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Identification of a novel Bves function: regulation of vesicular transport

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Initial Editorial Decision 29 May 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. I have now had an opportunity to read your manuscript carefully and I have also discussed it with an external Editorial Advisor with suitable expertise as well as my editorial colleagues and I am sorry to say that we cannot offer to publish the current study in The EMBO Journal.

We appreciate that you have identified VAMP-3 as a novel interaction partner of the cytoplasmic domain of Bves and show that the two proteins colocalise in polarized epithelial cells and in adherent tissues. Generation of MDCK cells that stably express a truncated version of Bves that cannot interact with VAMP-3 results in defects in transferrin receptor and integin internalization. In vivo, depletion of Bves by morpholino results in defects in integin dependent cell movement and polarity during gastrulation, which can be rescued by addition of a morpholino resistant Bves mRNA. Loss of Bves also affects cell adhesion and spreading on fibronectin.

After discussing this manuscript with an Editorial Advisor we have come to the conclusion that this is a potentially very interesting study. However, we find that at the current stage it is rather preliminary and we would require some further insight into how Bves affects VAMP-3 localization, function and a better description of its affect on integrin recycling. Therefore, if you are able to perform Bves siRNA knockdown experiments and quantitatively analyze the effects on integrin recycling (and assuming they have the expected outcome) we would be willing to send the manuscript out for review. However, unfortunately at this stage we find that further insight is required before we can further consider the manuscript for publication in the EMBO Journal.

Please note that we publish only a small percentage of the many manuscripts that we receive at the EMBO Journal, and that the editors have been instructed to only subject those manuscripts to external review which are likely to receive enthusiastic responses from our reviewers and readers. As in our carefully considered opinion, this is not the case for the present submission, I am afraid our conclusion regarding its publication here cannot be a positive one. I am sorry to have to disappoint you on this occasion.

Yours sincerely,

Editor
The EMBO Journal

Author's response 02 June 2009

Thank you for your input. The associate editor and you have suggested appropriate experiments and ones that we are in the process of conducting. We think these are perfectly reasonable additions to the study and would like to include if the experiments work and are insightful. I have to tell you that we have tried siRNA for several years on Bves (we discovered this gene) and have never gotten reasonable or reproducible knockdown results. We would like to conduct the suggested study but we have to tell you that the siRNA knockdown is not feasible at this time.

We would like to conduct the other suggested experiments and resubmit without the siRNA studies. Would it be possible to discuss this possibility with the associate editor? If this is not acceptable, we would have to move to another journal. We do want to publish in EMBO Journal and feel that the study is up to its high standards and would be interesting to its wide readership.

Thanks for your help in these matters.

Editor's reponse 05 June 2009

Thank you for your email, I have had an opportunity to discuss your letter with the editorial advisor and we have both come to the same conclusion that while the study is potentially interesting it is too preliminary to be further considered for the EMBO Journal without the knockdown experiments. I am sorry that we can not be more positive at this stage.

Author comment prior to resubmission 02 July 2009

Thank you for your previous email explaining the importance of presenting knockdown data to support the role of Bves in vesicular transport. We appreciate your interest in our work and took your comments seriously. We also agreed ith the comments that use of a second knockdown method of protein disruption was necessary to conclusively demonstrate Bves regulation of VAMP3-dependent vesicular transport. Therefore, we worked to produce a system where Bves function could be eliminated without expression of mutated forms of the protein.

Because no one studying Bves function has successfully used and reported siRNA knockdown of Bves, we have developed a new model system to study vesicular transport after Bves knockdown. In our previous work (Ripley et al, PNAS, 2006), we reported that Morpholino treatment of X. laevis at the two cell stage results in elimination of Bves protein in the developing embryo. With this information, we eliminated Bves from the developing embryo, isolated animal caps from these individuals, and developed a new in vitro assay to measure transferrin recycling. With new data gathered in response to your comments, we now show that
transferrin recycling is severely inhibited in these animal cap assays after Morpholino knockdown of Bves protein (new Figure 4, new Table I, and page 8). Importantly, expression of an engineered RNA missing the Morpholino target sequence completely rescues receptor recycling in Morpholino-treated animal caps.

These data provide the knockdown information you requested and greatly strengthen the finding that Bves regulates vesicular transport. We feel that with these new and highly supportive data, the study

is ready for review and will have significant impact for the broad readership of EMBO Journal.

1st Editorial Decision 22 July 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. Two referees, whose reports I enclose below, have evaluated your manuscript. As you will see from their comments the referees express potential interest in the role of the Bves-VAMP3 interaction and its effect on transferin uptake and integrin recycling and request some further experimental analysis to make the study suitable for publication in the EMBO Journal.

As you will see from the reports referee #1 finds that the current study preliminary and requires a more thorough analysis, referee #2 also raises several of these concerns. These include amongst other things further analysis of the localization data and how loss of Bves and VAMP3 affect the localization of the other protein. S/he also requires a more side-by-side comparison of the loss of Bves and Vamp3 on transferin uptake and integrin recycling and the effect of VAMP3 deletion in embryos. These issues should be addressed in the revised version of the manuscript, should you be able to address these criticisms, we could consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The manuscript demonstrates that Bves interacts with VAMP3 and it is suggested that via this interaction Bves plays a decisive role in the regulation of vesicle transport, thereby contributing to transferrin receptor uptake and integrin recycling.
Although the basic observation is potentially interesting the manuscript is in a very preliminary state and the analyses remain rather superficial. A main problem is that the data switch between two model systems: MDCK cells and Xenopus embryos. Most aspects are demonstrated in one system but not in the other and therefore it is difficult to assess if the data are conclusive. It is important to thoroughly test the findings in both model systems to assure that the findings are solid.

Major points:

1) Bves and VAMP3 co-localize
This is demonstrated in MDCK cells transfected with VAMP3 GFP and immunostained for Bves. As overexpression and GFP tagging might cause artefacts it would be desirable to see the localization of both endogenous proteins. Further it is claimed that both proteins localize in the basolateral areas. From the figure it rather appears that Bves is excluded from the basal area and that co-localization is apical. Analyzing confluent MDCK monolayers instead of non-confluent ones might be illustrative as these become more cuboidal and allow better distinction of compartments. It would also be essential to probe the interaction of Bves and VAMP3 more in the MDCK system. How does knockdown of VAMP3 affect Bves localization and vice versa. Does re-introduction of Bves that cannot bind VAMP3 lead to a dissociation of both molecules?

2) Bves affects transferrin uptake

It would be helpful to display the data from table I as a graph instead of showing the data in Fig. 4B. In addition VAMP3 should be knocked down and transferrin uptake measured to allow a side by side comparison.

3) VAMP-3 mediated integrin recycling is impaired in cells with mutant Bves
Again, displaying the quantitative data in a graph would be helpful.
Further, knockdown of Bves and VAMP-3 should be performed side by side and the integrin internalization should be quantified in both situations. It is also important to show how after Bves knockdown and or re-expression of the VAMP interaction deficient mutant the expression levels of integrins are affected.
Furthermore, wound healing assays should be performed with the same cells to see if impaired integrin recycling caused by Bves knockdown/mutation and VAMP3 knockdown affects migration of the cells.
It would also be important to know if the vesicles containing the internalized integrins are positive for Bves and VAMP3.

4) Defects in Bves depleted embryos
Once more, a side by side comparison with VAMP3 inhibition would be required to directly compare the phenotypes. Also the integrin expression levels of these cells should be measured and integrin recycling should be tested in these same cells (instead of only showing defective recycling in MDCK cells that are not tested for migration).

5) Defective spreading on FN
The data on cell spreading are poorly documented. It is important to display the dynamics of spreading in a way that can be evaluated by the reader. In example kymographic analyses of the spreading cells in differential interference contrast optics as seen in publications by Sheetz MP would be helpful.

Referee #2 (Remarks to the Author):

In this article the authors find that VAMP3 interacts with the transmembrane protein Blood vessel epicardial substance (Bves). They demonstrate that Bves regulates transferrin, integrin recycling and cell migration in vivo and gastrulation in Xenopus.
This is new finding demonstrating the role of Bves in membrane trafficking through the regulation of VAMP3.
The paper presents results of high importance in the field because it connnects a v-SNARE with a protein of importance during developement. I am only concerned by the lack of controls and details of some experiments and I think that the following questions need to be answered:
1-does Bves interact with other v-SNAREs? a control experiment is needed, particularly because VAMP3 KO mice do not show a developmental defect,
2-a true transferrin recycling experiment is needed because VAMP3 mediates the exocytosis part of the cycle,
3-does the lack of Bves block VAMP3 in a inadequate localization like it was shown in the case of BAP31?
4-how does the depletion of Bves compare with that of VAMP3 in Xenopus development?

1st Revision - authors' response 16 October 2009

We would like to thank the editors and reviewers for their support of our studies revealing that Bves interacts with SNARE protein, VAMP3, and regulates vesicular transport. We also want to thank the reviewers for their very good suggestions to improve the manuscript. As you will see, we have conducted all possible experiments suggested by both reviewers. We would like to note that we have produced 18 new figures (text figures 6 and 8; supplemental figures 1, 2, 4-12, and 14-18) and modified four of the original text figures (2, 4, 5 and 7) in this revised manuscript in response to the reviewers' comments. Only two points, subparts of larger comments, were not possible due to technical problems and are noted below. The revised manuscript has been greatly strengthened by

the work we did in response to both reviewers' comments. These are the specific responses we have made:

Reviewer 1:

A global comment from the reviewer, *A main problem is that the data switch between two model systems: MDCK cells and Xenopus embryos. Most aspects are demonstrated in one system but not in the other and therefore it is difficult to assess if the data are conclusive. It is important to thoroughly test the findings in both model systems to assure that the findings are solid.*

After considering this statement, we completely agreed with the reviewer. As seen in the revised manuscript, we have worked to conduct new experiments using both model systems for Bves and VAMP3. All new data using the MDCK cell and Xenopus laevis model systems consistently demonstrate Bves regulation of VAMP3-dependent processes and strengthen are overall argument for a role in vesicular transport. Additionally we compared VAMP3 function in all cases in side-by-side analysis, except for integrin uptake in early Xenopus embryos, where this was impossible for technical reasons. Our work and specific responses for this reviewer are outlined below.

1) *Bves and VAMP3 co-localize.* (We respond by breaking this into subparts.)

A. This is demonstrated in MDCK cells transfected with VAMP3 GFP and immunostained for Bves. As overexpression and GFP tagging might cause artefacts it would be desirable to see the localization of both endogenous proteins. Further it is claimed that both proteins localize in the basolateral areas. From the figure it rather appears that Bves is excluded from the basal area and that co-localization is apical. Analyzing confluent MDCK monolayers instead of non-confluent ones might be illustrative as these become more cuboidal and allow better distinction of compartments. It would also be essential to probe the interaction of Bves and VAMP3 more in the MDCK system.

This is a good comment; our data needed clarification. First, we now present localization studies on the endogenous proteins in confluent MDCK cell sheets in response to this comment (Figure 2). These images demonstrate the colocalization of Bves and VAMP3 in intracellular compartments and at the lateral surface of the cell membrane. The reviewer is correct, Bves is found on the lateral cell membrane in epithelia and is seen circumferentially in smooth, cardiac and skeletal muscle (Figure 3). We have added statements in the results clarifying this finding (page 6, lines 21-23; page 7, lines 2-7). We would note that we used four different commercially available antibodies against VAMP3 and while all of them showed staining at the membrane, none of them had extremely high affinity for the protein at this subcellular location. Thus, there is a difference in detection of endogenous VAMP3 when compared with the transfected gene protein.

B. *How does knockdown of VAMP3 affect Bves localization and vice versa. Does re-introduction of Bves that cannot bind VAMP3 lead to a dissociation of both molecules?*

Another good point. Thus, we examined whether "knockdown" of VAMP3 resulted in changes in Bves localization. We used the established MDCK model of inhibition of VAMP3 (Proux-Gillardeaux et al, 2005, PNAS) to examine this relationship. We show in new Supplemental Information Figure 2 that Bves localization at the cell membrane is greatly reduced with loss of VAMP3 function (described in the text on page 6, lines 25-28). Inhibition of Bves function appeared to result in similar loss of VAMP3 from the cell membrane but these images were not as consistent or as striking as that seen with disruption of VAMP3 activity. This may be due to the less avid reaction of the commercially available antibodies for VAMP3 in identifying the protein. We hope to further explore this relationship in the future.

2) *Bves affects transferrin uptake. It would be helpful to display the data from Table I as a graph instead of showing the data in Fig. 4B. In addition VAMP3 should be knocked down and transferrin uptake measured to allow a side by side comparison.*

In response to this valid criticism, we determined the effect of Bves and VAMP3 inhibition on transferrin uptake in new side by side analyses using the Xenopus laevis model system. We chose this system because this model enabled us to conduct a rescue experiment, which demonstrates the specificity of protein knockdown and effect on transferrin recycling. As seen in Figure 4, disruption

of Bves and VAMP3 function results in inhibition of transferrin uptake as compared to control Morpholino injected cells. Importantly, uptake is restored with expression of Bves and VAMP3 rescue mRNAs that are not inhibited by the Morpholino (Figure 4B). Thus, these new side by side experiments strongly support a role for Bves in VAMP3-mediated vesicular transport. This new data is displayed as a graph in Figure 4B, with the quantitative data in Table I. The results are described in the text on page 7, line 25 to page 8, line 12.

3) *VAMP-3 mediated integrin recycling is impaired in cells with mutant Bves*

A. *Again, displaying the quantitative data in a graph would be helpful. Further, knockdown of Bves and VAMP-3 should be performed side by side and the integrin internalization should be quantified in both situations. It is also important to show how after Bves knockdown and or re-expression of the VAMP interaction deficient mutant the expression levels of integrins are affected.*

As suggested by the reviewer, we repeated the integrin internalization experiment in side by side analyses with inhibition of both Bves and VAMP3, using the exact method of VAMP3 knockdown as previously reported in the initial assay by Proux-Gillardeaux et al (2005, PNAS). As seen in modified Figure 5, new Supplemental Information Figure 6, and Table II, inhibition of both Bves and VAMP3 function led to significant loss in integrin uptake. The loss of integrin uptake with inhibition of VAMP3 function agrees completely with the published work of Proux-Gillardeaux and serves as a highly important control. Loss of Bves function phenocopies the VAMP3 situation and strongly supports a role for Bves in VAMP3-mediated integrin internalization. As requested, we measured integrin levels and despite decreased integrin uptake levels with Bves inhibition, integrin expression levels remain the same, as shown by western blot in Figure 5H, as also described previously by Proux-Gillardeaux et al (2005). We have presented these data in graph form (Figure 5G) as requested by the reviewer. We describe the results on page 8, line 13 to page 9, line 5. Thanks to the reviewer for suggesting these studies.

B. *Furthermore, wound healing assays should be performed with the same cells to see if impaired integrin recycling caused by Bves knockdown/mutation and VAMP3 knockdown affects migration of the cells.*

We conducted wound healing assays with MDCK and Bves-inhibited MDCK cells (Bves118 cells) side by side with wild-type and mutated Tetanus toxin controls (WT TeNT and mut-TeNT, respectively) to determine the effect on cell migration. We found that this experiment was extremely complex for technical reasons and found that the results were not convincing enough to report here. The technical difficulties were that inhibition of Bves function disrupts cell-cell junctions in epithelial sheets and impairs Rho GTPase activity, which would have unknown affects on this particular assay. This was reported in work from our laboratory (Osler et al, 2005, Journal of Cell Science; Ripley et al, 2006, PNAS; Smith, Hager et. al., 2008, PNAS). The critical role of cell junctions and RhoGTPases in epithelial integrity and movement has been reviewed (Etienne-Manneville, 2008, Oncogene; Ridley et al., 2003, Science). Given the limitations and complications of this experimental model of migration and that analysis of cell spreading provided a more simple and highly controlled paradigm in which to test Bves function in an integrin-mediated process, we focused our analyses on single cell models of spreading (new Figures 6 and 8 and new Supplemental Information Figures 7-11 and 14-18; see below).

C. *It would also be important to know if the vesicles containing the internalized integrins are positive for Bves and VAMP3.*

This is an excellent suggestion. Using scratch injury of wildtype MDCK cells as described in the methods, we assayed the distribution of Bves, VAMP3 and internalized integrins. In new Supplemental Information Figure 5, we show that vesicles containing internalized integrins are also positive for Bves and VAMP3. These studies are described on page 9, lines 1-2. These new data with our functional studies on the regulation of integrin internalization strongly indicates a role for Bves in this process.

4) *Defects in Bves depleted embryos.*

A. *Once more, a side by side comparison with VAMP3 inhibition would be required to directly compare the phenotypes.*

Again, this is a great comment. We conducted extensive analyses of X. laevis development after knockdown of VAMP3 function (new Supplemental Information Figure 12 with accompanying Graph). We constructed new Morpholinos for VAMP3 and injected these translation-blocking agents into both cells of two cell X. laevis embryos. VAMP3 knockdown embryos were examined at the same stages as Bves-depleted embryos, as suggested by the reviewer. As seen with these new data, inhibition of VAMP3 function reveals defects in early development seen with Bves knockdown characterized by delay in blastopore closure (Supplemental Information Figure 12A and B; described on page 10, lines 3-7). It is interesting that both Bves and VAMP3 depleted embryos display similar defects during gastrulation, as this is when integrin-mediated adhesion is very important for migration across the blastocoel roof and subsequent blastopore closure (Marsden et al., 2001, Development). Furthermore, isolation of these migrating mesoderm cells reveals decreased cell adhesion over time in both Bves and VAMP3 depleted embryos (see below, new Figure 8 and Supplemental Information Figures 14-18; described on page 11, line 7 to page 12, line 20), indicating these molecules play a crucial role in integrin-mediated adhesion. Additionally, disruption of Bves and VAMP3 in X. laevis cells also inhibited transferrin uptake as noted above (Figure 4). Finally, it should be noted that Bves-depleted embryos had a more severe gross morphological phenotype when compared to VAMP3 depleted embryos. This maybe due to expression of VAMP2 at this stage (EST databases), whereas Bves is the only homologue expressed during early X. laevis development. Taken together, these experiments provide strong corroborating data for our in vitro findings.

Later in development, some variation between Bves and VAMP3 inhibition is observed as noted in Figure 7 and new Supplemental Information Figure 12 respectively. This is described on page 10, lines 17-20. This later variation is not unexpected due to non-overlapping functions in more mature organisms. Obviously, extensive analysis of the later VAMP3 phenotype in developing frog embryos and tadpoles will tell us more about the role of this protein in embryogenesis and is of great interest to us for future research. Clearly, detailed analysis of these phenotypes goes beyond the scope of this study and would require extensive documentation that would exceed the space limitations of the EMBO Journal. Overall, these new data are highly supportive of a conserved function of Bves and VAMP3 in X. laevis development.

B. *Also the integrin expression levels of these cells should be measured and integrin recycling should be tested in these same cells (instead of only showing defective recycling in MDCK cells that are not tested for migration).*

Good point. As suggested by the reviewer, we conducted western blot analysis of integrin expression in these embryos and found no change in integrin protein levels (Figure 7).

We were unable to complete experiments on integrin uptake in Xenopus laevis cells for technical reasons. We attempted to concentrate 8C8 antibodies from supernatants obtained from the Developmental Studies Hybridoma Bank. However, we were unable to purify sufficient amounts of IgG1 to directly label antibodies with a fluorophore. Next, we obtained hybridoma 8C8 cell lines and grew them in order to produce higher titers to purify the reagent. While we are in the process of purifying and labeling this reagent from supernatant, time does not permit further attempts to produce and characterize the reagent. After production of a newly conjugated antibody, repeated experiments would be needed to produce conclusive data. Production of ascites with cells was a viable alternative but could not be completed in the three months given to resubmit the manuscript. David Bader has an extensive track record of producing and analyzing monoclonal antibodies (beginning with Bader et al, 1982, J. of Cell Biology). Thus, we believe our methods to produce the reagent were properly conducted and we made a "good faith" effort.

5) *Defective spreading on FN. The data on cell spreading are poorly documented. It is important to display the dynamics of spreading in a way that can be evaluated by the reader. In example kymographic analyses of the spreading cells in differential interference contrast optics as seen in publications by Sheetz MP would be helpful.*

This is a great comment. The reviewer's criticism spurred us to examine the literature concerning analysis of cell spreading in general and specifically the studies of Sheetz and colleagues. We then conducted quantitative studies of cell spreading on FN using kymographic analyses. Additionally taking into account the reviewer's general observation that studies should be conducted using both model systems, we used both X. laevis and MDCK cells with and without inhibition of VAMP3 and Bves function (i.e. in side by side studies with controls). The result was that inhibition of Bves and VAMP3 in both systems leads to inhibition of cell spreading and/or maintenance of the spread phenotype. Specifically, inhibition of Bves and VAMP3 function in X. laevis head mesoderm cells results in loss of the maintenance of cell spreading. This is visualized with kymographs of live cells in new Figure 8, as well as new Supplemental Information Figures 14-18. Also, we used membrane-targeted GFP and RFP in experimental and control cells respectively to analysis the behavior of cells in the same dish (i.e. side by side; see Supplemental Information Figure 17). The text describing the methods (page 19, line 19 to page 20, line 9) and the analysis of the experiments and their interpretation is given on page 11, line 7 to page 12, line 20. These new data quantitatively demonstrate that both Bves- and VAMP3-inhibited cells have fewer lamellipodia as compared to control cells and have a reduced area of contact with the FN matrix.

Next, we conducted kymographic analysis of MDCK cells inhibited in both VAMP3 and Bves function along with their controls. In new Figure 6 and new Supplemental Information Figures 7-11, inhibition of VAMP3 and Bves function disrupts cell spreading. This is described on page 9, lines 6-25. These "side by side" morphometric studies using both systems as suggested by the reviewer provide very strong support for the hypothesis that Bves function is essential for integrin-dependent cell spreading on FN through interaction with VAMP3.

We would like to note that we had not considered kymographic analysis until the reviewer made her/his comment. This is a case where a reviewer's input really did improve the study significantly. As authors, we were taken with how the analyses unfolded and supported our central hypothesis.

Reviewer 2:

We thank the reviewer for her/his comments and for pointing out where necessary controls should be added and where additional data should be collected. We feel our responses to this reviewer's comments have greatly improved our studies.

1) *Does Bves interact with other v-SNAREs? a control experiment is needed, particularly because VAMP3 KO mice do not show a developmental defect*

This is an excellent point. We conducted new experiments to determine whether Bves interacts with other VAMPs. As seen in new Supplemental Information Figure 1 (described on page 6, lines 10-13), we show that Bves does indeed interact with VAMP2. Also, we have added a comment to the Discussion concerning the milder phenotype observed with VAMP3 knockdown in Xenopus embryos as compared to the Bves phenotype (page 15, lines 12-27), which may be explained by similar overlapping functions of VAMP proteins.

2) *A true transferrin recycling experiment is needed because VAMP3 mediates the exocytosis part of the cycle.*

Good point. We conducted a recycling assay using MDCK cells to determine if exocytosis is impaired with Bves disruption. We report in new Supplemental Information Figure 4 that Bves 118-expressing cells have reduced exocytosis of the transferrin receptor as compared to wild-type cells. This is presented on page 7, lines 21-23.

3) *Does the lack of Bves block VAMP3 in a inadequate localization like it was shown in the case of BAP31?*

This is a good question. We conducted new studies to determine whether expression of Bves 118 changed the localization of VAMP3. While we found that while Bves 118 expression inhibits VAMP3-mediated functions, intracellular distribution of the protein is not greatly affected. The converse experiment (inhibition of VAMP3 function) does reduce Bves localization at the cell

membrane (response to Reviewer 1, comment 1). As noted by the reviewer, expression of a truncated form of BAP31 inhibits the anterograde movement of specific proteins from the ER to the Golgi such as VAMP3/cellubrevin but allows the passage of other proteins such as the transferrin receptor (Annaert et al, J. Cell Biology, 1997). Thus, while expression of Bves 118 clearly inhibits VAMP3 function, this inhibition is not through an overt disruption of anterograde movement of the protein to the cell membrane.

4) *How does the depletion of Bves compare with that of VAMP3 in Xenopus development?*

Great point and one reiterated by reviewer 1. Please see our response to reviewer 1, point 4 as this is the same question.

2nd Editorial Decision 06 November 2009

Your revised manuscript has been reviewed by one of the original referees. Pending satisfactory minor revision, we are happy to publish your manuscript in the EMBO Journal.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.
When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

1) As Bves has been shown to activate Rac and Cdc42 via binding GEFT it remains somewhat unclear how much of the observed migration phenotype is caused by decreased Rac and consecutive increased Rho activity or by VAMP dependent impaired integrin recycling (presumably Bves 118 does also not bind GEFT). A strong contribution by increased Rho and decreased Rac activity seems especially likely as the cells in the movies undergo massive blebbing (this should be mentioned in the results section). This issue should be more explicitely and critically discussed in the discussion.

2) Integrin beta 1 is abbreviated inconsistently in the text

3) The figure legends rather interpret the results than describe the experiments. I think this should be changed.

3rd Revision - authors' response 11 November 2009

Please find appended our revised manuscript entitled, "Identification of a novel Bves function: regulation of vesicular transport." We have amended the text as recommended by the reviewer. Specifically, we have discussed the potentially overlapping roles of VAMP3 and GEFT and their downstream signal cascades in relation to Bves function (pg 15, line 31; pg 16, lines 1, 12-17). We have also revised the text so that -1 integrin is consistently abbreviated and have taken out interpretive sentences from the figure legends (pg 21, lines 1-2, 6, 16, 31; pg 22, lines 1, 4, 8-9, 18). These were all good comments and we appreciate the suggestions made by the reviewer. If you have

any questions for us, please feel free to contact me. Thank you again for your time and consideration in these matters.

