Identification of a Novel Intracellular Interaction Domain Essential for Bves Function

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Abstract

While Blood vessel epicardial substance (Bves) confers adhesive properties, the molecular mechanism of regulating this activity is unknown. No predicted functional motifs in this highly conserved integral membrane protein, other than the transmembrane domain, have been identified. Here, we report for the first time that Bves interacts with itself through an intracellular interaction domain that is essential for its intercellular adhesion activity. Glutathion-S-transferase (GST) pull-down and SPOTs analyses mapped this domain to amino acids 268-274 in the intracellular C-terminus. Site-directed mutagenesis revealed that lysines 272 and 273 are essential for homodimerization and cell adhesion. Human corneal cells transfected with wild-type Bves trafficked the protein to the cell surface, assembled junction complexes and formed epithelial sheets. In contrast, cells expressing Bves mutated at these positions did not form continuous epithelial sheets or maintain junctional proteins such as ZO-1 and E-cadherin at the membrane. A dramatic reduction in transepithelial electrical resistance was also observed indicating a functional loss of tight junctions. Importantly, expression of mutated Bves in epithelial cells promoted the transformation of cells from an epithelial to a mesenchymal phenotype. This study is the first to demonstrate the essential nature of any domain within Bves for maintenance of epithelial phenotype and function.

Introduction

Bves was discovered independently by Reese et al. [1] and Andree et al. [2] and is the prototypical member of the Popeye domain containing (popdc) gene family [2]. It is highly conserved and has been identified in a wide variety of vertebrate and invertebrates [1–5]. Both mRNA and protein of Bves are highly expressed in striated and smooth muscle and in various forms of epithelial cell types in the embryo and adult [1,2,5–11]. Biochemical analyses have determined that Bves is an integral membrane protein [12,13], while localization studies have found Bves at the lateral cell membrane and within vesicles of the Golgi apparatus [12,13]. Still, no molecular understanding of protein function is currently available.

Bves has the canonical structure of all predominant popdc gene products. This includes a short extracellular N-terminus with two invariant glycosylation sites, three transmembrane domains with two intervening loops and a long intracellular C-terminus [2,9,12]. While Bves has a highly conserved primary amino acid sequence among different species, there are no studies identifying any protein domain linked to any molecular or cellular function.

Phenotypic analyses of this gene family are only now emerging. Due to its subcellular localization and trafficking to points of cell-cell contact during epithelial sheet formation [9], we proposed that Bves might play a role in cell-cell adhesion. Transfection of Bves into normally non-adherent L-cells conferred adhesive activity [13] much like E-cadherin indicating that the transfected molecule confers adhesive properties [14,15]. Additionally, morpholino knockdown of Bves protein inhibited epithelial sheet formation and stability, and disrupted transepithelial electrical resistance (TER) [9]. While popdc1-null mice do not show an overt embryonic phenotype, presumably due to redundancy of expression with popdc2 and popdc3 genes, regeneration of skeletal muscle is delayed due to an inhibition of cell-cell adhesion/interaction [16]. Early inhibition of Bves function in Drosophila development results in disruption of pole cell migration [4], while gastrulation in X. laevis is severely restricted due to failure in epithelial morphogenesis [17]. Still, no reports have identified any functional domains within Bves or described the molecular basis of Bves function for adhesion or any other possible activities in tissue or organ morphogenesis.

Here for the first time, we report a Bves-Bves molecular interaction through its intracellular C-terminus that is essential for molecular regulation of cell-cell adhesion. This domain lies within the highly conserved Popeye region of the molecule, which heretofore has no ascribed function. Further we identify two amino acids in this sequence (K272 and K273) that are critical for homophilic binding. While transfection of wild type Bves promotes cell aggregation in L-cell assays, mutation or deletion of K272 and K273 abolishes this activity. Expression of these mutated transcripts dominantly interferes with normal Bves function in human corneal epithelial cells (HCE) resulting in loss of cell-cell adhesion, junction formation, TER and epithelial sheet integrity. Importantly, expression of mutated Bves leads to a change of cells from an epithelial to mesenchymal phenotype. This study is the first to...
identify a specific molecular mechanism by which Bves regulates cell-cell adhesion and to demonstrate that mutation of these sequences inhibits cellular functions attributed to this molecule.

**Results**

**Bves intermolecular interaction through the intracellular C-terminus**

The molecular basis of Bves adhesive function is unknown [13]. To determine the molecular mechanisms that underlie this function, we explored whether Bves-Bves intermolecular interactions could be detected. We generated an array of wild type and truncated Bves constructs to identify possible Bves-Bves interaction domains (Figure 1A). In a first set of experiments, Flag-tagged Wild Type (WT) Bves harvested from COS-7 cells was reacted with GST N- or C-terminal Bves produced in E. coli. As seen in Figure 1B, GST C-terminal Bves readily precipitated WT Bves, while GST N-terminal Bves and GST alone did not. Elimination of the two N-terminal glycosylation sites had no effect on C-terminal interaction (Figure 1B, lane g). These results do not exclude the possibility of N-terminal interactions but demonstrate direct association between molecules through the C-terminus of Bves. To further define sequences in the C-terminus responsible for this activity, a series of C-terminal truncations (shown in Figure 1A) were reacted in similar manner. Deletion of C-terminal up to aa 284 (Del-4 Bves) had no effect on precipitation efficiency (Figure 1C) but elimination of the next 33 amino acids (Del-3 Bves) completely abolished C-terminal interactions. This construct, Del-3 Bves, was used further as a non-interacting control in subsequent studies. These data are the first to identify Bves-Bves homodimerization.

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

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

Next, to determine whether specific amino acids are required for binding activity, alanine substitution of individual amino acids across this core element was conducted and the resulting peptides were reacted with WT Bves. Figure 2D shows that lysine to alanine substitution at positions 272 and 273 eliminates Bves binding to the core element peptide. Interestingly, peptides 11 to 13, which are reactive in SPO Ts analysis, are the only targets that contain both K\(^{272}\) and K\(^{273}\). These data indicate that aa 268-274 are required for at least one Bves-Bves interaction and that K\(^{272}\) and K\(^{273}\) are critical for this function.

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**Figure 1. Bves constructs and GST pull-down assay.** A, Diagram of GST-fusion proteins and deletion constructs. GST C- and N-terminal Bves proteins and serially deleted Flag-tagged Bves proteins. B, GST pull-down assay with C- or N-terminal Bves. Bacterial lysates of GST-Bves fusion proteins were reacted with COS-7 cell lysates transfected with Flag-tagged WT Bves and analyzed with Western blots using an anti-Flag antibody. The potential effects of glycosylation were also tested. Control band (WT Bves protein with or without glycosylation) are shown on lane a and b. Only GST C-terminal Bves reacted in this assay regardless of glycosylation state (lanes d and g). C, Serial deletion analysis of C-terminal interaction. GST C-terminal Bves was reacted with Del-5 Bves, Del-4 Bves and Del-3 Bves and processed for Western blotting to detect interactions. Del-5 Bves and Del-4 Bves (lanes a and b) interact with the GST C-terminal Bves while Del-3 Bves does not.

doi:10.1371/journal.pone.0002261.g001
The intracellular interaction domain is essential in Bves-mediated cell-cell adhesion

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

Expression of mutated Bves inhibits formation and stability of epithelial sheets

We next determined whether expression of mutated Bves molecules would disrupt native adhesive properties in epithelia using a human corneal epithelial cell line (HCE). Multiple stable HCE cell lines expressing WT Bves, KK-Mutant Bves (Figure 3C) and KK-Deletion Bves (Figure 3D). Both of these transfected cell lines failed to aggregate above levels seen in non-transfected parental cells (compare 3A, C and D). Taken together, these data demonstrate that mutation of the newly-identified Bves-Bves intracellular interaction domain abolishes the adhesive function.

Figure 2. SPOTs protein mapping.

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

doi:10.1371/journal.pone.0002261.g002

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intracellularly (Figure 6G, H). It should be noted that all images were exposed and electronically processed identically. Thus, the reduced intensity of staining in Figure 6F and J can be compared to the greater signal observed in Figures 6G and H. In addition, the expression of a KK-Mut Bves inhibits trafficking and/or accumulation of endogenous Bves at the cell surface (Figure 6K).

Still, in isolated areas, anti-Bves staining without overlapping anti-Flag staining was observed at the cell surface between adjacent cells indicating the presence of endogenous protein in the absence of transfected Bves. Anti-Flag immunoreactivity around the entire cell was rarely observed. In KK-Del Bves transfected cells distinct circumferential anti-Bves staining was also difficult to detect. These cells appeared smaller and had a significant intracellular accumulation of transfected proteins.

To determine whether mutations of Bves at positions 272 and 273 disrupt juncional adhesive complexes, cultures were assayed for the distribution of known juncional proteins. As seen in Figure 7, ZO-1 and E-cadherin, components of the tight and adherens junctions, respectively, are drastically redistributed in cell lines expressing exogenously mutated forms of Bves. ZO-1 staining is seen in small patches but not with that of the altered forms of the protein. Circumferential staining of ZO-1 and E-cadherin were rarely if ever detected in cells expressing KK-Mut Bves or KK-Del Bves.

TER is a standard measurement of epithelial function and tight junction integrity. In order to determine the effect of mutation or substitution of K272 and K273 on tight junction activity, HCE cell lines stably transfected with WT Bves, KK-Mut Bves and KK-Del Bves were grown to confluence and assayed for TER (Figure 8). Transfected cultures were compared to the non-transfected parental cell line. A resistance of ~350\(\Omega\) cm\(^2\) was observed with non-transfected controls while transfection with WT Bves produced elevated resistance. When we measured TER in HCE cell lines expressing KK-Mut Bves or KK-Del Bves, values were dramatically reduced to near background levels. TER for KK-Mut Bves [p-value<0.0001, CI (0.07, 0.14)] and KK-Del Bves [p-value<0.0001, CI (0.07, 0.15)] are significantly reduced compared to the control parental group, while TER for WT Bves is significantly greater than those of the control parental group [p-value = 0.006, CI (1.08, 2.28)]. Taken together, these data suggest the expression of Bves with mutation in the putative intracellular interaction domain produced marked disruption of epithelial structure and function.

Expression of mutated Bves leads to changes in epithelial cell phenotype

Alteration of cell-cell adhesion/interaction can lead to changes in cell phenotypes, most notably through processes involving epithelial-mesenchymal transition [18]. Control parental and WT Bves-transfected cells expressed cytokeratin (Figure 9) and other markers of the epithelial phenotype with no expression of mesenchymal phenotype. In contrast, cells stably transfected with KK-Mut Bves expressed and accumulated vimentin, a marker of mesenchymal phenotype [19]. The number of cells with this phenotype was constant but low (Figure 9G). In contrast, a high percentage of KK-Del Bves expressing cells exhibited a mesenchymal phenotype even under culture conditions that favor maintenance of the HCE epithelial phenotype (Figure 9H). These data suggest that mutation of the Bves-Bves intracellular interaction domain leads to changes in cells consistent with a mesenchymal phenotype.

Discussion

Bves is a highly conserved transmembrane protein with cell-cell adhesion function that is expressed in a variety of epithelia and
muscle from flies to humans. Previous studies suggest that the function of the popdc gene family of which Bves is a prototypical member has great implications for development and disease. Still, the molecular mechanisms underlying this function are completely unknown. Additionally, no protein motif or domain has been identified to account for any activity of this gene family. Here for the first time, we demonstrate Bves-Bves interaction and identify a functional domain in the Bves molecule that regulates this process.

Figure 4. Phase contrast of transfected human corneal epithelial (HCE) cells. Non-transfected parental cells (panel A) form confluent epithelial sheets, as do HCE cells transfected with WT Bves (panel B). However, in WT Bves transfected cells, areas of tight adherence are readily observed (arrow head). In HCE cells transfected with mutant Bves, a contiguous monolayer is rarely formed. KK-Mut Bves transfected cells are more rounded and moderate gaps are viewed (panel C, arrows), while KK-Del transfected cells display distinct gaps.

doi:10.1371/journal.pone.0002261.g004

Figure 5. Distribution of transfected WT Bves in HCE cells. A stable cell line expressing WT Bves was grown to confluence and examined for Bves expression and distribution. Panel A demonstrates distribution of anti-Bves staining while B displays anti-Flag staining. Note that most staining overlaps (Panel C, merge) but small areas are observed that are positive for anti-Bves and negative for anti-Flag (arrows in A–C).

doi:10.1371/journal.pone.0002261.g005
Figure 6. Detection of endogenous and transfected Bves in human corneal epithelial cells (HCE). Anti-Bves staining (top panels, A–D), anti-Flag staining (middle panels, E–H) and merge (bottom panels, I–L) are shown. A, Endogenous Bves is detected at the parental cell membrane in confluent monolayers. E, Parental cells do not react with anti-Flag. B, Endogenous Bves is expressed in WT Bves transfected cells. F, Transfected WT Flag-tagged Bves traffics to the cell membrane, where it co-localizes with anti-Bves staining, which detects endogenous protein. C, G, K, Transfection of KK-Mut Bves and D, H, L, KK-Del Bves show a general loss of membrane staining for both endogenous and transfected protein. Inset K, importantly, sporadic green staining at the membrane is seen in merged images with transfected KK-Mut Bves suggesting localization of endogenous but not transfected protein. (Scale Bar 100 μm)
doi:10.1371/journal.pone.0002261.g006

Figure 7. Expression of ZO-1 and E-cadherin in HCE cells. A, E, Parental HCE cells B, F, HCE cell lines transfected with WT Bves, C, G, KK-Mut Bves, and D, H, KK-Del Bves were stained with ZO-1 in panels A through D and E-cadherin in panels E through H. Note the loss of peripheral ZO-1 and E-cadherin staining in KK-Mut Bves and KK-Del Bves cells. (Scale Bar 100 μm)
doi:10.1371/journal.pone.0002261.g007
Further, we show that mutation or deletion of specific amino acids within this domain abolishes the cell-cell adhesion mediated by the molecule and leads to predictable changes in cell phenotype. These novel studies identifying Bves-Bves interaction and its regulation have larger implications for the understanding of Bves function in development and disease.

Identification of an intracellular interaction domain in Bves

While members of the popdc family are highly conserved, neither Bves nor any other family member harbors a predicted protein motif that would account for its cell-cell adhesive function [1,2,5]. However, the presence of an intracellular Bves-Bves interaction domain responsible for membrane clustering of the molecule had been previously suggested by Professor Thomas Brand [20]. Here, our truncation analysis of the C-terminus demarcates a region (aa 252-284) that mediates Bves-Bves intermolecular interaction and is sufficient for at least one homophilic binding event (Figures 1 and 2). It should be noted that the present study does not preclude the presence of additional Bves-Bves C-terminal interactions. Within this newly identified domain, lysines at positions 272 and 273 are critical for molecular interaction and are conserved in Bves protein sequences in mouse, human and chicken (NCBI). The identification of an intracellular domain that is essential for intercellular adhesive activity is not unprecedented, as an intracellular juxtamembrane domain in E-cadherin is essential for its intercellular adhesion function [21–23]. As no previous studies have ascribed function to any motif within Bves, identification of...
this intracellular domain is critical for an understanding of the function of this molecule.

**Mutation of Bves disrupts cell-cell adhesion**

Expression of mutated Bves does not promote cell clustering in L cells in contrast to expression of WT Bves controls (Figure 3). Also, expression of mutant constructs greatly inhibits adhesion properties in normally adherent HCE cells (Figure 4–6). These assay systems provide strong corroborating data that suggest a functional significance for this intracellular interaction domain in cell-cell adhesion. We propose that over-expression of KK-Mut Bves or KK-Del Bves acts in a dominant-negative or interfering fashion in adherent cells that normally express Bves.

Additionally, immunofluorescence analysis of HCE cells expressing transfected WT Bves detects the molecule at the cell surface along with components of the adherens and tight junctions (Figure 7) and expression of WT Bves increases TER in transfected cells (Figure 8). In contrast, over-expression of either mutant form of Bves inhibits a function of the endogenous Bves protein as epithelial sheet integrity is compromised. It is interesting to note that only small puncta of endogenous Bves are observed at the cell surface (Figure 6K). Additionally, surface staining of adhesion molecules such as ZO-1 is punctate and also greatly diminished in cells expressing mutated Bves (Figure 7). These results indicate that: 1) the KK-containing domain is important for proper trafficking and/or stability of Bves since KK-mutated Bves disturbs membrane localization of endogenous Bves, and/or 2) the KK-containing domain is involved in regulating the membrane localization of other adhesion molecules. Therefore it is plausible that inhibition of Bves-Bves intracellular interaction in turn disrupts the generation and/or maintenance of cell-cell adhesion. Bves is one of the first molecules to traffic to points of cell-cell contact and it interacts with ZO-1 a known mediator of cell-cell adhesion [9,13]. Disruption of either one or both of these functions could inhibit the assembly or stability of forming junctions. This may lead to the loss of adhesion protein localization at the membrane and the observed drop in TER in epithelial cells expressing mutated Bves. These data support the hypothesis that Bves is a critical regulator of cell-cell interaction and clearly delineates essential intra-cellular interaction in the process. From these new data, we postulate that the intracellular interaction domain is critical for clustering of Bves molecules and that this aggregation is important for intercellular interaction functions of Bves and/or association with other protein components of cell-cell junctions.

**Inhibition of Bves function leads to changes in cell phenotypes**

A key finding in the present study is that cells expressing mutated Bves exhibit an altered phenotype. Many, but not all, epithelial cells expressing this molecule take on varying morphologies and express vimentin, a marker of the mesenchymal phenotype [19]. These HCE cells, which normally do not exhibit mesenchymal behavior, take on this fate with altered Bves function and the lack of membrane localization and/or stability of the protein. This finding mimics the situation observed for Bves during coronary artery differentiation. There we have observed that, adherent epithelial cells of the developing epicardium express Bves at the lateral cell surface [13]. When individual cells undergo epithelial-mesenchymal transition, Bves is removed from the cell surface along with other adhesion molecules and cells assume a mesenchymal phenotype. It is possible that expression of mutated Bves inhibits cell-cell adhesion resulting in the production of mesenchyme not unlike that observed during those processes observed in vivo. Thus, it is possible that the regulation of cell-cell adhesion is controlled in part through inter-molecular association governed by the Bves intracellular interaction domain.

**Materials and Methods**

**Bves constructs**

Specific regions of the Bves molecule were cloned using Polymerase Chain Reaction (PCR) strategies for biochemical and cellular analysis. Primers are listed in Table 1. N-terminal (amino acid (aa) 1-36) or C-terminal (aa 115-357) regions of Bves were cloned into the EcoRI and Xhol site of pGEX-2X-1 (Amersham, Piscateway, NJ) for bacterial expression and the Sal I and Not I site of pCNeo (Promega, Madison, WI) for eukaryotic expression. Wild type (WT) Bves and a C-terminal deletion series [Del-5 Bves (aa 1-309), Del-4 Bves (aa 1-284) and Del-3 Bves (aa 1-251)] were Flag-tagged (YVKDDDDK) on their C-termini and inserted into the Sal I and Not I site of pCNeo. Alanine substitution and deletion of K272 and K275 of Flag-tagged Bves were produced by a sequential PCR method using primers listed in Table 1 and cloned into pCNeo. Sequences of all constructs were confirmed in the DNA sequencing core at Vanderbilt University.

**Cells, transfection and production of stable cell lines**

COS-7 cells (ATCC, Manassas, VA) and L-cell (CCL 1.3, ATCC) were cultured in Dulbecco’s Modification of Eagle’s Medium (DMEM, Mediatech, Herndon, VA) with 4mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose and 1.0 mM sodium pyruvate supplemented with 10% fetal bovine serum (FBS, Atlanta biological, Lawrenceville, GA) and 10 µg/ml Penicillin-Streptomycin solution (Mediatech). Human corneal epithelial cells (HCE) were originally obtained from Dr. K. Araki-Sasaki (Osaka, Japan), maintained as previously described [24], and were grown in Defined Keratinocyte-SFM with growth supplement (Invitrogen, Grand Island, NY). L-cells were transfected with WT Bves, C-terminal Bves, N-terminal Bves, KK-Mut Bves and KK-Del Bves. Two µg of each construct were used for transfection with the FuGENE6 transfection reagent (Roche, Indianapolis, IN). After 72 hours, positive clones were selected in growth medium containing G418 (0.4 mg/ml) and resistant cells were maintained in medium with G418 (0.2 mg/ml). HCE cells were also transfected with WT Bves, KK-Mut Bves and KK-Del Bves as described above. Stably transfected cell lines were obtained by using medium with G418 (0.02 mg/ml). Production of Bves protein was confirmed by immunoechemical staining with anti-Bves (B846, [13]) and anti-Flag (M2, Sigma, St. Louis MO) antibodies.

**GST pull-down assay**

GST N-terminal, GST C-terminal Bves or GST proteins were prepared using standard methods [9] and mixed with WT Bves produced in COS-7 cells and incubated overnight at 4°C. Replicate WT Bves transfected COS-7 cells were incubated with tunicamycin (2 mg/ml) to produce WT Bves protein devoid of glycosylation products. Reactions were then applied to glutathione beads and rocked at 4°C for four hours. Beads were washed three times with Phosphate Buffered Saline (PBS) and the protein was eluted with SDS sample buffer. Eluted proteins were analyzed by SDS-PAGE and Western blot using anti-Flag antibody [1]. The same experiments were repeated with a series of C-terminal deletion constructs to identify the precise region required for Bves-Bves homophilic interaction. Dilutions of antibodies were: primary antibodies (anti-Flag, M2, Sigma 1:1000), secondary antibody (anti-mouse IgG alkaline phosphatase (AP) -conjugated, Sigma...
Alanine Substitution analysis

**Table 1. Sequences of primers used to generate Bves constructs**

| Primer Name | Direction | Sequence (5’→3’) | T an (°C) |
|-------------|-----------|-----------------|----------|
| 5’ N-term   | S         | TTGACAGAATCTAGGACACTACGGGCAATCAGC | 57 |
| 3’ N-term   | AS        | CAGATATGCTGGGTCTTCTTCCAGATTCG | 57 |
| 5’ C-term   | S         | TTGACAGAATCTAGGACACTACGGGCAATCAGC | 57 |
| 3’ C-term   | AS        | CAGATATGCTGGGTCTTCTTCCAGATTCG | 57 |
| 5’ Sali Bves | S         | AGAGCTGTTGGCGACATTTTACGTCTGTCGTCCTTTGTCATTTAAGG | 55 |
| 3’ Not I/Flag WT | AS | TACATATGCTGGGTCTTCTTCCAGATTCG | 57 |
| 3’ Not I/Flag Del-5 | AS | TACATATGCTGGGTCTTCTTCCAGATTCG | 57 |
| 3’ Not I/Flag Del-4 | AS | TACATATGCTGGGTCTTCTTCCAGATTCG | 57 |
| 3’ Not I/Flag Del-3 | AS | TGTACCTATATGGGGCGGACACTCTGTACGTCTGTCCTTTGTCATTTAAGG | 57 |
| 5’ Mut/Del | S         | ATCTGTTTTCCAGTGGGCA | 55 |
| 3’ Mut/Del | AS         | GAACTGGAAACATAAAATGAGT | 55 |
| 5’ KK-Mut | S         | CCTTAAATGACAAGGCCTCA-ATTGATCGGCAGCCAAGTCT | 55 |
| 3’ KK-Mut | AS         | AGACTTGGCTGGGTCAATCAGGCGGTGACCTGTCTTGAATGACG | 55 |
| 5’ KK-Del | S         | CTTAATAGCAGAGGCTCA-ATTGATCGGCAGCCAAGTCT | 55 |
| 3’ KK-Del | AS         | AGACTTGGCTGGGTCAATCAGGCGGTGACCTGTCTTGAATGACG | 55 |

Note: Bold and underline characters show the mutation sites and hyphen shows the deletion site

Sequences of primers used to generate Bves constructs

**L-cell aggregation assay**

Cellular adhesion activity of non-transfected control and stable L-cell lines transfected with Bves constructs (WT Bves, KK-Mut Bves, and KK-Del Bves) was compared in standard hanging drop suspension cultures [14,15]. Images were acquired using an inverted image microscope (Olympus IX70), an object lens (LCPlan FI 20X/0.40 Ph1), a camera (OPTRONICS MagnaFire-Model S60800) and software (MagnaFire 2.1A) at room temperature.

**Immunofluorescence assay**

Immunofluorescent analysis of protein expression and distribution was similar to previously published studies [9,13,23]. HCE cells transfected with WT Bves, KK-Mut Bves and KK-Del Bves or non-transfected parental cells were seeded on a four well-chamber slide (Lab-Tek II, Naige Nune, Naperville, IL) and immunostained using following antibodies. Primary antibodies were: anti-Bves (B846, [13], 1:200), anti-Flag (M2, Sigma, 1:150), anti-ZO-1 (Zymed, South San Francisco CA, 1:200), anti-E-cadherin (Sigma, 1:200), anti-cytokeratin (DAKO, Carpinteria CA, 1:200), and anti-vimentin (AMF17b, Developmental Hybridoma Bank, Iowa City IA, 1:200). Anti-rabbit IgG conjugated with Alexa 488 (Molecular Probe, Eugene OR, 1:3000) and anti-mouse IgG conjugated with Cy3 [Jackson ImmunoResearch Lab, West Grove PA, 1:3000] were used as secondary antibodies. DAPI (Molecular Probes) was used at 1:5000 to stain nuclei. Images were acquired using a fluorescence microscope (Olympus AX70 TRF), an object lens (Olympus UPlan APO 40X/0.85), a camera (OPTRONICS MagnaFire-Model S60800) and software (MagnaFire 2.1A) at room temperature.

**Transepithelial electrical resistance (TER)**

To examine tight junction activity in confluent cultures, the TER was measured. Non-transfected and transfected HCE cells were used in this study. Cells (2×10^5 cells/cm^2) were seeded on a six well transwell chamber (cell culture insert, 0.4 µm pore,
Falcon/BD lab ware, Franklin Lakes, NJ) and cultured for two weeks. TER was measured using an Epithelial volt-ohm-meter (EVOM) (World Precision Instruments, Sarasota, FL). TER was calculated as follows: TER = [Reading of EVOM - reading of blank] × surface area of the membrane (4.2 cm²). The data are log-transformed to stabilize variances, Analysis of variance was applied to test for overall difference, followed by Dunnett’s method to compare the three experimental groups to the parental group while controlling the family-wise type I error rate at 5%. Simultaneous 95% confidence intervals of the group mean ratios were obtained using Dunnett’s method. The reported p-values and confidence intervals are adjusted for multiple comparisons [26].

Acknowledgments
We thank Samyukta Reddy for expert assistance with cell culture and Michael Ray and Yoshiya Kawaguchi for technical advice. We thank Bettina Wilm, Travis K Smith, and Megan E Osler for critical review of the manuscript.

Author Contributions
Conceived and designed the experiments: DB MK AW MC HH. Performed the experiments: MK AW MC HH. Analyzed the data: DB MK AW TK MC HH. Contributed reagents/materials/analysis tools: DB MK MC. Wrote the paper: DB MK MC HH.

References
1. Reese DE, Zavaljevski M, Streiff NL, Bader D (1999) bves: A novel gene expressed during coronary blood vessel development. Developmental Biology 209: 159–171.
2. Andree B, Hillemann T, Kessler-Icekson G, Schmitt-John T, Jockusch H, et al. (2000) Isolation and characterization of the novel popeye gene family expressed in skeletal muscle and heart. Developmental Biology 223: 371–382.
3. Hitz MP, Pandur P, Brand T, Kahl M (2002) Cardiac specific expression of Xenopus Popeye-1. Mechanisms of Development 115: 123–126.
4. Lin S, Zhao D, Bownes M (2007) Blood vessel/epicardial substance (bves) expression, essential for embryonic development, is down regulated by Gsk3/EGFR signalling. International Journal of Developmental Biology 51: 37–44.
5. Reese DE, Bader DM (1999) Cloning and expression of bves, a novel and highly conserved mRNA expressed in the developing and adult heart and skeletal muscle in the human. Mammalian Genome 10: 913–915.
6. Breher SS, Mavridou E, Brenness C, Froese A, Arnold HH, et al. (2004) Popeye domain containing gene 2 (Popdc2) is a myocyte-specific differentiation marker during chick heart development. Developmental Dynamics 229: 695–702.
7. DiNangelo JR, Vaaavada TK, Cain W, Duncan MK (2001) Production of monoclonal antibodies against chicken Pop1 (BVES). Hybrid Hybridomics 20: 377–381.
8. Osler ME, Bader DM (2004) Bves expression during avian embryogenesis. Developmental Dynamics 229: 658–667.
9. Osler ME, Chang MS, Bader DM (2005) Bves modulates epithelial integrity through an interaction at the tight junction. Journal of Cell Science 118: 4667–4678.
10. Osler ME, Smith TK, Bader DM (2006) Bves, a member of the Popeye domain-containing gene family. Developmental Dynamics 235: 586–593.
11. Vaaavada TK, DiAngelo JR, Duncan MK (2004) Developmental expression of Pop1/Bves. Journal of Histochemistry & Cytochemistry 52: 371–377.
12. Knight RF, Bader DM, Backstrom JR (2003) Membrane topology of Bves/Pop1A, a cell adhesion molecule that displays dynamic changes in cellular distribution during development. Journal of Biological Chemistry 278: 32072–32079.
13. Wada AM, Reese DE, Bader DM (2001) Bves prototype of a new class of cell adhesion molecules expressed during coronary artery development. Development 128: 2085–2093.
14. Nakada MT, Amin K, Christofidou-Solomidou M, O’Brien CD, Sun J, et al. (2000) Antibodies against the first Ig-like domain of human platelet endothelial cell adhesion molecule-1 (PECAM-1) that inhibit PECAM-1-dependent homophilic adhesion block in vivo neutrophil recruitment. Journal of Immunology 164: 452–462.
15. Thorson MA, Anastasiadis PZ, Daniel JM, Iretton RC, Wheelock MJ, et al. (2000) Selective uncoupling of p<sub>120</sub>(catalytic) from E-cadherin disrupts strong adhesion. Journal of Cell Biology 148: 189–202.
16. Andree B, Fleige A, Arnold HH, Brand T (2002) Mouse Pop1 is required for muscle regeneration in adult skeletal muscle. Molecular & Cellular Biology 22: 1504–1512.
17. Ripley AN, Osler ME, Wright CV, Bader D (2006) Xbves is a regulator of epithelial movement during early Xenopus laevis development. Proceedings of the National Academy of Sciences of the United States of America 103: 614–619.
18. Reese DE, Mikawa T, Bader DM (2002) Development of the coronary vessel system. Circulation Research 91: 761–768.
19. Hay ED (2003) The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. Developmental Dynamics 23: 706–720.
20. Brand T (2005) The Popeye domain-containing gene family. Cell Biochemistry & Biophysics 43: 95–103.
21. Nagafuchi A, Takeichi M (1988) Cell binding function of E-cadherin is regulated by the cytoplasmic domain. EMBO Journal 7: 3679–3684.
22. Ozawa M, Kemler R (1998) Altered cell adhesion activity by pervanadate due to the dissociation of alpha-catenin from the E-cadherin-catenin complex. Journal of Biological Chemistry 273: 6166–6170.
23. Ozawa M, Kemler R (1998) The membrane-proximal region of the E-cadherin cytoplasmic domain prevents dimerization and negatively regulates adhesion activity. Journal of Cell Biology 142: 1603–1613.
24. Araki-Sasaki K, Ohashi Y, Sasabe T, Hayashi K, Watanabe H, et al. (1995) An SV40-immortalized human corneal epithelial cell line and its characterization.[see comment]. Investigative Ophthalmology & Visual Science 36: 614–621.
25. Ripley AN, Chang MS, Bader DM (2004) Bves is expressed in the epithelial components of the retina, lens, and cornea. Investigative Ophthalmology & Visual Science 45: 2475–2483.