Dbl family of GEFs have since been identified (Rossman et al. 2005).

Dbl family GEFs, for the most part, share two conserved domains: the Dbl homology domain (DH) and the pleckstrin homology domain (PH). DH domains catalyze the exchange of GDP for GTP by Rho-family GTPases by promoting a "nucleotide-free" state, thereby stimulating GTP binding due to the relative higher cytosolic GTP concentration. The DH domain elicits this functional activity by interaction with the switch regions of Rho-family GTPases, altering the nucleotide-binding pocket and disrupting the proper structural conformation necessary for GDP binding (Rossman et al. 2002). While some Dbl GEFs are promiscuous in their binding to GTPases, others are specific to one GTPase, and this specificity is likely determined by uniqueness within the DH domain (Schmidt and Hall 2002).

PH domains serve to localize Dbl GEFs to plasma membranes. Through control of localization to sites where nucleotide exchange activity occurs, and allosteric interactions, PH domains help to regulate Dbl GEF activity. The PH domain is always found C-terminal to the DH domain in Dbl GEFs, although

instances of Dbl GEFs with no PH domain do exist (Rumenapp et al. 2002). Although the PH domains do not catalyze GTPase activity themselves, their presence is important for proper GEF activity. Experiments have shown that DH-PH fragments of Dbl GEFs stimulate a higher rate of nucleotide exchange activity than the respective DH domains alone (Ron et al. 1991; Whitehead et al. 1995; Liu et al. 1998; Rossman and Campbell 2000). In some cases, investigators demonstrated the necessity for PH-domain membrane targeting in GEF function by replacing the PH domain with other sequences that direct protein localization to the plasma membrane. Interestingly, PH domains of Dbl GEFs show little affinity or specificity for phospholipids, indicating that interaction with the lipid bilayer alone is insufficient GEF localization to the membrane (Snyder et al. 2001) and that interaction with other membrane associated proteins may be critical for proper localization of Dbl GEFs to sites where they are needed for GTPase regulation. Here I demonstrate that Bves, an integral membrane protein, interacts directly with the Dbl-family GEF GEFT. This Bves-Geft interaction may be one of these interactions that serve to localize a Dbl GEF to the proper site of Rho GTPase activity in order to properly control Rho GTPase signaling in a variety of cellular events.

## Approach to this project

As outlined in this chapter, Bves is a novel protein that is largely uncharacterized. The domain of expression of this protein is still debated in the

literature, the function is undefined, and molecular mechanism of any Bves action is completely unknown. The ability of Bves to affect cellular behaviors during epithelial wound healing (Osler et al. 2005), *Xenopus* development (Ripley et al. 2006), and skeletal muscle regeneration (Andree et al. 2002) has been reported, yet no plausible molecular mechanism for these phenotypes has been offered at this time. In fact, direct interaction with any protein has not been reported, although interaction with a protein complex containing ZO-1 has been demonstrated (Osler et al. 2005).

Given that the expression pattern of Bves was disputed, and that no directly interacting proteins had been discovered; little evidence for making hypotheses of Bves function and molecular mechanism of function existed. The absence of conserved protein motifs also provided no clues with which a hypothesis could be logically formulated regarding Bves function. With this knowledge, I first set out to remove the controversy regarding the expression pattern of Bves from the literature. The generation of a new panel of monoclonal antibodies specific to Bves, the characterization of these reagents, and the definition of the expression pattern of Bves during mouse embryogenesis are described in Chapter II.

We next hypothesized that *definition of Bves-interacting proteins will reveal the function of Bves*. Utilizing a yeast two-hybrid screen with an embryonic mouse heart library, I discovered a direct interaction between the intracellular carboxyl-terminus of Bves and GEFT, a Rho-family GTPase GEF.

Characterization of this interaction, as well as our investigation of the functional significance of this interaction, is provided in Chapter III. The relevant discussion, our conclusions from the entirety of this data, and potential future avenues of research are presented in Chapter IV.

# CHAPTER II

# CHARACTERIZATION OF BVES EXPRESSION DURING MOUSE DEVELOPMENT USING NEWLY GENERATED IMMUNOREAGENTS

## Introduction

Bves was isolated independently by two laboratories (Reese et al. 1999; Andree et al. 2000) using subtractive hybridization screens for heart enriched gene products. Additional members of the gene family (Popdc2 and Popdc3) were also isolated (Andree et al. 2000). Sequence analysis of Bves revealed no conserved or predicted functional motifs and no homology to any previously identified protein. Three hydrophobic regions near the N- terminus were identified that have since been determined to be functional in anchoring the protein to the plasma membrane (Wada et al. 2001; Knight et al. 2003). Orthologous genes have been identified in numerous invertebrates and vertebrates (Reese and Bader 1999; Reese et al. 1999; Andree et al. 2000; Hitz et al. 2002; Ripley et al. 2006).

Controversy about the expression pattern of the Bves protein persists in the literature. Detection of Bves message through *in situ* hybridization, Northern blotting (Andree et al. 2000), or *lacZ* knock-in (Andree et al. 2002) do not agree with detection of the Bves protein using multiple anti-Bves immunological reagents (Reese et al. 1999; DiAngelo et al. 2001; Wada et al. 2001; Osler and

Bader 2004; Ripley et al. 2004; Vasavada et al. 2004). While *in situ* hybridization and *lacZ* knock-in analyses have been interpreted as indicating that Bves is expressed preferentially in cardiac and skeletal muscle, analyses of protein expression indicate that Bves is expressed in many epithelial cell types as well. The first polyclonal antibody generated by our laboratory, D033, revealed expression in the proepicardium, migrating epicardium, epicardial-derived mesenchyme and smooth muscle cells of the cardiac arteries of the developing chicken heart (Reese et al. 1999). A second polyclonal antibody, B846, also revealed Bves expression in cardiac muscle and all epicardial/epicardially derived tissues listed above (Wada et al. 2001), as well as expression in various epithelial cell lines (Wada et al. 2001), epithelia of all three germ layers during early chick development, epidermis, gut endoderm (Osler and Bader 2004), and epithelia of the lens, retina, and cornea (Ripley et al. 2004). A subsequent antibody against the *X. laevis* ortholog of Bves was developed, and has revealed highly similar expression in the frog (Ripley et al. 2006). A monoclonal antibody generated against the chicken Bves protein (DiAngelo et al. 2001) also demonstrated that Bves is expressed in skeletal muscle, cardiac muscle, brain, and epicardium (Vasavada et al. 2004). The monoclonal antibody generated by Duncan and colleagues clearly reacts with the chicken Bves protein in cardiac myocytes and transiently in the epicardium, but has not been reported to react with chicken Bves protein in other epithelial cell types (DiAngelo et al. 2001; Vasavada et al. 2004).

Here, I describe the generation of multiple new α- mouse Bves monoclonal antibodies that display reactivity with cardiac muscle, skeletal muscle, and epithelial cell types throughout embryonic development, as well as cultured epithelial and muscle cell lines. I also thoroughly examine the developmental expression profile of the mouse Bves protein using these and other previously generated α-Bves reagents. Thus, I provide a comprehensive description of Bves expression at the protein level in the mouse, which is lacking in the literature at this time. Our data clearly demonstrate that the Bves protein is present in developing muscle and epithelial cell types derived from all three germ layers. These studies are essential for a meaningful understanding of Bves function and to determine the role of Bves in mouse embryogenesis.

## Materials and Methods

*Generation of α-Bves monoclonal antibodies*

Antibodies were generated against the peptide DPTLNDKKVKKLEPQMS (amino acids 266-283 of mouse Bves) in collaboration with QEDBioscience (San Diego, CA) using standard methodology (Bader et al. 1982). Antibodies were initally screened using ELISA against the original peptide. Reactive clones were selected from this screen and were then subjected to screening using secondary immunofluoresence against COS-7 cells transfected with Bves expression constructs. Reactive clones were further characterized using standard

immunoblotting procedures against GST-fused Bves, Popdc2, and Popdc3. Once isolated, hybridomas were cultured and also injected into the peritoneal cavity of mice to generate ascites fluid. Five independent clones were used to generate ascites, and all five of these hybridoma lines will be deposited in the Developmental Studies Hybridoma Bank.

*Antibodies*

Primary antibodies against E-cadherin (Chemicon), ZO-1 (Zymed), sarcomeric myosin (MF20, DSHB), c-myc (Sigma), cytokeratin (Sigma), and GST (Amersham) were applied according to manufacturer's specifications. Alexa-488 and Alexa-568 conjugated secondary antibodies (Molecular Probes) were used at 1:4,000 dilutions for indirect immunofluoresence, and alkaline phosphatase conjugated secondary antibodies (Sigma) were dilluted 1:10,000 for immunoblotting. DAPI (4', 6-diamidine-2phenylidole-dihydrochloride; Roche) was used to visualize nuclei per manufacturer's specifications. When direct labeling of antibodies was necessary, Zenon Alexa Fluor labeling kit (Molecular Probes) was employed to label primary anitbodies according to manufacturer's specifications. The polyclonal antibody B846 has been previously described (Wada et al. 2001; Osler and Bader 2004). Newly generated $\alpha$-Bves monoclonal antibody ascites fluids were used at a 1:2,000 dilution for immunohistochemistry with both tissue sections and cultured cells. Samples were incubated in primary antibodies at 4°C overnight in a humidified chamber.

*Western Blotting analysis*

Hearts were excised from adult (~8 weeks) ICR mice (Jackson Labs), and dissected into small pieces. Tissue was then homogenized using extraction buffer (1x PBS, 1% Igepal CA-630 (Sigma), 0.5% sodium deoxycholate, 0.1% SDS) containing mammalian protease inhibitors (Roche Complete). Samples were then centrifuged at 21,000 x g for 30 minutes at 4°C. Supernatant was collected, and protein concentration assayed using Bradford assay (Biorad). 40 ug protein was diluted in SDS-PAGE sample buffer, and electrophoresed on a 10% SDS-PAGE gel at 125 V. Protein was then transferred to Immobilon-P membrane (Millipore), and then blocked in blocking solution (10% nonfat dry milk (Carnation), 100mM Tris Cl pH 7.5, 150 mM NaCl, .25% TritonX-100 (Sigma)) overnight at 4°C. Primary antibody (SB1, anti-Bves monoclonal Ab) was applied to blot at 1:2000 dilution (~1 ug/mL) for one hour at room temperature. Blot was washed 3 times with 1x TBST (100mM Tris Cl pH 7.5, 150 mM NaCl, .25% TritonX-100 (Sigma)), and then incubated with alkaline phosphatase conjugated secondary antibodies (Sigma) at manufacturer's recommended concentrations in blocking buffer for one hour at room temperature. Blot was washed 3 times with 1x TBST, and binding of secondary antibodies visualized using NBT/BCIP (Roche) according to manufacturer's specifications.

*Tissue/cell preparation*

Tissues were harvested, washed in cold PBS and then incubated in 20% sucrose in PBS overnight at 4°C. Tissues were then processed for frozen sectioning and immunohistochemical staining of using standard methodology (Bader et al. 1982; Wada et al. 2003). Cultured cells were transfected with expression constructs using Fugene (Roche) transfection reagent according to manufacturer's instructions.

*Immunofluoresence methods for $\alpha$-Bves monoclonal antibodies*

As this is the first communication employing these immunochemical reagents, a protocol generated for their use is provided. Briefly, tissue sections or cultured cells were fixed for 10 minutes in cold 70% methanol, washed three times with PBS, and permeabilized with 0.25% Triton X-100 in PBS for 10 minutes. Sections/cells were then washed with PBS three times, and non-specific binding was blocked by incubation with 2% bovine serum albumin in PBS for one hour at room temperature. Ascites fluids were applied at 1:2,000 dilutions for 1-16 hours at room temperature. No variation in background or staining intensity was observed over that period. Alexa-conjugated secondary antibodies (Molecular Probes) were added for one hour at room temperature according to manufacturer's specifications. Subsequent washing was standard.

*Generation of mammalian and bacterial expression constructs*

Mammalian and bacterial expression constructs were generated using PCR amplification followed by cloning into pCI-neo (Promega) and pGex 5x-3 (Amersham). All expression constructs express the portion of the respective protein 3' to the hydrophobic transmembrane domains of Bves. A c-myc epitope tag was also added to the carboxyl terminus of all mammalian expression constructs to aid in monoclonal antibody characterization.

*Culture of neonatal mouse myocytes*

Hearts of N2 mice were harvested, mechanically dissociated, and placed in cold PBS. Hearts were placed in 5 mL 0.25% Trypsin-EDTA (Cellgro) for two minutes with rapid stirring using mechanical stir bar. 3.5 mL of Trypsin-EDTA was removed after two minutes, and replaced with 3.5 mL fresh Trypsin-EDTA. The first two fractions of Trypsin-EDTA were discarded, with fractions 3-8 retained and placed into a 10x volume of myocyte medium (DMEM: 4.5 g/L glucose, 0.0025M thymidine, 10 U/mL penicillin, 10 mg/mL streptomycin, 100 μg/mL gentamycin, 15% NuSerum (Collaborative Biomedical Products)). Myocytes were gently pelleted at 650 x G for seven minutes, then resuspended in growth media, counted using a hemacytometer, and plated on 100 mm plastic dishes at $3x10^6$ cells per dish.

## Results

*α-Bves monoclonal antibodies recognize Bves specifically*

Immunochemical reagents against mouse and chicken Bves have previously been generated and characterized (Reese et al. 1999; DiAngelo et al. 2001; Wada et al. 2001; Osler and Bader 2004; Ripley et al. 2004; Vasavada et al. 2004). Recently, DiAngelo et al. (2001) have generated a monoclonal antibody against the intracellular C-terminus of chicken Bves. To this point, no monoclonal reagents against murine Bves exist and the characterization of protein expression in any organism is incomplete.

Specificity of α-Bves reactivity was initially established using transfection of COS-7 cells with a c-myc-tagged Bves expression construct followed by co-immunofluoresence using α-c-myc and putative Bves monoclonal antibody supernatants. All ten of the antibody-secreting clones that passed the initial screening process were reactive with transfected Bves (SB1 is given as an example in Figure 6A, i-iii). Anti-Bves antibodies were then tested for cross reactivity with other members of the gene family (Figure 6A, iv-ix) and a non-related protein, LEK1 (Figure 6A, x-xii) by transfection of COS-7 fibroblasts with c-myc tagged Bves, Popdc2, and Popdc3. All antibodies generated were found to react in a similar manner: specifically with Bves and not with other Popdc family members or the unrelated LEK1 protein.

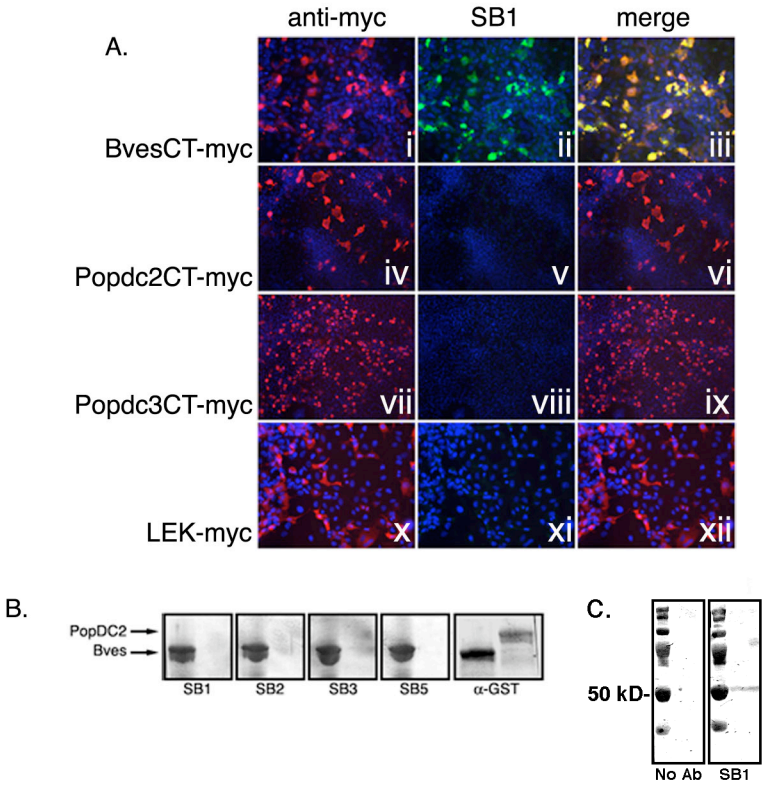



Figure 6: Characterization of α-Bves monoclonal antibodies. SB1 seen to be reactive with COS-7 cells transfected with c-myc tagged Bves expression constructs, but no labeling of cells transfected with other members of the protein family (PopDC2 and PopDC3) is observed (Fig. 6A). SB family monoclonal reagents are also Bves specific in immunoblotting assays against GST-tagged fusion proteins. No reactivity with PopDC2 (Fig. 6B) or PopDC3 (data not shown) is observed. SB1 reacts with a single protein in immunoblotting assays of adult mouse heart (Fig. 6C). Reactive band is visualized at approximately 53 kD.

α-Bves monoclonal antibodies were then tested for specificity in immunoblotting assays. GST-fusion expression constructs of each Bves family member were generated, and purified protein was subjected to standard immunoblotting procedures. SB1 is used here as an example. Again, α-Bves monoclonal antibodies were found to react specifically with Bves, and were unreactive with Popdc2 (Figure 6B) and Popdc3 (data not shown).

α-Bves monoclonal antibodies were also found to react with an approximately 53 kD protein in immunoblotting assays using protein samples from adult mouse hearts (Figure 6C). These data demonstrate the reactivity and specificity of these reagents for Bves amongst Popdc family members.

*α-Bves monoclonal antibodies detect Bves in epithelial cell lines*

Analyses of Bves expression in the chicken utilizing *in situ* hybridization techniques differ from results obatined using immunochemical methods (Reese et al. 1999; Andree et al. 2000; Wada et al. 2001; Osler and Bader 2004; Vasavada et al. 2004). While both methods provide evidence for expression in muscle cell types, *in situ* hybridization assays and immunochemical assays do not agree concerning expression in epithelial cell types. Utilizing cell lines allows the examination of Bves expression in a clonal cell population consisting of a single cell type. Having verified specificity of antibodies SB1-SB5, I then tested the newly generated antibodies on a variety of cell lines (Figure 7). α-Bves monoclonal antibodies revealed expression in cell lines derived from the rat

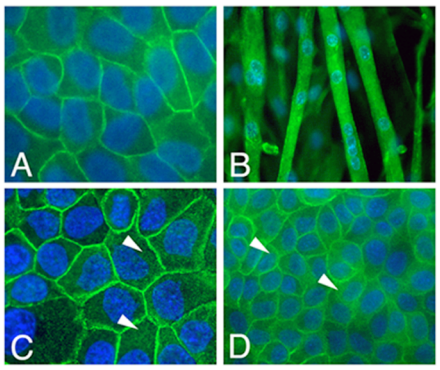



Figure 7: Bves is expressed in muscle and epithelial cell lines. Bves expression (green) is observed using indirect immunofluoresence and monoclonal antibody SB1 (green) with DAPI (blue) staining for nuclei. Peripheral expression is observed in the EMC (epicardial mesotheial cells, Fig 2A) cell line, the HCE (human corneal epithelial, Fig. 7C) cell line, and the MDCK (Maldin Darby Canine Kidney, Fig. 7D) cell line. A broader staining pattern is seen in the differentiated mouse C2C12 mouse myoblast cell line (Fig. 7B).

epithelial epicardium (line EMC, Figure 7A), differentiated mouse skeletal myoblasts (line C2C12, Figure 7B), human corneal epithelium (line HCE, Figure 7C), and canine kidney epithelial cells (line MDCK, Figure 7D). Intense staining in the lateral compartment of the cell membrane is observed in all epithelial cell lines tested thus far. Punctate intracellular staining is also observed in these cell lines (see arrowheads, Figure 7C) and in the C2C12 muscle cell line (Figure 7D). These patterns are in general agreement with the subcellular distribution detected with polyclonal reagents (Reese and Bader 1999; Wada et al. 2001; Osler and Bader 2004). Therefore the SB monoclonal series recognizes Bves in a variety of cell types across a spectrum of mammalian species and cell lines, in agreement with previously generated reagents.

*Bves is expressed in mouse epidermis throughout development*

While epithelial and muscle cell lines clearly express Bves, these cell lines may not reflect embryonic expression of the protein. To test whether Bves protein is present in developing epithelia derived from ectoderm, frozen sections of mouse embryos at various stages of development were examined for Bves expression using the SB1 antibody. Expression of Bves protein was observed in multiple epithelial tissues. Using epithelial markers cytokeratin and ZO1 to verify staining patterns, I determined that Bves is expressed in the epidermis of the mouse throughout development (Figure 8). At E12.5, Bves is expressed in the apical portion in the epidermal layer. At low power magnification in Figure 8, the

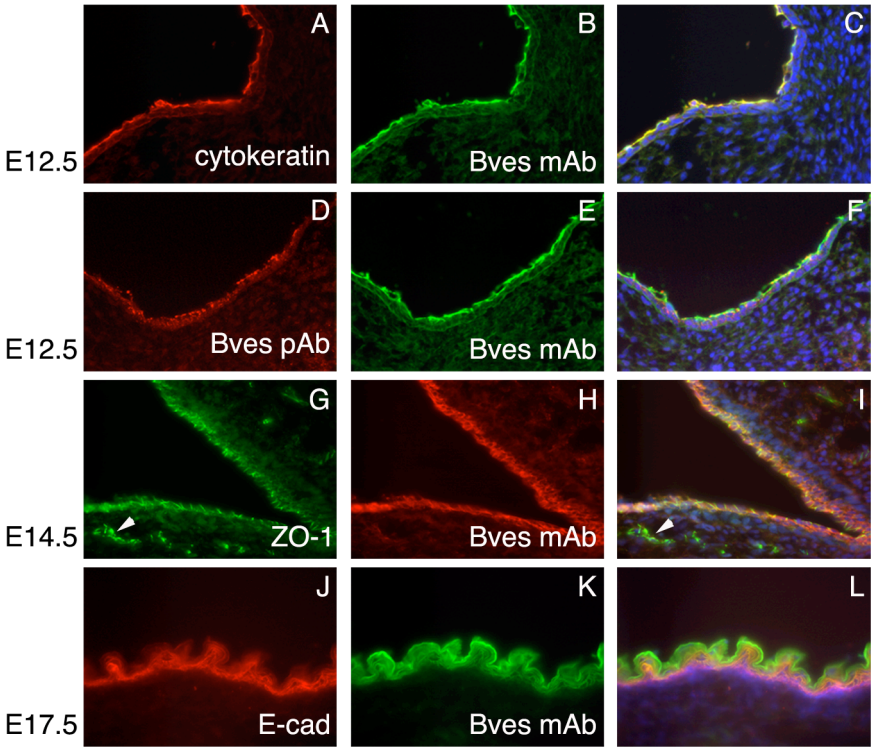



Figure 8: Bves expression in the epidermis during gestation. Bves expression is seen in the epidermis throughout development, as observed by colocalization with cytokeratin, ZO-1, and E-cadherin markers at various developmental stages. Colocalization of Bves (Fig. 8B) and cytokeratin (Fig. 8A) at E12.5 shows expression of Bves in keratin producing cells, which continues throughout gestation. Expression of Bves in subdermal blood vessels is not observed (arrowheads, Fig. 8G-8I). Near the end of gestation, expression of Bves (Fig. 8K) is seen in the epidermis at a position apical to that of E-cadherin (Fig. 8J). Polyclonal anti-Bves antibody (B846, Fig. 3D) and monoclonal anti-Bves antibody (SB1, Fig. 8E) show a similar distribution in the epidermis during development. DAPI counterstain used to visulalize nuclei in blue (Fig. 8A-L).

general colocalization of SB1 and cytokeratin is observed (Figure 8, A-C). At E12.5, Bves is also detected in the apical regions of the developing epidermis. Interestingly, while both SB1 and the polyclonal B846 both recognize Bves protein in the epidermis (Figure 8, D-F), the distribution pattern of SB1 labeling is somewhat broader in the epidermis than that of the polyclonal antiserum. As development proceeds, a high degree of colocalization of Bves and ZO1 is observed in the epidermal layer in the lateral membrane (Figure 8, G-I), while no Bves expression is observed in the endothelium of the subdermal blood vessels (see ZO1 positive cells indicated by arrowheads Figure 8, G and I). Near the end of gestation, an increase in intensity of staining is observed, and the pattern of Bves distribution appears to be wider than at earlier points of development. The monoclonal antibody SB1 appears to be reactive with Bves protein in very apical portions of epidermal cells. This is exemplified by comparison of the epidermally-expressed E-cadherin and Bves (Figure 8, J-L). While both are clearly expressed in this epithelium and exhibit domains of overlap, the major deposition of Bves is apical to E-cadherin. Taken together, the data demonstrate Bves expression in ectodermally derived epidermis of the mouse.

*Bves is expressed in developing cardiac and skeletal muscle*

The first generation polyclonal antibody D033 (Reese et al. 1999) did not react with cardiac muscle. However Andree et al (2000) clearly demonstrated that Bves mRNA was expressed at high levels in cardiac myocytes using *in situ*

hybridization. In addition, DiAngelo et al (2002) used an α-Bves monoclonal antibody to show protein expression in avian cardiac myocytes. Subsequent polyclonal antisera from our laboratory also demonstrated Bves expression in the heart (Osler and Bader 2004). To determine the expression pattern and subcellular localization of the Bves protein in the mouse heart, an analysis at various embryonic stages using SB1 was undertaken. At E12.5, Bves staining using the SB1 antibody is observed in a uniform subcellular pattern in cardiac myocytes (Figure 9A). At later stages and in the adult, staining is most intense at the myocyte periphery (Figure 9B). Polyclonal B846, which has previously been used to study Bves expression in epithelial cells (Osler and Bader 2004), also reacts with cardiac myocytes. As previously seen in epidermis (Figure 8), the pattern of localization revealed by B846 in myocytes varies from that of SB1. Consistent with these results, SB1 recognizes a broader distribution of Bves in the myocardium, labeling the entire periphery of the cell (lateral and longitudinal surfaces). A similar pattern is seen in the chicken during embryonic stages using the polyclonal antisera B846. Bves is seen to localize around the entirety of the myocyte (data not shown). However, B846 only recognizes Bves at the intercalated disc, and not on lateral surfaces (Figure 10A). These results are consistent with observations in epithelia, where B846 appears only to recognize Bves protein at points of cell-cell contact, as the intercalated disc is the only point of cell-cell contact for the cardiac myocyte. Additionally, Bves localizes to nascent points of cell-cell contact in reaggregating myocytes (Figure 10B). This

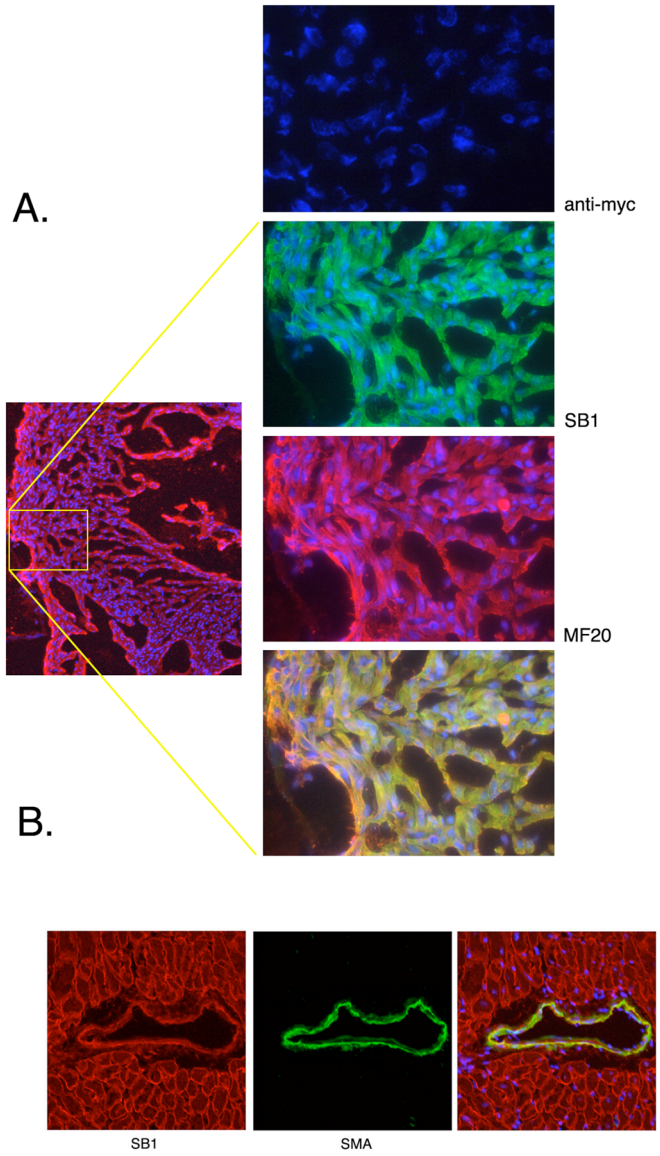



Figure 9: Bves expression in the heart. Broad expression of Bves is observed in the heart at E17.5 (Fig. 9A) as observed by labeling with SB1 (anti-Bves, green) and MF20 (anti-sarcomeric myosin, red). Negative control using anti-myc antibody shows no background reactivity (Fig. 9A). Nuclei are visualized using DAPI (Fig. 9A, 9B) In the adult heart, subcelllular localiaztion of protein becomes more restricted (Fig. 9B). Bves (red) is observed to localize to periphery of cardiac myocytes in definitive myocardium. Expression of Bves in vascular smooth muscle of the coronary arteries is also observed using SB1 (anti-Bves, red) and $\alpha$-smooth muscle actin (green).

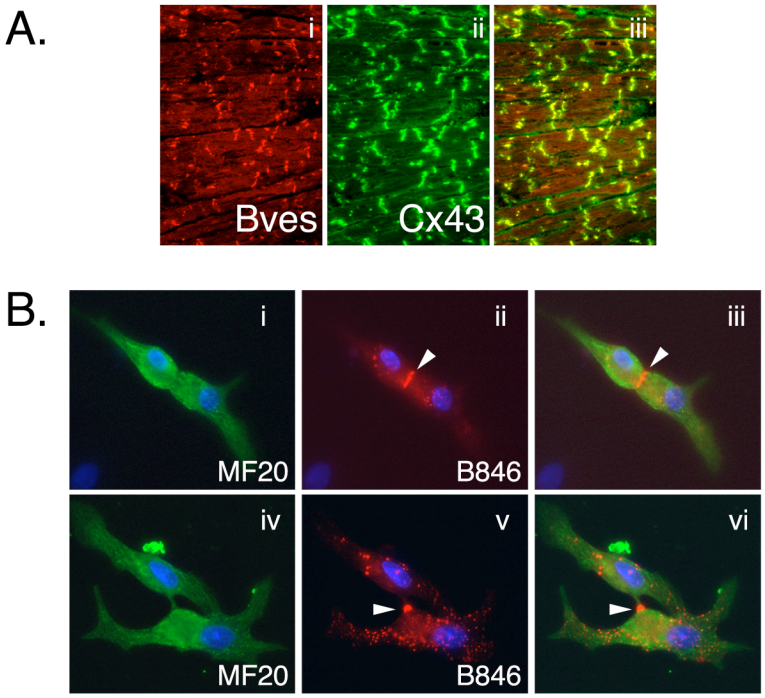



Fig 10: Bves localizes to points of myocyte-myocyte contact *in vivo* and *in vitro*. Anti-bves antibody B846 (Fig. 10A, red) shows Bves colocalizes with Cx43 (Fig. 10B, green) to the intercalated disc structure in adult mouse heart. Bves (Fig 10B ii and Fig 10B v, red) also localizes to myocyte-myocyte contact points in primary cultures of mouse N2 myocytes (arrowheads). DAPI used to visualize nuclei in all panels, and anti-sarcomeric myosin antibody MF20 used to label myocytes (Fig. 10B i and iv, green).

result in highly consistent with observations regarding Bves localization during nascent contact formation in cultured epithelia (Osler et al. 2005).

It is of interest to note that SB1-5 also detect Bves expression in not only in cardiac myocytes, but also in smoooth muscle cells of the coronary system (as seen by colocalization with $\alpha$-smooth muscle actin, Figure 9B) in agreement with our previous studies (Reese et al. 1999; Wada et al. 2001). Andree et al (2000) report Bves mRNA expression in somites and delveoping skeletal muscle. In agreement with those studies, SB1-5 also detects Bves protein in developing skeletal muscle (Figure 11). In skeletal myocytes, as confirmed by colocalization with the $\alpha$-MHC monoclonal antibody MF20, SB1 reveals strong Bves expression. The polyclonal $\alpha$-Bves reagent B846 also recognizes this Bves expression in skeletal myocytes. Note that the epithelial epidermis (arrowheads, Figure 11) is positive for SB1 while MF20 labeling is negative; however both antibodies are positive for adjacent skeletal muscle. Interestingly, Bves expression is also seem in the epithelial somite early during musculogenesis (data not shown).

Expression of Bves in the epicardium has been debated and remains controversial at this time. While *in situ* hybridization studies did not yield significant signal in the epicardium (Andree et al. 2000), monoclonal (DiAngelo et al. 2001) and polyclonal antibodies (Reese et al. 1999; Wada et al. 2001; Osler and Bader 2004) detect transient or sustained expression of Bves in the epicardium. Thus, it is important to note that our initial observations using the

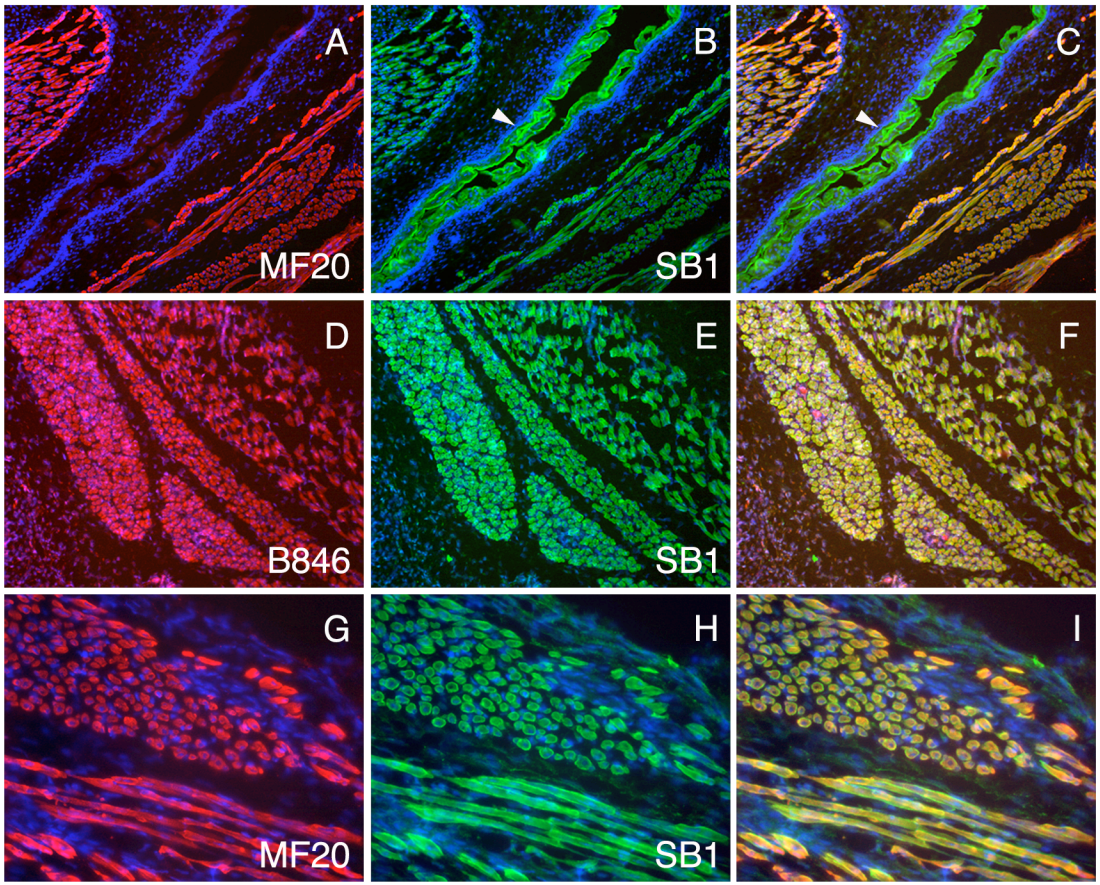



Figure 11: Bves expression in developing skeletal muscle at E17.5. Bves expression is visualized using SB1 antibody (Fig. 11B and 11H, green) and MF20 antibody is used to visualize sarcomeric myosin (Fig. 11A and 11G, red). Co-labeling with MF20 demonstrates expression in skeletal muscle. Note that the epithelial epidermis (arrowheads) is positive for SB1 while MF20 labeling is negative; while both antibodies are positive for adjacent skeletal muscle. Polyclonal antiserum (B846, Fig. 11D) also recognizes Bves expression in skeletal muscle in a similar pattern to anti-Bves monoclonal antibody (SB1, Fig. 11E). DAPI used to visualize nuclei in blue (Fig. 11A-I).

reagents and methods described here do not detect Bves protein in the definitive epicardium, even though these antibodies are highly reactive with the epicardially derived EMC cell line (Figure 7A).

*Bves is expressed in epithelia of the lung and esophagus during development*

We next tested SB1 antibody reactivity in endodermally derived epithelium using lung and gut epithelium as examples of this cell type. Bves is detected in the epithelial components of the digestive tract and lung during development. Particularly, strong expression in the esophagus and main bronchi is observed at E14.5 (Figure 12). As confirmed by co-expression of E-Cadherin, these cells are the epithelial linings of these passageways. However, the expression of Bves in the respiratory system appears to be restricted to the trachea and larger bronchi, while expression in smaller airways is not observed at high levels. Comparison of Bves and E-cadherin staining demonstrates that Bves protein distribution is not uniform in the epithelium of the respiratory system, while expression in the esophagus appears to be more evenly distributed. These data clearly demonstrate the expression of Bves in endodermally derived epithelium.

## Discussion

For a meaningful understanding of protein function during development or in the adult, it is essential to determine the domain of expression of the protein. The expression of Bves has been a topic of debate since the initial experiments

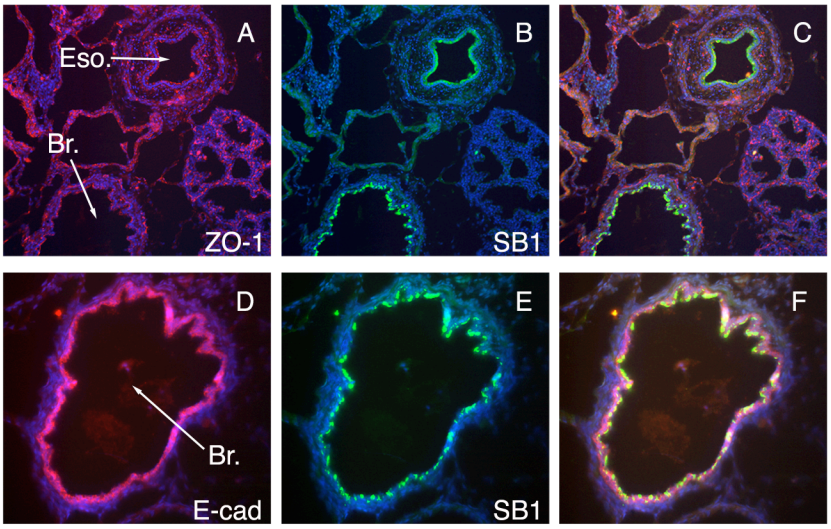



Fig 12: Bves expression in epithelial components of the digestive and respiratory tracts. Bves labeled using SB1 antibody(Fig. 12B, green) is observed in epithelia of the esophagus as seen by colocalization with ZO1 (Fig. 12A, red). Expression of Bves (Fig. 12D, green, SB1 antibody) in a subset of epithelial cells that line bronchi is demonstrated through colocalization with E-cadherin (Fig. 12C, red). Nuclei are visualized in blue using DAPI (Fig. 12A-F). Note that not all epithelial cells in respiratory passageway are Bves positive, and that Bves expression is absent in smaller airways. (Eso. = esophagus, Br. = bronchi)

characterizing the gene. The expression of Bves has been previously examined using polyclonal antisera in the developing chick and frog (Reese et al. 1999; Wada et al. 2001; Osler and Bader 2004; Ripley et al. 2004; Vasavada et al. 2004; Ripley et al. 2006), *in situ* hybridization in the develoing chick (Andree et al. 2000) and frog (Hitz et al. 2002; Ripley et al. 2006), *lacZ* knock-in (Andree et al. 2002; Andree et al. 2002), RT-PCR (Wada et al. 2003; Osler and Bader 2004), and Northern blotting (Reese et al. 1999; Andree et al. 2000; Andree et al. 2002). However, despite the utilization of these many methods in several model systems, disagreement in the literature still persists regarding the expression pattern of this protein. While some reports indicate that the expression of the protein was either restricted to or highly enriched in cardiac, skeletal, and smooth muscle (Andree et al. 2000; Andree et al. 2002; Andree et al. 2002; Hitz et al. 2002); other reports have strongly supported a broader expression pattern that extends to many epithelial cell types (Reese et al. 1999; Wada et al. 2001; Wada et al. 2003; Osler and Bader 2004; Ripley et al. 2004; Vasavada et al. 2004). Here, using newly generated monoclonal reagents, I show that Bves is expressed in tissues derived from all three germ layers, various epithelia and in epithelial cell lines, and in smooth and striated muscle.

My goal was to develop reagents to more precisely determine the expression pattern of the Bves protein, and to examine the expression of Bves during mouse development. As expected, expression of Bves was observed in skeletal and cardiac muscle (Figure 11 and Figure 9, respectively). This is in

agreement with previous analyses of Bves mRNA expression and with antibody studies from the Duncan and Bader laboratories (Reese et al. 1999; Andree et al. 2000; DiAngelo et al. 2001; Wada et al. 2001; Osler and Bader 2004). In contrast, we have reported that our original D033 polyclonal serum, while clearly reacting with the protein (Reese et al. 1999; Osler and Bader 2004), does not recognize Bves in heart muscle. In addition to striated muscle staining, the SB antibody series recognizes Bves expression in some but not all smooth muscle populations. Clearly, coronary smooth muscle is intensely stained by SB antibodies (Figure 9) in agreement with our previous polyclonal data.

The major point of disagreement at this time concerning the expression of Bves is whether it is present in developing and adult epithelia. Using newly generated monoclonal antibodies, I was able to clearly demonstrate protein expression in many endodermally- and ectodermally-derived epithelia. Still, not all epithelia in the developing or adult organism exhibit antibody reactivity. This may be due to variation in isoform production and/or the simple lack of expression. The localization of protein revealed by the SB1 antibody is highly similar to that observed using previously characterized mono- and polyclonal antibodies with the notable exception of epicardial staining. Additionally, our present studies definitively detect the protein in numerous cell lines of epithelial origin (Figure 7). The subcellular distribution of Bves protein revealed by the SB series generally but not completely follows the pattern of staining observed with polyclonal B846 (Wada et al. 2001; Osler and Bader 2004). This staining is

abundant at points of cell-cell contact, consistent with the hypothesis that Bves plays a role in cell-cell adhesion/interaction (Wada et al. 2001; Osler and Bader 2004). I have also observed Bves at points of myocyte-myocyte contact in reaggregation assays, which further supports this hypothesis.

The expression pattern revealed by investigation of Bves mRNA expression and *lacZ* knock-in is often different from the pattern revealed through examination of the protein expression. Both *in situ* analyses (Andree et al. 2002) and *lacZ* knock-in assays (Andree et al. 2002) reveal very little epithelial expression. However, three independently generated polyclonal antisera (against chick Bves, mouse Bves, and frog Bves) from our laboratory reveal Bves expression in a variety of epithelial tissues, along with striated and smooth muscle types. In addition, the Duncan laboratory has generated a monoclonal antibody against chick Bves that also recognizes expression of Bves in the epicardium of the chicken at E6. Examination of Bves expression in other epithelia using this antibody has not yet been published. Still, the present data clearly detect Bves in a variety of epithelial cell types. The discrepancy between mRNA expression analysis and analyses using immunoreagents may be due to a low level of Bves message in non-muscle cell types that makes mRNA detection difficult. Accordingly, when using either immunochemical or riboprobe assays to determine expression of a protein, negative results should be interpreted cautiously as many factors can affect the results of these assays independent of message/protein presence.

These antibodies clearly demonstrate that Bves is expressed in a variety of epithelial and muscular cell types, and that Bves protein expression extends to derivatives of all primordial germ layers. Understanding of the expression pattern of the protein is necessary for developing and understanding the function of the protein. Such broad expression within the organism and in species ranging from invertebrate to human indicates that the function of the Bves protein will likely be more general in nature, including muscle and non-muscle cell types.

# CHAPTER III

# BVES INTERACTS WITH GEFT AND MODULATES RAC1/CDC42 SIGNALING

## Introduction

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

Several indications 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 an interaction with a protein complex containing 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). Additionally, mice null for the *bves* gene are delayed in regeneration of skeletal muscle upon injury (Andree et al. 2002). Despite these indications of Bves function, no direct molecular mechanism for any of these phenotypes exists at this time.

Many of the preliminary indications of Bves function seem to involve cell movement, interaction, and adhesion. Using a yeast-two hybrid screen for interacting proteins, I identified GEFT (guanine nucleotide exchange factor T) (Guo et al. 2003) as a protein that interacts with the cytoplasmic portion of the Bves protein. GEF proteins modulate activity of small GTPases, specifically the Rho family of GTPases in the case of GEFT (Guo et al. 2003; Bryan et al. 2004; Bryan et al. 2005; Bryan et al. 2006). The activity state of GTPases is controlled by GEFs and GAPs (GTPase-activating proteins). GEFs stimulate exchange of GDP for GTP, thereby activating Rho-family small GTPases. GAPs stimulate the activity of GTPases, thus favoring the inactive GDP-bound state of these proteins. The Rho-family of small GTPases have myriad effects on cell behavior; including control of proliferation, differentiation, cell motility, and gene expression (Bishop and Hall 2000; Etienne-Manneville and Hall 2002). Considering previously observed organogenesis and cellular phenotypes observed when Bves function is inhibited, this relationship was examined further.

Here, I show that Bves interacts with GEFT. I also demonstrate 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. This study provides evidence that Bves directly affects the activity levels of Rac1 and Cdc42, and that the phenotypes previously observed when Bves function is altered may be a result of modulation of Rho GTPase activity via a GEFT-Bves interaction.

## Materials and Methods

*Yeast two-hybrid*

The cytoplasmic portion (amino acids 115-358) of mouse *bves* was PCR amplified from a full-length mouse *bves* clone (aa 1–358) containing restriction sites and ligated into pGBKT7 for use in the Matchmaker Y2H System 3 (BD Biosciences Clontech, San Jose, CA) (Figure 13). The bait was mated with a yeast strain pretransformed with a mouse heart embryonic day 17.5 cDNA library. Yeast colonies that survived on Quadruple Dropout Medium (QDO; SD/–Ade/–His/–Leu/–Trp/X-a-Gal) and exhibited lacZ expression were subjected to further testing. Colonies were streaked several times to ensure plasmid segregation. Library plasmids were isolated, and the inserts were sequenced by the Vanderbilt Sequencing Core Facility and identified using NCBI Blast (Altschul et al., 1990Go). For each identified protein product, false-positive tests involving

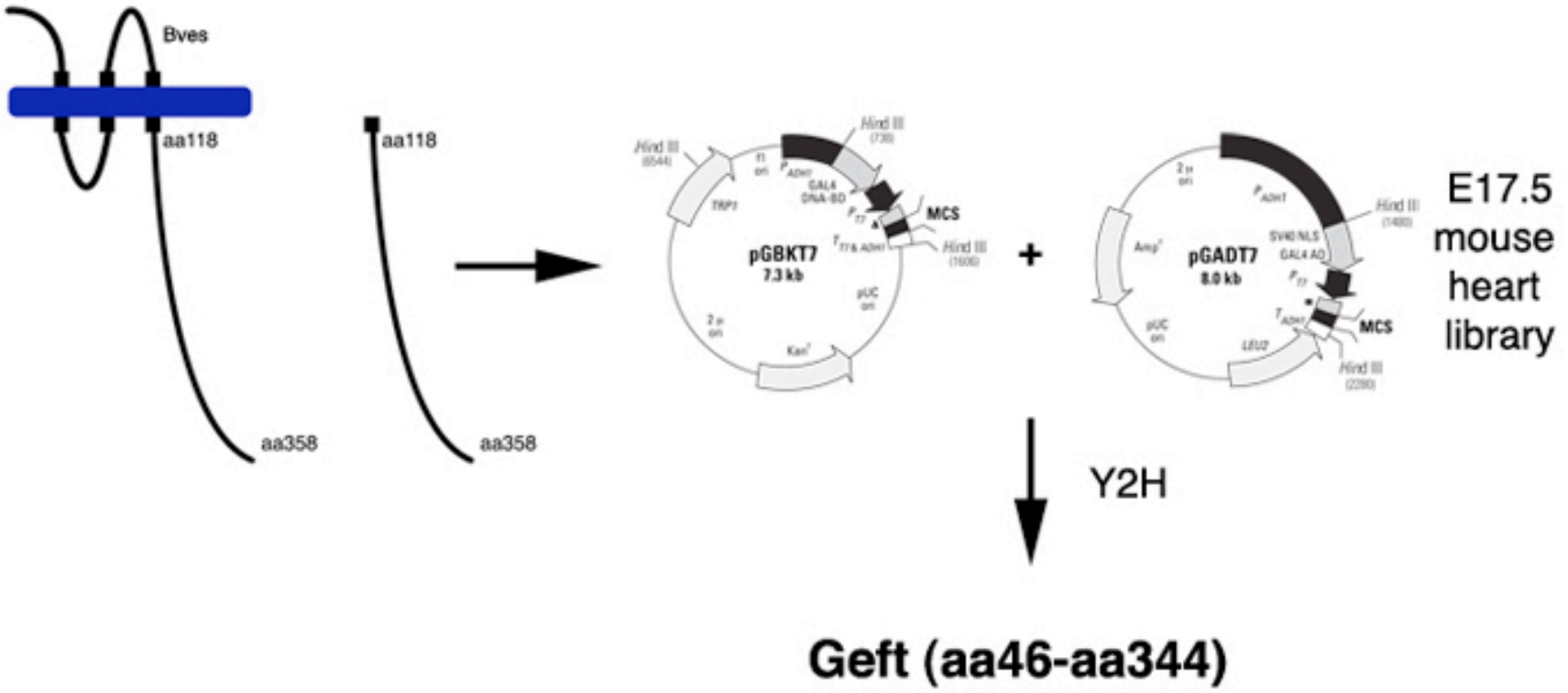



Figure 13: Yeast two hybrid approach to isolation of Bves-interacting proteins. The cytoplasmic carboxyl-terminus of mouse Bves (amino acids 118-358) were cloned into pGBKT7 via a PCR-based approach and transformed into yeast. This plasmid was then mated with a pretransformed E17.5 mouse heart library in a yeast two-hybrid screen. Two independent clones were isolated that contained GEFT cDNAs

empty vector and random protein matings were conducted to eliminate spurious interactions according to manufacturer's recommendations.

*Deletion analysis*

Deletion constructs of the bait used for the initial yeast two-hybrid screen were made by PCR amplification from the original bait plasmid, pGBKT7-mbves CT. Deletion constructs of GEFT were generated by PCR amplification from pCMVTag-2b-mGeft (a kind gift from M. Liu) and cloned into pGADT7. Deletion constructs generated are seen in Figure 14. Bves and GEFT deletion constructs were transformed into AH109 and Y187 yeast, respectively, for matings. Colonies were grown on QDO medium and tested for lacZ expression to determine viable interactions.

*Cell culture*

COS-7 cells were grown on 10-cm dishes and in DMEM (Cellgro). Cells were transfected using Lipofectamine 2000 (Invitrogen) transfection reagent according to manufacturer's specifications. Protein was extracted with 1 ml of extraction buffer (20 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.05% SDS, 1 mg/ml BSA, 1 mM DTT) and 100 $\mu$l of protease inhibitor (Sigma, P8340). Extracted protein was subject to SDS/PAGE analysis followed by immunoblotting. Lysate (10 $\mu$g per lane) was used to confirm protein expression.

*Glutathione bead preparation*

GST fusion proteins were generated by PCR from the C-terminal tail (aa 115-347) and the N-terminal tail (aa 1-36) of murine Bves and cloned into the pGEX bacterial expression vector. GST-N terminal Bves, an ~34 kDa protein, consists of the GST tag 5' of the extracellular N-terminal region of Bves. GST-C terminal Bves, an ~66 kDa protein, contains the GST tag followed by the intracellular C-terminal tail of Bves. Constructs were transformed into BL21 E. coli bacterial strain and protein was induced with isopropyl-ß-D-thiogalactopyranoside (IPTG) using standard methods (Amersham). Bacterial lysates were prepared by 5 cycles of freeze/thaw, followed by brief sonication, and were stored at –80°C until use.

Preparation of glutathione-Sepharose 4B for pull-down was performed as follows. A 50% slurry of glutathione-Sepharose 4B was prepared from a commercially available 75% slurry (Amersham). An aliquot of 1 ml of bacterial lysate expressing the GST fusion proteins was cleared by centrifugation (14,000 g) prior to the addition of 40 $\mu$l of 50% slurry. Cleared lysate was incubated with glutathione-Sepharose 4B for at least 2 hours; the Sepharose was then washed three times with 100 $\mu$l of PBS, and resuspended in 100 $\mu$l of PBS. These glutathione-Sepharose 4B beads, now bound to the GST, GST N-terminal Bves, or GST C-terminal Bves, were used for interaction assays described below. In order to verify that the amounts of glutathione-Sepharose 4B-bound GST proteins

being used for pulldown experiments was consistent, 20 μL samples of these reagents were boiled in 2x SDS-PAGE sample buffer, and eluted proteins were subjected to SDS-PAGE followed by colloidal blue staining.

*GST-pulldown of GEFT*

Mouse GEFT 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).

COS-7 cells transfected with pEGFP-mGeft or pEGFP-mNudeL1 were grown to confluence in 10-cm dishes. Protein was extracted with 1 ml of extraction buffer (20 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.05% SDS, 1 mg/ml BSA, 1 mM DTT) and 100 μl of protease inhibitor (Sigma, P8340). Cells were incubated on ice for 30 minutes 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 and the antibody concentrations used were as listed below. Blots were developed by using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche) and scanned into digital format (Hewlett–Packard).

*Antibodies*

Monoclonal anti-GFP (JL8, Invitrogen) was used at a dilution of 1:5000; monoclonal anti-Rac1 (Abcam) and polyclonal anti-Cdc42 (Abcam) were used at 1:500 dilutions according to manufacturers directions. Appropriate alkaline-phosphatase conjugated antibodies (Sigma) were used at 1:30,000 dilutions for immunoblotting.

For immunostaining, monoclonal antibodies to Bves (Smith and Bader 2006) were used at a 1:2000 dilution. Polyclonal antibody against the GEFT protein (a kind gift of M. Liu) was used at a 1:1000 dilution. Secondary detection was performed using Alexa-568 and Alexa-488 conjugated specific antibodies specific to the primary antibodies used.

*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 Rac1/Cdc42 Assay

Reagent Kit (Upstate Cell Signaling) (Taylor and Shalloway 1996). 10 μg of Rac1/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). Full length Bves was cloned in frame with GFP of the pEGFP-C1 plasmid to generate GFP-Bves. 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. Initial assays of cell motility analyzing the effects of full length Bves transfection were performed by the Lo laboratory at the National Institutes of Health as described previously (Xu et al. 2006). 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 a 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 $4\pi$ (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 (Figure 13). The cytoplasmic carboxyl terminal portion of Bves (aa115-358) was used for this screen. This region of Bves contains the uncharacterized popdc domain (Breher et al. 2004; Brand 2005; Osler et al. 2006). Utilizing a yeast two-hybrid screen with cDNAs expressed in the embryonic mouse heart, I 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 (Ripley et al. 2004; Osler et al. 2005; Ripley et al. 2006), I 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 14).

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 450 is necessary for interaction with Bves (Figure 14). 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 necessary, but 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. The cytoplasmic portion of Bves has previously been shown to contain cysteines through which intramolecular disulfide binding occurs (Knight et al. 2003). The region of Bves shown here to be responsible for interaction with GEFT contains a cysteine at amino acid 283, indicating that an intramolecular disulfide bond through this cysteine may be critical for Bves protein structure that dictates interaction with GEFT. It should also be noted that the Dbl homology (DH) domain (Hoffman and Cerione 2002), which is the domain of GEFT responsible for nucleotide exchange activity with GTPases, falls within the region

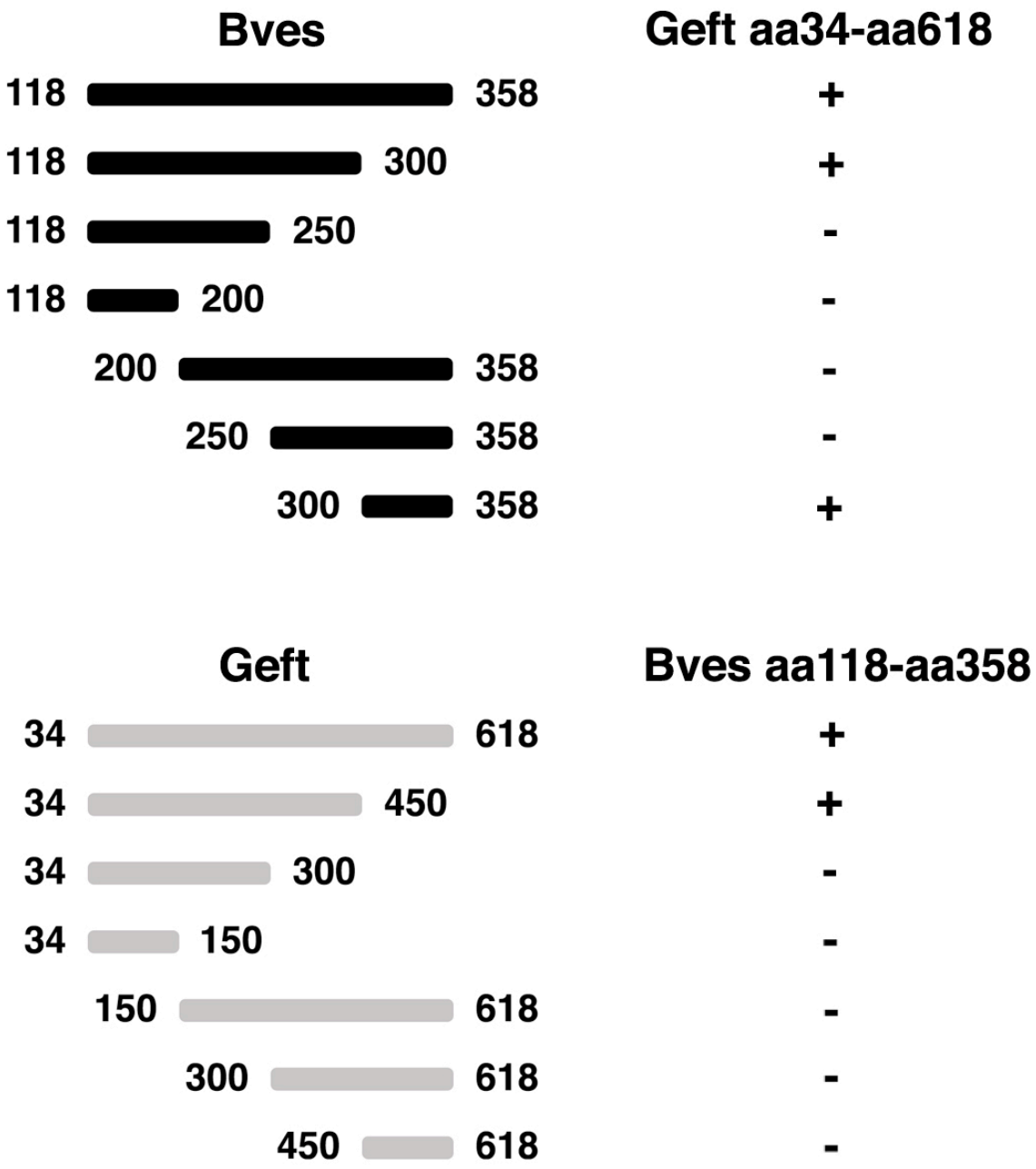



Figure 14: Deletion analysis strategy. Bves and GEFT truncations were generated using PCR-based strategies for further definition of interacting domains. Bves truncations (black bars) were screened against full-length GEFT (aa34-618) for interaction. GEFT truncations (gray bars) were screened against aa118-358 of Bves. Results of matings listed on right, + signifies growth on selective media, while – indicates no growth observed upon mating.

that I have found to be necessary for GEFT-Bves interaction. This domain is present in all Dbl family members, and our findings may indicate that Bves is capable of interaction with other Dbl family members. Our laboratory is currently investigating this possibility. Taken together, the data presented here demonstrate that the aa 250-aa 300 region of Bves and the aa 300-450 region of GEFT are necessary for the interaction between these two proteins.

*Biochemical verification of mBves-mGeft interaction*

Utilizing a GST-pulldown strategy, I biochemically confirmed the Bves-Geft interaction revealed by the genetic screen. Prokaryotic GST-fusion protein expression constructs of Bves were generated, while GEFT-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 15 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 (Liang et al. 2004; Li et al. 2005). Thus, I demonstrate that mBves specifically interacts with mGeft in this assay and corroborate the yeast two-hybrid analyses.

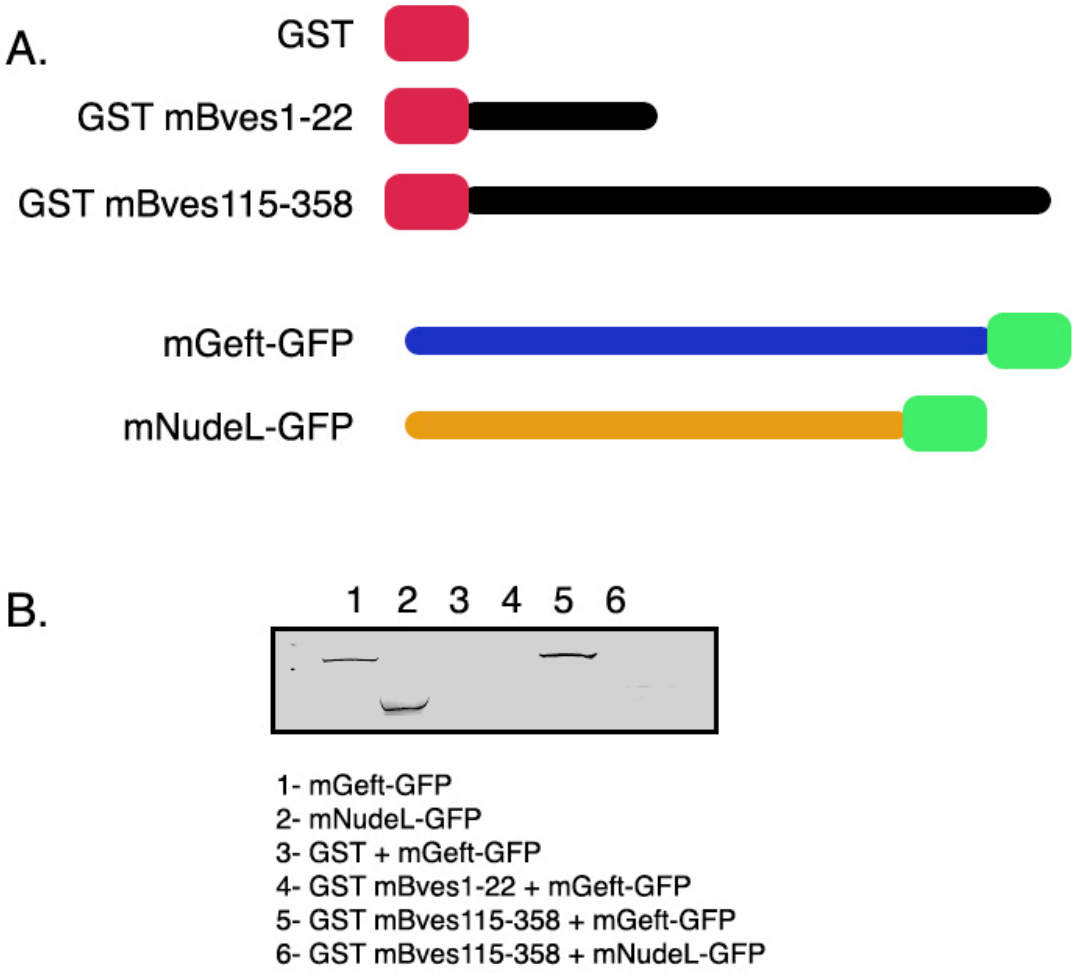



Figure 15: GST-Bves pulldown of GEFT. GST-Bves fusion proteins representing the extracellular N-terminus and cytoplasmic C-terminus of Bves were tested for interaction with GEFT-GFP and NudeL-GFP (Figure 15A). Representative mobilities of mGeft-GFP (Figure 15B, lane 1) and mNudeL-GFP (Figure 15B, lane 2) are provided. No reactivity is observed in lanes containing isolates from GST/mGeft-GFP (Figure 15B, lane 3), GST-mBves1-22/mGeft-GFP (Figure 15B, lane 4), or GST-mBves 115-358/mNudeL-GFP (Figure 15B, lane 6) pulldowns, 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.

*Exogenous expression of mBves affects activation of Rac and cdc42.*

Having demonstrated that mBves interacts with mGeft, I next sought to determine if mBves expression changes activity levels of the Rac1 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), I utilized a PAK-21 pulldown approach to assay for GTPase activity upon transfection of mBves 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 16, 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.

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

Having determined that expression of the intracellular carboxyl terminus of

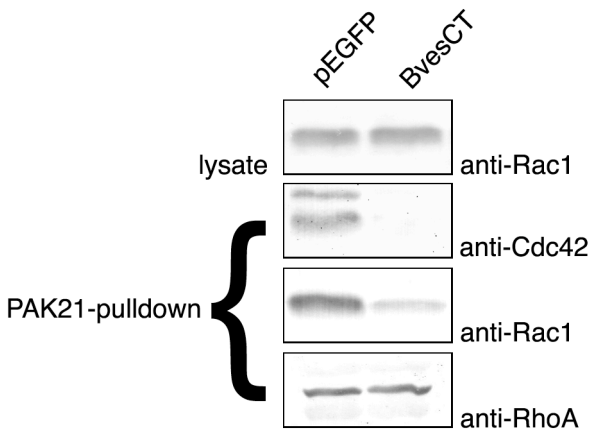
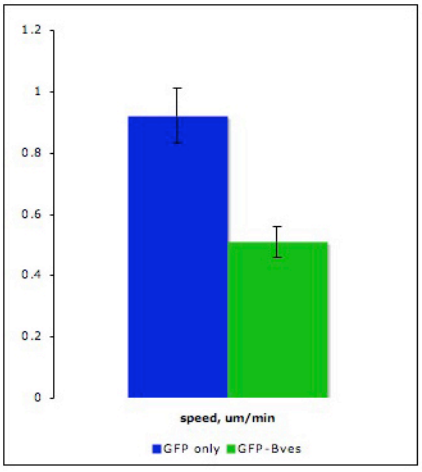


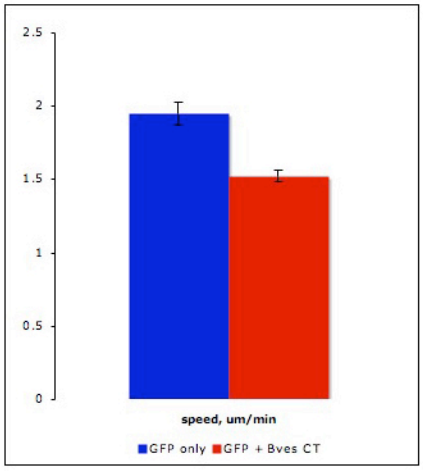

Figure 16: Transfection of the carboxyl-terminus of Bves reduces Rac1 and Cdc42 activity in NIH 3T3 cells. Cells were transfected with either pEGFP (control) or pEGFP-BvesCT (amino acids 118-358 of mouse Bves). Lysates were harvested, and PAK-21 pulldowns were performed. Samples from pulldowns were loaded and blotted with $\alpha$-Rac1, $\alpha$-Cdc42, and $\alpha$-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 appears unchanged. Cell lysates were loaded and blotted with $\alpha$-Rac1 to verify equivalent amounts of total Rac1 was present in samples used for assay.

Bves reduces the amount of active Rac1 and Cdc42 in NIH 3T3 cells, I next sought to determine if transfection of full-length Bves and truncated Bves has an effect on cellular motility. Previous studies have determined that reduction of Rac1 and Cdc42 activity results in a decrease in cell movement (Kraynov et al. 2000; Etienne-Manneville and Hall 2001; Itoh et al. 2002). Co-transfection of a GFP marker plasmid with the carboxyl terminus of GFP, or transfection of full-length murine Bves fused with GFP allowed real-time imaging of cell movement in collaboration with the Lo lab at the National Institutes of Health. As seen in Figure 17, transfection of the full-length mBves-GFP construct markedly (~45%) reduces the speed of cellular migration (total path length/time) (Figure 17) in comparison to the control cells which were transfected with a GFP-only expression plasmid. As Rac1 and Cdc42 signaling are well-characterized activators of cellular motility (Etienne-Manneville and Hall 2002), this decrease in cell movement would be expected due to the previously observed decrease in active Rac1 and Cdc42 (Figure 16). No significant change in directionality of movement (net path length/total path length) was detected in this experiment (data not shown).

These experiments also allowed us to analyze the effect of exogenous Bves expression on cellular roundness. Using the measured area and perimeter length of a cell, it is possible to quantify how efficiently the measured perimeter encompasses the cellular area. The maximum roundness measurement would be obtained from a cell that was perfectly circular. Therefore more protrusions




| Mean | GFP only | GFP-Bves |
|---|---|---|
| speed (um/min) | 0.92 | 0.51 |




| Mean | GFP only | GFP-Bves CT |
|---|---|---|
| speed (um/min) | 1.95 | 1.52 |

| Comparison | difference | t | P value |
|---|---|---|---|
| GFP vs. GFP-Bves CT | 0.43 | 3.204 | P<0.005 |
| GFP vs. GFP-Bves | 0.4109 | 4.548 | P<0.001 |

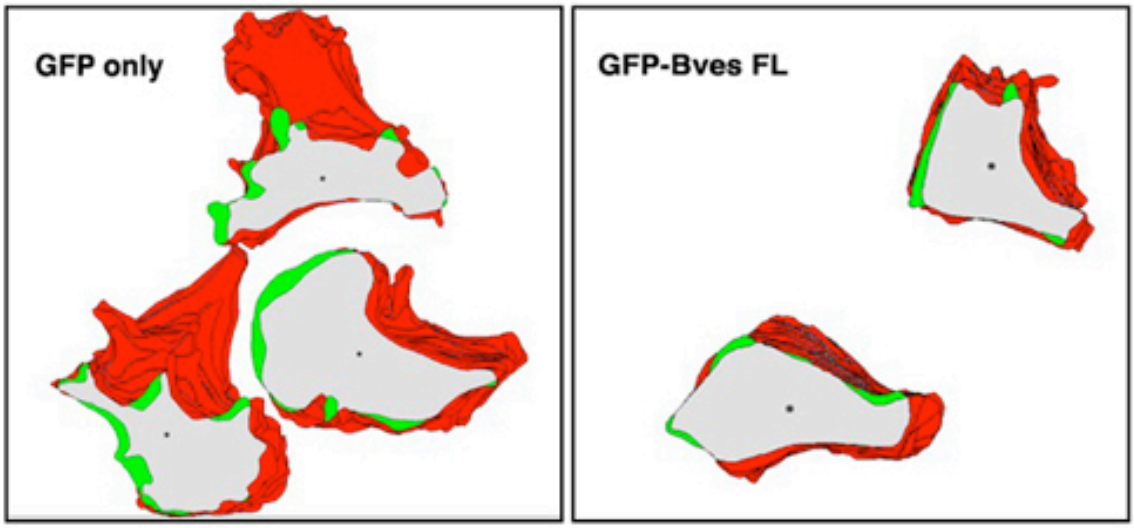
Figure 17: Transfection of Bves and truncated Bves reduces motility of NIH 3T3 cells. Cells were transfected and motility measured as described in Materials and Methods. Upon transfection of either full-length (green bar) or truncated Bves (aa 118-358, red bar), a significant reduction in motility speed is observed in comparison to cells transfected with a GFP-only expressing plasmid (blue bars). Error bars represent SEM, significance determined using standard Student-T test.

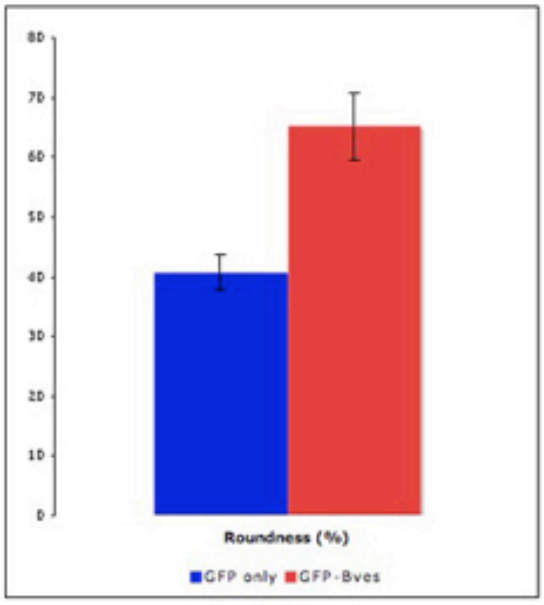
(lamellipodia and filopodia) a cell has, the lower the cell's measured roundness will be. As Rac1 and Cdc42 drive lamellipodial and filopodial extension (Fukata et al. 2003), respectively, cells with higher levels of active Rac1 and Cdc42 would have lower roundness measurements than cells with relatively lower levels of active Rac1 and Cdc42. Consistent with our previous findings that exogenous Bves expression negatively regulates the amount or active Rac1/Cdc42, this experiment found that exogenous expression of Bves caused cells to be ~25% more round than cells transfected with a GFP marker plasmid alone (Figure 18). Again, this indicates that overexpression of Bves leads to a repression in overall Rac1/Cdc42 signaling activity.

Subsequent experiments conducted at Vanderbilt by our laboratory further support our findings. As shown in Figure 17, cotransfection of the carboxyl terminus of mBves with a GFP marker plasmid also decreases the rate of cell movement in this assay by ~22% in comparison to cells transfected with the marker plasmid alone. Taken together, 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. It has previously been demonstrated that Bves affects the trans-




| Mean | GFP only | GFP-Bves |
|---|---|---|
| Roundness (%) | 40.8 | 65.2 |

| Comparison | difference | t | P value |
|---|---|---|---|
| GFP vs. GFP-Bves | 24.4 | 3.533 | P<0.01 |

Figure 18: Transfection of truncated Bves decreases protrusive activity. Roundness of cells measured in real-time as described in Materials and Methods. Software analysis of cellular area and perimeter allows determination of roundness (sample of images rendered for analysis presented in top panels). Upon transfection of truncated Bves (aa118-358), an increase in roundness (red bar) of ~25% is observed in comparison to cells transfected with GFP only (blue bar). ). Error bars represent SEM, significance determined using standard Student-T test.

epithelial resistance of cultured cells, possibly via an interaction with a protein complex containing the tight junction protein ZO1. Several other phenotypes have been reported when Bves protein is decreased, but no molecular mechanism for these observations has been determined to this point. The presented data are the first to establish a direct 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), and upon knockdown of Bves expression in cultured epithelia defects in wound healing have also been reported (Ripley et al. 2004). The described interaction with a component of the Rac1/Cdc42 signaling pathway may provide the first molecular mechanism to explain the cellular/embryonic phenotypes observed upon alteration of Bves expression levels previously described in the literature.

*Bves interacts with GEFT, a modulator of Rho-family GTPase signaling*

Here, I demonstrate that Bves interacts with GEFT, a GEF for small Rho-family GTPases. GEFT 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 Rho-family GTPase activity. The motility of

cells has been shown in numerous studies to be controlled by Rho-family GTPases through the control of processes such as filopodial and lamellipodial extension, as well as polymerization of actin (Etienne-Manneville and Hall 2002). Here, I show that when wildtype Bves or a Bves truncation is transfected into NIH 3T3 cells, movement and roundness of these cells is dramatically affected. I 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 support our hypothesis that Bves modulates the Rac1/Cdc42 activity through an interaction with GEFT. Potential models for the experimental results presented here are discussed later in this chapter.

*Modulation of Rac1/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 Rac1/Cdc42 during gastrulation and convergent extension of *Xenopus* (Habas et al. 2003; Tahinci and Symes 2003; Miyakoshi et al. 2004; Kwan and Kirschner 2005; Ren et al. 2006). Our laboratory previously described a defect in epithelial migration upon knockdown of Bves expression using morpholino oligonucleotides on developing *Xenopus* embryos. Perturbation of Rac1/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 have been shown to be critical players in cellular activity required for wound healing and epithelial sheet integrity (Fenteany et al. 2000; Nobes 2000; Kofron et al. 2002; Malliri et al. 2004; Stramer et al. 2005; Woolner et al. 2005; Kimura et al. 2006). Again, the phenotypes observed upon disregulation of normal Bves levels are consistent with a role for Bves in control of Rac1/Cdc42 signaling.

Additionally, the Brand laboratory noted that in Bves-null animals, skeletal muscle regeneration is delayed upon injury. Rac1/Cdc42 has been shown to affect skeletal muscle regeneration (Chen et al. 2003; Bryan et al. 2005) and regeneration is dependent on process extenstion and myoblast motility (Carlson 1973; Carlson and Faulkner 1983). This previously observed phenotype is also seemingly consistent with a role for Bves in control of Rac1/Cdc42 signaling.

*Potential mechanisms of Bves modulation of Rac1/Cdc42 activity*

The discovery of an interaction between Bves and GEFT leads us to several 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. Several other questions are brought to light by our discovery of this interaction, including the question of whether Bves interacts with

other GEFs, or is an interacting protein specific to GEFT. If it is determined that Bves does interact with other GEFs, the interaction presented here may represent an entirely new pathway for cellular regulation of GTPase activity.

The first potential model is one where Bves controls the nucleotide binding ability of GEFT, and possibly other GEFs. As shown previously, Bves preferentially localizes to the plasma membrane (Wada et al. 2001; Osler et al. 2005; Smith and Bader 2006). 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-GEF interaction may serve as a negative regulator of GEF activity, thereby leading to decreased activation of GTPase signaling. Thus, the overexpression of truncated or full-length Bves may lead to aberrant blockage of this active site, causing the experimental results presented here. This model is also consistent with previously reported “Bves-knockdown” phenotypes of delay in skeletal muscle regeneration, aberrant cell movement during *Xenopus* gastrulation, and altered epithelial sheet integrity and migration. If Bves controls the nucleotide binding activity of GEFT and potentially other

GEFs, disregulation of GTPase signaling control would likely result, potentially generating the previously published phenotypic abnormalities.

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 and 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 (and potentially other GEFs) to this leading edge, allowing them to catalyze nucleotide exchange of GTPases (Figures 19 and 20). This model is also consistent with the results described here. Exogenous overexpression of full-length and truncated Bves may disrupt this controlled localization, resulting in a decrease in overall GTPase activity at the leading edge due to decreased GEF presence in the area. As transfected full-length Bves has been reported to accumulate in the Golgi/ER and not localize to the membrane (Knight et al. 2003)(Smith and Bader unpublished results) and the truncated Bves used here has a broad cytoplasmic distribution (Smith and Bader 2006), exogenous expression of these proteins could serve as a “sink” that prohibits localization of GEFT to the leading edge of motile cells. Again, this model is also consistent with the previously observed knockdown/inactivation phenotypes. If Bves is critical for proper localization of GEFT and possibly other GEFs to sites of

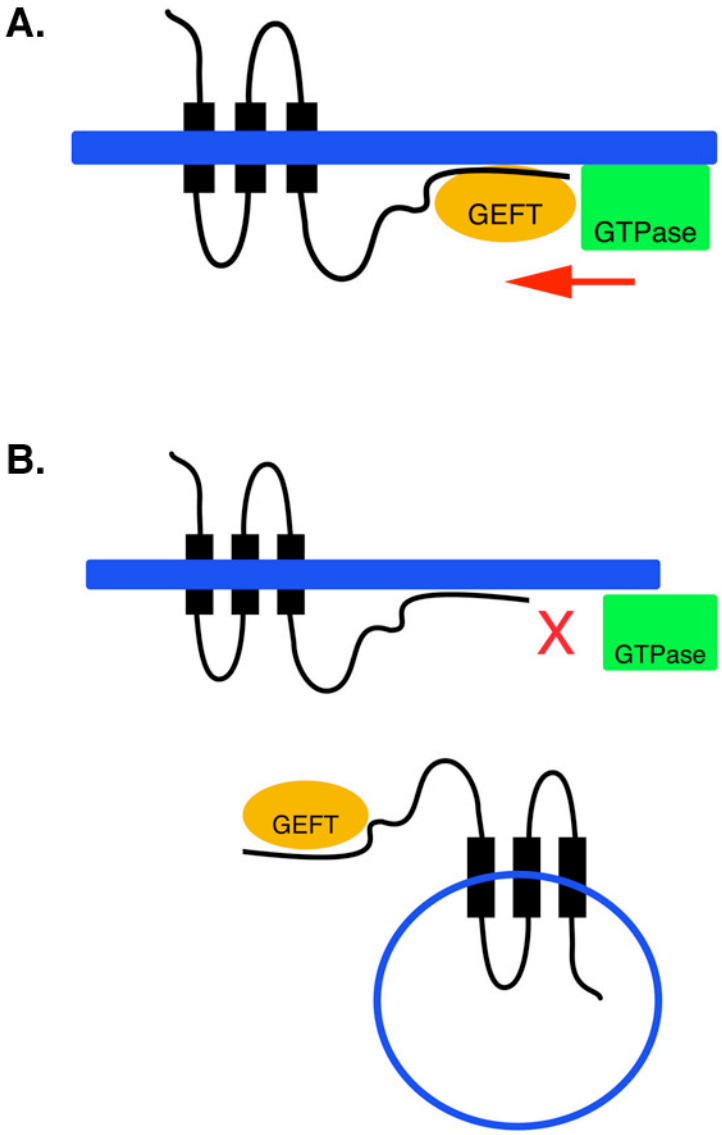



Figure 19: Potential model for Bves function. In a normal cell, Bves acts as a "scaffolding" protein, facilitating interaction with membrane-associated GTPases with GEFT (Figure 20A), However, when either full-length (Figure 20B) or truncated Bves (not shown) is exogenously expressed, GEFT may be sequestered from the site of activity at the membrane.

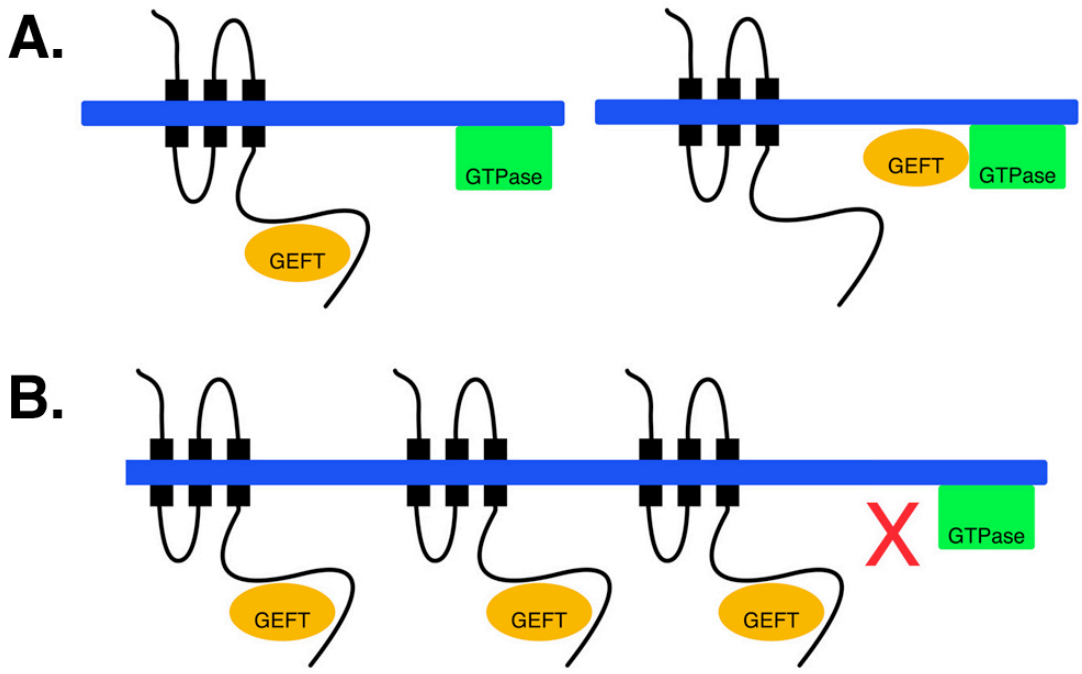



Figure 20: Potential model for Bves function. In a normal cell, Bves normally acts to sequester GEFT from interacting with membrane-associated GTPases (Figure 21A). A signal from the cell stimulates release of GEFT, and GEFT then moves to the membrane and interacts with GEFT causing stimulation of GTPase activity. When full-length or truncated Bves is exogenously expressed (Figure 21B), aberrant interaction of Bves and GEFT leads to improper localization of Bves, and overall repression of GTPase activity.

activity, decreased Bves levels would disregulate GTPase signaling cascades through improper localization of the necessary catalytic proteins.

Similarly, Bves may block interaction of GEFT and potentially other GEFs with another protein(s) at the plasma membrane that are necessary for proper localization of GEFs during GTPase activation. Bves may serve to block interaction between GEFs and membrane anchors during times when Rac1/Cdc42 activity is downregulated. Accordingly, this blockage would then be somehow removed during periods of GTPase activity upregulation. Overexpression of the proteins used in these experiments may block these GEF-anchor protein interactions from occurring, leading to an overall decrease in GTPase activity due to improper localization of GEFT. As with the previously described models, this model is also consistent with results observed upon depletion of Bves protein. Decreased Bves protein in a cell in this model would allow GEFT and possibly other GEFs to be constantly available for stimulation of GTPase activity, and lead to overall deregulation of the tightly controlled GTPase signaling cascades necessary for proper control of cellular movement.

In summation, I have determined that Bves interacts with GEFT, a member of the Dbl family of GEFs. I report that exogenous expression of full-length and truncated Bves constructs *in vitro* leads to a decrease in active levels of Rac1 and Cdc42, and results in phenotypic changes consistent with downregulation of Rac1 and Cdc42 activity. 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. The results presented here are consistent with previously observed phenotypes in other experimental systems, and provides direction to the fields continuing investigation of the function of Bves.

## Current Investigations

Investigation of the Bves-Geft interaction is still underway in our laboratory. We are currently attempting to determine the colocalization of GEFT and Bves in a variety of tissues and cell types using confocal microscopy. I have examined the localization of both the GEFT and Bves proteins in differentiated C2C12 myoblasts (Figure 21). In this cell type, I have not observed extensive colocalization of Bves and GEFT to this point. This result is not necessarily inconsistent with the previously presented data, however. As localization of these proteins changes during different cellular events, we will likely need to assay a variey of cell lines undergoing different cellular processes to determine at what time and place Bves and GEFT colocalize, and subsequently determine the localization of these proteins. For example, in two different myotubes shown in Figure 19, different protein localizations are observed. A significant portion of the protein is seen to be cytoplasmic in distribution (white arrowheads) in some areas. In other areas, very little cytoplasmic Bves is seen, and the vast majority of the Bves protein localizes to the plasma membrane of the cell (white arrows).

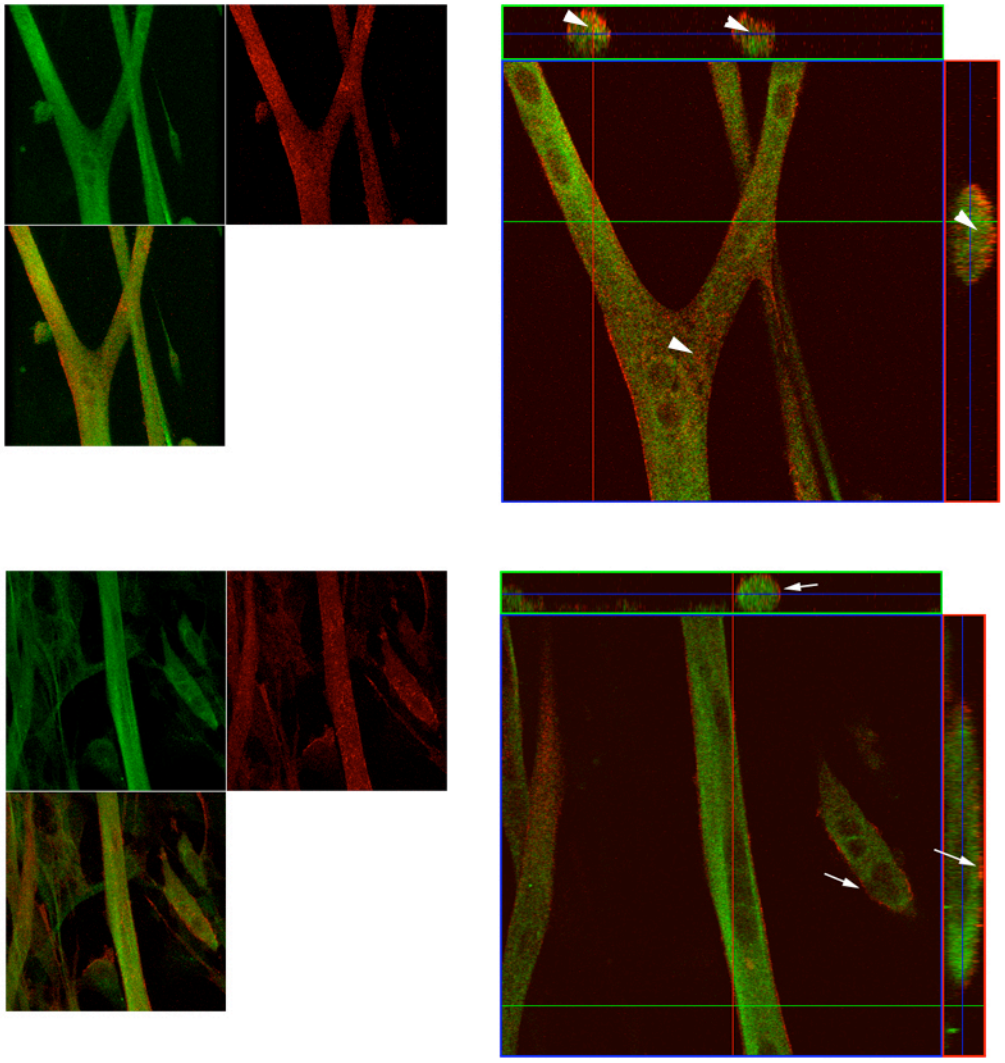


Figure 21: Localization of Bves and GEFT in differentiated C2C12 myotubes. Differentiated C2C12 myotubes were labeled with antibodies against GEFT (green) and Bves (red). Confocal microscopy was used to examine the localization of these proteins in a three dimensional manner. Bves is observed to localize to both the intracellular space (arrowheads), and also to be enriched at the plasma membrane surrouding myotubes (arrows).

# CHAPTER IV

# CONCLUSIONS AND FUTURE DIRECTIONS

## Conclusions

*Summary*

Bves was isolated by the Bader lab in 1999, and has since been the subject of more than 20 peer-reviewed articles. As with many recently discovered molecules, the earliest work on Bves was centered on defining the genomic organization of the *bves* gene. Subsequent work focused on the definition of expression of *bves* in the chicken and mouse. The two groups actively studying Bves at this time, the Brand group and the Bader group, utilized two different methodologies for analysis of Bves expression. The Brand group focused on definition of Bves expression using protocols that defined the presence of *bves* message, and reported *bves* was expressed nearly exclusively in striated muscle and non-vascular smooth muscle. Our group chose to examine Bves expression using immunoreagents, and generated polyclonal antibodies against the chicken Bves protein. Using these antibodies, the Bader group found Bves to be expressed in the proepicardium, epicardium, and components of the coronary vasculature. Interestingly, these reagents also

indicated that Bves is expressed in multiple epithelia throughout the embryo such as the gut and eye.

These divergent findings led to an important question in the Bves field. While the Bader group focused on studying Bves in the developing coronary vasculature, the Brand group directed their efforts towards analysis in striated muscle types. In fact, the expression of Bves in epithelial cell types has been the center of disagreement since the initial publications describing Bves. While immunochemical detection methods showed the presence of Bves in epithelial cell types as well as striated muscle, examination of Bves expression using RT-PCR, in-situ hybridization, and northern blotting showed that Bves was expressed only in striated and non-vascular smooth muscle.

Definition of the domain of expression of a gene is a critical step in its study. The knowledge of where a protein is expressed and where it is absent provides clues to potential function. For example, knowing that a protein is expressed only in muscle cell types would indicate that this protein might have a "muscle-specific" function. Likewise, knowing that a protein is absent from certain cell types allows exclusion of some possible functional hypotheses. Accordingly, I sought to properly define the expression pattern of Bves using newly developed monoclonal antibodies to the Bves protein. The results presented in Chapter II of this dissertation conclusively demonstrate that Bves is expressed not only in striated muscle and some non-vascular smooth muscle, but is also expressed in multiple epithelial cell types throughout the developing

mouse embryo as well as vascular smooth muscle of the coronary vasculature (Smith and Bader 2006). These findings are critical for the analysis of Bves, as they not only resolve the persistent conflict in the literature, but also indicate that Bves likely has a function that is not specific in nature to muscle.

However, the most important question in the Bves field still had no answer: *What is the molecular function of Bves?* As Bves contains no conserved protein-protein interaction motifs, and appears to have been subject to gene duplication during evolution (Andree et al. 2000; Brand 2005), candidate or inactivation approaches were excluded from consideration by our laboratory. Early in this project, I developed the hypothesis that *determining the protein(s) that interact with Bves will reveal the function of Bves*. I reasoned that being able to place Bves into a molecular pathway would enable us to postulate about Bves function in a more focused manner, and directly test these hypotheses to determine the functional significance of the Bves protein. To this end, I conducted a yeast two-hybrid screen to find interacting protein(s), and isolated GEFT. As described in Chapter I, GEFT modulates Rho-family GTPases. In Chapter III, data was presented that clearly demonstrates that Bves biochemically interacts with GEFT. I also presented data that Bves overexpression *in vitro* represses Rac1 and Cdc42 activation, and also produces cellular phenotypes consistent with modulation of Rho-GTPase signaling.

Thus, the data presented in this thesis not only resolve important questions in the literature concerning the expression pattern of Bves, but also

describe the first direct molecular interaction with another protein. In addition, modulation of a defined cellular signaling pathway has been demonstrated, placing Bves into a molecular context that will enable the entire field to more directly examine the molecular function of this novel protein. The following discussion addresses several points about the previously presented data, and discusses several potential models of function along with experiments to investigate these models.

*The importance of Bves expression in non-muscular tissues*

The generation of the monoclonal α-Bves immunoreagents described in Chapter II is an important event in the field of Bves study. Before the publication of this work, dispute about the localization of Bves was one of the primary topics in the literature of the field. Expression studies using polyclonal antibodies clearly showed expression in non-muscle cell types (Reese and Bader 1999; Reese et al. 1999; Wada et al. 2001; Osler and Bader 2004; Ripley et al. 2004; Vasavada et al. 2004). However, some of these antibodies were not reactive with mammalian forms of the protein, and all were beginning to be in short supply due to their polyclonal nature. To address these issues, I generated a panel of monoclonal reagents against the mouse Bves protein and conducted an assay of expression during mouse embryogenesis that clearly demonstrates widespread non-muscular expression of Bves (Smith and Bader 2006). In the literature at this point, there are 3 unique polyclonal antisera (Reese et al. 1999; Wada et al.

2001; Ripley et al. 2004), one monoclonal against avian Bves (Vasavada et al. 2004), and the antibodies described in Chapter II. All of these antibodies recognize Bves in non-muscular cell types.

While the antibodies described in Chapter II do not recognize Bves in either of these structures at the developmental stages examined, they clearly label Bves in the smooth muscle of the coronary vasculature of the mature heart. It should be noted that the antibodies described in Chapter II recognize Bves in the EMC (Epicardial Mesenchymal Cell) line, which is a cell line derived from epicardium. Additionally, all 5 of the antibodies I generated and described in Chapter II recognize Bves in a variety of epithelial cells lines and epithelia in developing and mature organisms. A more thorough investigation of the expression of Bves in the epicardium is still needed to determine if the antibodies developed here recognize Bves in the epicardium at other stages than those examined, but it is clear from the data presented that Bves is expressed in multiple epithelial tissues and in coronary smooth muscle, which is a product of the proepicardium/epicardium.

Beyond the knowledge of where and when a protein is expressed, determining the localization pattern of a protein is important for other reasons. For instance, expression of a protein can serve to mark a specific population of cells for experimental purposes. For example, the protein Sca-1 is commonly used to identify hematopoietic stem cells in a variety of experimental protocols (Spangrude et al. 1988; Spangrude et al. 1989). Using this marker, investigators

can isolate populations stem cells or study the localization and activity of these cells *in vivo*. Similarly, expression of Bves is now used in the field of coronary vessel development as a marker for the epicardium or epicardially-derived cells. More importantly for this discussion, however, is how the expression pattern of Bves can be used to hypothesize about the function of the protein itself. If Bves were found to be muscle-specific, it would naturally lead an investigator to hypothesize that the protein might have a role in contraction, fusion of myoblasts, or regulation of muscle-specific genes. However, understanding that Bves is actually expressed in a variety of non-muscle cell types, as well as striated muscle, they would likely be led to a different set of possible hypotheses when considering the function of the protein. The finding that the Bves protein was not specific to striated muscle led us to hypothesize that Bves must play a role in a process conserved between epithelial and muscular cell types.

*Bves interacts with GEFT, a modulator of Rho-GTPase signaling*

In Chapter III, I describe the discovery and characterization of an interaction between Bves and the Rac1/Cdc42 specific GEF GEFT. As this protein-protein interaction was isolated using yeast two-hybrid technology, by nature of the experimental method the interaction between GEFT and Bves is direct. This is the first reported direct interaction of Bves with any protein, and thus represents a major landmark in the study of the Bves. I also present data from cell culture models consistent with Bves modulation of Rac1/Cdc42 control.

While there have been several phenotypes reported when expression levels of Bves are experimentally downregulated, there has been no definition of the molecular mechanism behind these phenotypes. While the data presented here do not address the molecular mechanism behind these phenotypes, a depression in Rac1/Cdc42 signaling could account for the previously reported experimental results.

To this point, all reported mutant phenotypes that occur when levels of Bves are experimentally manipulated exhibit abnormalities consistent with defects in cellular movement and/or adhesion. Rho-family GTPases have been demonstrated to be one nexus between coordination of cellular adhesion and adhesion (Evers et al. 2000; Noren et al. 2000; Teramoto et al. 2003; Noritake et al. 2005). Rho-family GTPases have been shown to be necessary for cadherin-based adhesion in multiple cell types (Braga et al. 1997; Hordijk et al. 1997; Kuroda et al. 1997; Kodama et al. 1999). The control of cellular adhesion by Rho-family GTPases has been shown to be direct, and not merely a by-product of GTPase control of actin-based motility. IQGAP, a downstream target of GTPase signaling, negatively regulates cell-cell adhesion by interacting with ß-catenin, which causes $\alpha$-catenin to dissociate from the cadherin-catenin complex (Kuroda et al. 1998; Fukata et al. 1999). Thus, phenotypes that indicate disruption of cell motility and/or cell adhesion are entirely consistent with defects in Rho-family GTPase signaling.

Brand and colleagues reported a delay in skeletal muscle regeneration when Bves is globally inactivated in the mouse through gene targeting (Andree et al. 2002). Interestingly, it has been recently reported that GEFT is a potent regulator of mesenchymal cell behavior during skeletal muscle regeneration in mammalian models (Bryan et al. 2005). In this model, either control virus or GEFT virus was injected into the anterior tibialis muscle with cardiotoxin. In this standard skeletal muscle regeneration assay, cardiotoxin kills mature skeletal muscle, and the resident mesenchymal satellite cells then proliferate and differentiate to regenerate muscle cells (Carlson 1973; Carlson and Faulkner 1983). Upon viral overexpression of GEFT, a powerful promotion of skeletal muscle regeneration was seen (Bryan et al. 2005). Thus, Bves and GEFT, which I have shown here to be interacting proteins, have both been experimentally demonstrated to be involved in regulation of skeletal muscle regeneration.

Later, our laboratory presented data demonstrating that Bves plays a role in control of epithelial sheet integrity and in regulation of wound healing *in vitro* (Ripley et al. 2004). When levels of expressed Bves are reduced by morpholino antisense oligonucleotides, a decrease in epithelial integrity of cultured corneal cells was observed. Similar treatment of these cells in wound healing models induced an increase in cell movement at the wound surface but regeneration of an intact epithelium was ultimately impeded (Ripley et al. 2004). Proper regulation of GTPases has been previously demonstrated to be critical for proper regulation of corneal epithelial wound healing (Kimura et al. 2006; Lee and Kay

2006). Again, our laboratory has reproduced an experimental result by perturbing Bves function that has also been reported to be a result of disruption of Rho-GTPase function. This finding also further supports the hypothesis that Bves modulates Rho-GTPase activity.

Finally, our laboratory recently reported that experimental downregulation of Bves expression in developing *Xenopus laevis* results in major defects in movement of epithelial cells (Ripley et al. 2006). When Bves knockdown is experimentally induced at the two-cell stage, gastrulation is arrested by disruption of epiboly and involution. When a clonal knockdown of Bves is performed by injection of morpholino into the A1 blastomere, progenitors of this blastomere move completely randomly throughout the embryo and cell intercalation during gastrulation is abnormal (Ripley et al. 2006). As before, this phenotype is highly consistent with other published phenotypes described when Rho-family GTPase signaling is perturbed. Rac1 signaling has been demonstrated to be necessary for convergent extension movements and cell intercalation during gastrulation (Tahinci and Symes 2003), and Cdc42 has also been shown to be a regulator of gastrulation and convergent extension (Choi and Han 2002; Penzo-Mendez et al. 2003). These phenotypes are consistent with the *Xenopus* phenotype, and suggest a disruption in a common molecular pathway.

The alteration of cell motility upon transfection of Bves and truncated Bves presented in Chapter III also supports the hypothesis that Bves modulation of Rho-GTPase signaling may be responsible for the previously described

phenotypes. In these experiments, overexpression of either full-length or truncated Bves reduced the ability of NIH 3T3 cells to migrate abnormally (Figure 17). Wound healing, skeletal muscle regeneration by satellite cells, and migration of cells during gastrulation of *Xenopus laevis* are all processes that require highly regulated cellular movement. The knockdown experiments in cell culture and *Xenopus* models discussed above both demonstrate exhibit defective cell migration. In the Bves $^{-/-}$ mouse model, the movement ability or speed of satellite cells was not measured, and it was not noted if motility of satellite cells appeared to be affected.

In summary, the interaction between Bves and a modulator of Rho-family GTPase signaling could represent a molecular explanation for previously observed phenotypes exerted by experimental manipulation of Bves expression levels. While the data presented here demonstrate that Bves directly affects Rac1 and Cdc42 activity levels, experiments to determine whether alteration of Rac1 and Cdc42 activity occurs in the described mutant phenotypes have yet to be conducted. Potential models of Bves modulation of these signaling cascades are discussed in Chapter III. These models represent the first actual models for experimental testing that focus directly on one particular cellular pathway. Although many components of the Bves/Rho-family GTPase relationship remain to be defined, the importance of the discovery of an interaction between Bves and a characterized member of a known molecular pathway cannot be overstated. Where investigators were previously left to investigate whole

organisms or whole cells upon manipulation, this interaction now provides a specific, characterized target for investigators to focus upon when evaluating experiments that manipulate Bves activity.

## Future Directions

In the final component of this thesis, potential future experiments and avenues of investigation will be presented. By necessity, early examinations of Bves function have focused on "large-scale" assessment of experimental manipulation of Bves. The product of the current work, however, represents a turning point in this field. In the future, investigators will be able to focus on a particular cellular pathway when designing experiments and evaluating results.

First, further identification of interacting partners is necessary for a thorough explanation of Bves activity. Pursuit of this avenue of investigation has already begun in our laboratory. The interaction described in Chapter III is the result of a yeast two-hybrid screen I conducted. Naturally, this screen generated many potential interacting proteins, several of which passed all false positive screens. These proteins are shown in Table 4. These proteins should also further analyzed, as I analyzed GEFT. It is likely that interaction between one or more of these proteins can be biochemically verified, and may reveal important functional knowledge regarding Bves. In addition to the yeast two-hybrid screen described here, I have performed a split-ubiquitin screen for Bves-interacting proteins. The recently developed split-ubiquitin modification of the yeast two-

Table 4: Summary of genes isolated from yeast two-hybrid screen against embryonic heart library. Right column represents number of clones isolated in original screen.

| Gene Name | Number of clones |
|---|---|
| Aorta and Vein protein | 3 |
| K+ Channel tetramerization domain | 1 |
| RES4-25 protein homolog | 1 |
| TRIP-1 | 1 |
| Nexin 6 | 1 |

hybrid system is superior for isolation of interacting partners of membrane-associated and membrane-bound proteins (Fetchko and Stagljar 2004; Thaminy et al. 2004; Iyer et al. 2005). Using this technology, I have isolated an interaction between Bves and Ndrg4 (N-myc regulated downstream gene 4) (Smith and Bader, unpublished results). Ndrg4, like Bves, is highly expressed in developing neural tissues and cardiac muscle (Zhou et al. 2001). Interestingly, Ndrg4 has been experimentally demonstrated to control MEK and ERK phosphorylation (Hongo et al. 2006). MEK and ERK are also downstream targets of Rho-family GTPase signaling (Zugasti et al. 2001; Rul et al. 2002). Taking into account the GEFT-Bves interaction, the interaction of Bves with another component of Rac1/Cdc42 signaling provides further support for our findings that Bves modulates Rac1/Cdc42 signaling. Further investigation and characterization of the Ndrg4-Bves interaction, as well as interaction between Bves and other proteins will undoubtedly provide valuable data for those interested in the molecular mechanisms underlying Bves activity. As with the previously described yeast two-hybrid screen, many results from the split-ubiquitin screen that passed all tests for false-positives were not further examined. These proteins, shown in Table 5, should definitely be further studied for potentially important interactions with Bves.

Along with identification of additional interacting proteins, it will be important to determine whether Bves is capable of interactions with other members of the Dbl family of GEFs. As shown in Chapter III, Bves interacts with

Table 5: Summary of genes isolated from split-ubiquitin screen against adult mouse heart library. All of these proteins were isolated from original screen, and tested for potential false positive interaction. All clones passed false positive screening.

| Gene name |
|---|
| Gpsn2 |
| Cklfsf7 |
| Ndrg4 |
| Vamp3 |
| PtplA |
| Brain MY047 |
| Fundc1 |
| Fundc2 |

GEFT through a region of GEFT that contains the highly conserved DH domain. While the Bves-Geft interaction may be an interaction unique to this member of the Dbl family, the possibility exists that this interaction is shared with other family members. If Bves were found to interact with other GEFs, it would indicate that Bves might have a broad function in control of GTPase signaling, as opposed to the rather specific function of interaction with one particular GEF. In fact, careful consideration of the PAK-21 pulldown assay of GTPase activation presented in Chapter III (Figure 16) indicates that Bves may modulate GTPase signaling through modulation of more than one GEF. As seen in Figure 16, the amount of Cdc42 activity in this assay is almost entirely abolished by transfection of truncated Bves, and the amount of Rac1 activity is reduced by more than 50%. As more than one GEF is likely involved simultaneously in GTPase signaling in most cell types (Overbeck et al. 1995), the amount of activity reduction seen indicates that truncated Bves may be affecting the activity of more than one GEF at a time. Thus, it is critical to determine whether Bves interacts promiscuously with multiple members of the Dbl GEF family, or is specific to GEFT, in order to truly elucidate the molecular mechanism underlying Bves control of GTPase signaling.

Potential roles for the other members of the Popdc gene family should also be studied to determine any role in regulation of GTPase signaling during cell movement and developmental processes. As there is a very high degree of conservation between all family members in the intracellular carboxyl-terminus,

shown here to be important for interaction with GEFT, it is reasonable to hypothesize that some or all other Popdc family members may interact with GEFT or other GEFs. At this point, no function has been ascribed to any of the other Popdc family members (Osler et al. 2006). Additionally, functional redundancy seems to be indicated by the lack of a strong phenotype in Bves $^{-/-}$ mice. All or some members of the Popdc family may interact with GEFs to regulate myriad cellular processes by affecting the activity states of GTPases. Alternatively, it has been shown that homophillic Bves protein-protein interaction occurs (Knight et al. 2003), therefore it is also possible that Bves is capable of interacting with other Popdc family members. If this were the case, other Popdc family members may interact with and regulate the activity of Bves, thereby indirectly controlling GTPase activity. In any case, it is clear that the relationship between Bves and other Popdc family members needs to be examined more closely, as well as the individual and perhaps unique characteristics of each family member.

Next, experimental definition of Rac1/Cdc42 activity levels in the knockdown/knockout phenotypes previously reported is necessary. Examination of Rac1/Cdc42 levels in the satellite cells that regenerate skeletal muscle in the Bves$^{-/-}$ mouse (Andree et al. 2002) and comparison of these levels to that of a wildtype animal would strongly indicate that the observed phenotype is a direct result of disruption of proper Rac1/Cdc42 signaling induced by Bves inactivation. Similarly, determination of relative Rac1/Cdc42 activation states in the *Xenopus*

*laevis* model system utilized by our laboratory is necessary for similar reasons. Further investigation of these models, as well as the NIH 3T3 model system described in Chapter III, will also allow investigators to directly address the models presented above. In these systems, definition of the subcellular localization of Bves, Rac1, Cdc42, and GEFT and potentially other GEFs is possible. If experimental manipulation of Bves were found to alter the localization of other components of the Rho-family GTPase signaling pathway, this would strongly indicate that Bves is critical for proper localization of proteins involved in this pathway. A variety of methods to address the localization of these proteins are available, but the most likely to be used involve usage of the immunoreagents described in Chapter II. Additionally, now that these reagents are available for use, and have been demonstrated to be specific for the Bves protein, re-examination of previously published experiments should be performed. For example, the visualized ultrastructural localization of Bves should be compared with the previously published electron micrographs that used the B846 polyclonal antisera (Osler et al. 2005). As noted in Chapter II, the Bves protein has a wider localization when visualized with the SB panel of monoclonal antibodies than when visualized with the B846 antisera.

In summation, the work presented in this thesis represent not only a significant advance for the field of Bves study, but provide critical direction and tools for investigation to continue and advance. The immunoreagents described in Chapter II not only helped to resolve long-standing discrepancies in the

literature, but also provide tools that will be used in the future. These antibodies will undoubtedly be used to determine the subcellular localization of Bves in the future experiments described above, and also will provide valuable tools for immunoprecipitation-based experimental protocols necessary for investigation of new Bves-interacting proteins. Furthermore, the identification of the first protein known to directly interact with Bves not only places Bves into a molecular context, but also provides focus and direction for future experiments investigating the function of Bves in modulation of Rho-family GTPase signaling. Additionally, the screens for interating proteins that were performed as a part of this research have also isolated many other candidate proteins that interact with Bves in a genetic screen. These interacting proteins may open more new and exciting avenues of investigation of the Bves protein, just as the identification of a direct interaction between Bves and Geft does in this work.

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