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The SNARE protein Vti1a functions in dense-core vesicle biogenesis

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(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.)

Editor: Andrea Leibfried

1st Editorial Decision 07 January 2014

Thank you for submitting your manuscript entitled 'Vti1a functions in dense-core vesicle biogenesis'. I have now received the three reports on your paper. Please excuse again the delay in getting back to you.

As you can see below, all three referees appreciate your data and conclusions very much. They propose some amendments and additional experiments to support your data and claims, all of which are clearly outlined in the reports. Referee #1 also raises the important point that it currently remains unclear how a TGN SNARE mediates granule biogenesis. I do not know what kind of data you might already have at hand to address this point, but we would very much appreciate additional insight into this regulation.

Given the clear comments provided, I would like to invite you to submit a revised version of the manuscript, addressing the concerns of the referees. Please do not hesitate to contact me in case of further questions.

I should also add that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to address the concerns raised at this stage.

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, please visit our website:
http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE REPORTS

Referee #1:

This paper addresses in one of the best systems to study exocytosis, the chromaffin cell, the role of the SNARE protein Vti1 in calcium-induced secretion. The paper convincingly demonstrates that Vti1 is not on chromaffin granules and is not directly involved in exocytosis which are important findings given the confusion in the field about the role of Vti1 in synaptic vesicle exocytosis. The paper also demonstrates that the biogenesis of chromaffin granules is impaired in Vti1a-deficient chromaffin cells, and that this phenotype is not further aggravated by the additional deletion of the other Vti1 isoform, Vti1b.

Overall, these are important findings and in my opinion the paper is technically excellent and biologically significant, rendering is eminently suitable for EMBO J. My only criticism regards the definition of the precise nature of the phenotype in Vti1a-deficient chromaffin cells. I did not see a compelling co-localization of Vti1a with any particular marker in the data shown in the paper. I think the authors are probably right that Vti1a is localized to the TGN, but it would be helpful if this was documented further. It would also be interesting, even important, to gain insight into why a TGN SNARE should be invovled in chromaffin granule biogenesis. Is a fusion step involved in this biogenesis, and do deficiencies in other SNAREs have a similar phenotype? Does the Vti1 deletion also impair biogenesis of other secretory organelles in the TGN? What is the fusion step involved? It would make this paper even more interesting and important if the authors could provide some hints about the underlying biology here, especially if they were able that an as yet uncharacterized membrane fusion event is required for chromaffin granule biogenesis.

Finally, a minor criticism - I think the abstract could be formulated a little more crisply and more concretely describe the secretory defect in the Vti1a-deficient chromaffin cells.

Referee #2:

Previous studies have shown a role of vti1a in synaptic and Glut4 vesicles trafficking in neurons and 3T3-adipocytes, respectively. Walter and colleagues now provide a comprehensive analysis of vti1a function in adrenal chromaffin cells. They demonstrate that vti1a is largely absent from VAMP2-containing structures, representing LDCVs, but is enriched in the TGN area. Cells lacking vti1a show reduced exocytosis of LDCV. The data suggest that vti1a is involved in the formation/maturation of secretory vesicle, but not in the vesicle priming or fusion processes at the plasma membrane. Although these results are to some degree confirmatory, the paper contains several findings, which will be of interest in the field of membrane trafficking further clarifying transport step-specific vti1a functions. Interestingly, the absence of vti1a not only affects the LDCV number but also the vesicle size, which may indication a role of vti1a in vesicle-vesicle fusion. In contrast to vti1a, vti1b does neither affect LDCV vesicle trafficking nor LDCV size. The phenotype of the double vti1a/vti1b knock out seems to be largely similar to the single vti1a null, indicating that both vti1 isoforms have distinct functions.

Specific issues:

Based on the immunofluorescence analyses the authors report reduced syntaxin-6 levels. It would be helpful to confirm this reduction by Western blot analysis and to include other SNARE proteins (VAMP2, 3, 4, syntaxin 16, SNAP-25 homologues ....) to test for potential compensatory mechanisms (SNARE overexpression), which may accompany the vti1a knock out. For example, to ensure the survival of vti1a -/- mice other SNAREs may at least partially compensate the transport defects caused by the lack of vti1a. Thus, a vti1a knockdown in chromaffin cells using siRNA or shRNA in a cell culture system may actually show a more dramatic phenotype and eliminate potentially compensatory mechanisms.

Although, the localization of Vti1a to the TGN is obvious, the high background signal (speckles) may preclude a detection of Vti1 on LDCVs. Is the antibody sensitive enough to allow the detection of Vti1a on vesicles? Figure 2A shows only a few bright vti1a spots. However, such spots are also present on in Figure 2B (vti1a knock out). Does Figure 2C indicate that Vti1a is in general absent from vesicles (at least at the cell's foot print area)? It would be helpful to further substantiate the presence vesicular structures positive for vti1a. (Despite of this potential limitation of the localization studies, the functional experiments clearly indicate that Vti1a is not directly involved in the fusion process at the plasma membrane.)

Surprisingly, the vti1a null also results in reduced sodium and calcium currents, which may be explained by trafficking defects of these ion channels or other components. Did the authors further investigate this finding?

The authors observe a reduction of LDCV diameter and a reduced syb-2 staining. Are these reductions directly proportional or does it result in a change of surface density of syb-2 on the vesicle.

Compared to the vti1a knock out, the double vti1a/vti1b double knock out shows rather a minute reduction in the amperometric current (compare figures 4A and 7Ai). The authors should comment on this result.

The authors' argument that a long-term expression (2 days) of vti1a, required to rescue regulated exocytosis, suggests an involvement of vti1a in an upstream step in the secretory pathway is unclear and should be further explained. (A direct comparison of the membrane protein vti1a, which has to travel through parts of the secretory path to reach its functional destination and the cytosolic Rab3 proteins might not be appropriate).

Scale bars are missing in figures 6C, 7Bi, 7Biii, and 8A.

Referee #3:

The authors analyze dense-core vesicle release in chromaffin cells lacking the endosomal SNARE vti1a. This molecule has been long thought to be involved in homotypic endosomal fusion, or in fusion of endosomes or endosome-derived vesicles to the Golgi apparatus. Several recent high-profile reports have claimed a role for vti1a in synaptic vesicle exocytosis, an issue which is now highly controversial: several groups claim the involvement of this molecule in neuronal exocytosis, while others deny it.
The current manuscript addresses this issue in detail, in dense-core vesicles. I found the manuscript clear, convincing, and thorough. I suggest its publication, pending correction of a few minor points.
1. The authors indicate several times that syntaxin 6 is a marker for immature secretory vesicles. This is not a view shared by many investigators. Syntaxin 6 is present in a variety of endosomes and participates in homotypic early endosome fusion, as well as in fusion of endosomes to the trans-Golgi. The authors should make this issue clearer in their manuscript. This issue should be dealt with carefully especially in the first section of Results.
2. The following phrase of the abstract is confusing: "CypHer-tagged synaptotagmin-1 antibody loading suggest that normal secretion in chromaffin cells can be maintained without recycling release machinery constituents, suggesting that vti1a participation in recycling does not cause the

secretory phenotype". I am now able to understand it, having read the entire manuscript. But I suggest that the authors clarify their meaning, indicating which machinery constituents they refer to.
3. The authors should add several phrases to the last paragraph of their Introduction, in which they clarify why the references on synaptic vesicle recycling are relevant for a study on chromaffin dense-core vesicles. This will be useful especially for readers from the hippocampal neuron/synaptic vesicle field.
4. The authors seem to largely ignore an extremely interesting issue, the lower size of the calcium currents in vti1a-deficient cells (Fig 3). They should at least discuss this issue in more detail.
5. I find the experiments involving the recycling of CypHer-coupled antibodies convincing. However, I encourage the authors to add a control experiment in which they incubate the cells with antibodies that have been pre-incubated with the antigenic peptide, and which therefore can no longer bind the epitope. Alternatively, they could use antibodies targeting a protein not present in chromaffin cells. This would convince the readers of the specificity of antibody uptake.
6. What do the experiments employing pH 5.5 solutions test (Fig. 6D)? The authors suggest that no change in fluorescence is reported, since the antibodies are already in acidified organelles. I can equally suggest that no change in fluorescence is seen, since the antibodies are in intracellular organelles, acidified or not, which are simply not affected by bathing the etracellular membrane of the cell with pH 5.5 buffers. The authors may want to clarify this issue.
7. One of the most interesting points of the manuscript is the need for long-term (but not short-term) expression of vti1a for compensating the vti1a-null phenotype. The authors may want to include this finding in their abstract.

1st Revision - authors' response 07 April 2014

Response to reviewers

We would like to thank all reviewers for their encouraging and constructive comments that have helped us improve the manuscript. To address the reviewers' concerns we worked on our text and figure arrangements. Furthermore, we added a number of experiments: we have further characterized vti1a-localization by co-staining with a classical TGN-marker, investigated expression levels of cognate SNAREs biochemically, quantified the levels of Ca2+-channels by immunostainings and fluctuation analysis and investigated the mechanism of antibody-coupled cyanine dye uptake. Our point-by-point responses are listed below.

Referee #1:
This paper addresses in one of the best systems to study exocytosis, the chromaffin cell, the role of the SNARE protein Vti1 in calcium-induced secretion. The paper convincingly demonstrates that Vti1 is not on chromaffin granules and is not directly involved in exocytosis which are important findings given the confusion in the field about the role of Vti1 in synaptic vesicle exocytosis. The paper also demonstrates that the biogenesis of chromaffin granules is impaired in Vti1a-deficient chromaffin cells, and that this phenotype is not further aggravated by the additional deletion of the other Vti1 isoform, Vti1b.
Overall, these are important findings and in my opinion the paper is technically excellent and biologically significant, rendering is eminently suitable for EMBO J. My only criticism regards the definition of the precise nature of the phenotype in Vti1a-deficient chromaffin cells. I did not see a compelling co-localization of Vti1a with any particular marker in the data shown in the paper. I think the authors are probably right that Vti1a is localized to the TGN, but it would be helpful if this was documented further.

We thank the reviewer for her/his positive feedback. The reviewer suggests to further study the precise sub-cellular localization of vti1a in chromaffin cells. Our tentative conclusion of a TGN-localization was based on the vti1a-positive staining of compartments adjacent to the cis-Golgi marker GM130. To some degree this indirect interpretation was due to limitations in the antibody-combinations we could use, as we relied on a vti1a rabbit antibody. To follow the reviewer's suggestion we tested a battery of TGN and vti1a specific antibodies and found a monoclonal mouse vti1a antibody that resulted in similar stainings as our rabbit antibody, and a rabbit TGN38 antibody, which gave positive staining in chromaffin cells (a number of TGN-antibodies did not yield good

stainings in chromaffin cells). We combined these antibodies in a double-staining. As it turned out, TGN38 did not co-localize with vti1a, but localized in a compartment next to it (Fig. 1D in revised manuscript). Likewise, TGN38 was localized next to syntaxin-6 staining (Fig. 1E in revised manuscript). Therefore, the only staining, which overlaps with vti1a is syntaxin-6, even though the reviewer is right that the colocalization is not perfect. Overall, based on this analysis and the co-staining with other markers for cellular organelles (Lamp1, Synaptobrevin, Chromogranin B (also added in revision, EFig. 1), GM130) we conclude that vti1a is localized at a syntaxin-6 positive compartment localized next to TGN38. Such a compartment has been described before: it is involved in the recycling of GLUT4 (Shewan et al, 2003) and recently PICK1 was found in the same compartment and shown to drive formation of vesicles in Growth Hormone producing cells (Holst et al, 2013). The compartment was referred to as a 'subdomain of TGN' (Shewan et al, 2003), or 'immature vesicles' (Holst et al, 2013). We prefer not to make a too strong statement about the immature vesicles, since this is controversial and would seem to require immuno-EM, but we think our data are consistent with the presence of such a specialized compartment for vesicle generation. We want to thank the reviewer for calling for the extra staining, which established that we are looking at this particular compartment.

It would also be interesting, even important, to gain insight into why a TGN SNARE should be invovled in chromaffin granule biogenesis. Is a fusion step involved in this biogenesis, and do deficiencies in other SNAREs have a similar phenotype? Does the Vti1 deletion also impair biogenesis of other secretory organelles in the TGN? What is the fusion step involved? It would make this paper even more interesting and important if the authors could provide some hints about the underlying biology here, especially if they were able that an as yet uncharacterized membrane fusion event is required for chromaffin granule biogenesis.

We appreciate the reviewer's suggestion to further investigate the vti1a-dependent fusion step responsible for the vti1a null phenotype. Indeed we would very much like to understand this better. As pointed out in our discussion, we speculate that vti1a-loss leads to a reduced efficacy of a membrane fusion step involved in LDCV biogenesis, leading to a reduction of vesicle number and a decrease in vesicle size and synaptobrevin content. Nonetheless, this fusion reaction is not fully abolished in vti1a nulls and we thus suspect compensation by other SNARE proteins, possibly by members of the SNAP25-family that could provide a Qb (and a Qc) SNARE motif. To investigate this reaction molecularly, we checked for possible up-regulation of known interaction partners or candidate proteins in the vti1a nulls which might indicate compensatory function. To this end we quantified the expression levels of syntaxin 16, SNAP23, -25, -29, -47 and VAMP4 by Western blot. These data are now shown in Figure 2 of our manuscript, but no changes in protein levels were detected.
We don't have more information about the fusion step, but we have now integrated our present work on vti1a with previous work from Sharon Tooze's laboratory on syntaxin-6 and immature vesicle fusion and recent work on PICK1 from two groups into one model for vesicle generation (Fig. 9D). This model also accounts for the finding that Vti1a requires long-term reexpression for rescue, whereas rescue of the knockouts of integral vesicle membrane proteins synaptobrevin-2/VAMP2 and synaptotagmin-1 – which are directly involved in fusion – is complete within a few hours. We would like to mention that although not included in the present manuscript, we have solid data to show that PICK1-rescue also requires days, which is consistent with this model (Pinheiro et al., ms under revision). The model is only tentative, but we think it might be helpful as an inspiration for future work.

Finally, a minor criticism - I think the abstract could be formulated a little more crisply and more concretely describe the secretory defect in the Vti1a-deficient chromaffin cells.

We thank the reviewer for pointing out this shortcoming and have worked to improve the abstract.

Referee #2:
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plasma membrane. Although these results are to some degree confirmatory, the paper contains several findings, which will be of interest in the field of membrane trafficking further clarifying transport step-specific vti1a functions. Interestingly, the absence of vti1a not only affects the LDCV number but also the vesicle size, which may indication a role of vti1a in vesicle-vesicle fusion. In contrast to vti1a, vti1b does neither affect LDCV vesicle trafficking nor LDCV size. The phenotype of the double vti1a/vti1b knock out seems to be largely similar to the single vti1a null, indicating that both vti1 isoforms have distinct functions.

We thank the reviewer for her/his encouraging remarks.

Specific issues:
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We agree with the reviewer that screening for compensation by upregulation of other SNAREs in the vti1a knockout is very relevant. To address this issue, we analyzed protein levels by Western blots from whole adrenals, including the adrenal cortex (dissecting out the adrenal medulla of glands from new-born animals was not possible). The blots were repeated 4 times (for each blot, we pooled glands from 5 animals of each genotype to get enough material), and we present example blots and quantification in the new Fig. 2Bi-ii. We tested the levels of syntaxin 6, syntaxin 16, SNAP23, -25, -29, -47, VAMP2/synaptobrevin2 and VAMP4/synaptobrevin4.

Western blot analysis confirmed the reduction of the vesicular SNARE protein synaptobrevin-2. In contrast, in the Westerns we did not find a reduction of the vti1a SNARE partner syntaxin 6, even though a clear reduction was seen by immunostaining. This might be due to the fact that for Western blot analysis whole glands (including the adrenal cortex) were used, whereas the immunofluorescence quantification was limited to chromaffin cells only. Therefore, the levels of syntaxin 6 (which is ubiquitously expressed) in the adrenal cortex might have dominated the signal in the Westerns and precluded the detection of subtle changes. In contrast, VAMP2/synaptobrevin-2 is only expressed in the adrenal medulla, making it easier to compare the experiments. No changes in protein levels were detected for the other proteins, indicating that there is no upregulation of these SNAREs in the absence of vti1a (with the caveat that most of these SNAREs would also be expressed in the adrenal cortex). We have now included these data. Because the reduction in syntaxin-6 was not found by Western blot, we have toned down the conclusions on this point.
The reviewer also suggested siRNA-knockdown of vti1a to study effects of vti1a-protein levels more acutely. Unfortunately this approach is difficult to apply in primary cultures of chromaffin cells, since transfection of these cells is very difficult and has to be done by viral infection, combined with the fact that we can only keep the cells in primary culture for 4 (sometimes 5) days. Since we find that several days of reexpression of vti1a is necessary to rescue the null, we would similarly expect that the knockdown would have to be fully established for several days for the phenotype to appear. This would be borderline possible/impossible within this short timeframe, because of the time lag when using viral infections and because the protein would have to turn over. Therefore, establishing efficient knockdown in these cells would take much longer than the timeframe of resubmission. Finally, previous studies in other cells have concluded that even under conditions of almost complete knockdown, the remaining (a few %) vti1a retained its functionality in homotypic endosome fusion, raising the concern whether the knockdown approach would work at all (Bethani et al, 2009). Given this previous study, we think that the use of a genetic knockout is an advantage of our investigation.

Although, the localization of Vti1a to the TGN is obvious, the high background signal (speckles) may preclude a detection of Vti1 on LDCVs. Is the antibody sensitive enough to allow the detection of Vti1a on vesicles? Figure 2A shows only a few bright vti1a spots. However, such spots are also present on in Figure 2B (vti1a knock out). Does Figure 2C indicate that Vti1a is in general absent from vesicles (at least at the cell's foot print area)? It would be helpful to further substantiate the

presence vesicular structures positive for vti1a. (Despite of this potential limitation of the localization studies, the functional experiments clearly indicate that Vti1a is not directly involved in the fusion process at the plasma membrane.)

To the first part of the question, regarding the sensitivity of the antibody: the line profiles shown in panel A (Figure 3A in the revised manuscript) already suggest that vti1a in these examples is largely absent from synaptobrevin-2 positive compartments. However, as the reviewer points out, it is hard to conclude whether vti1a is generally absent from synaptobrevin-2 positive LDCVs based on single images alone. One of the problems is that even in the absence of specific staining SIM images appear "speckled". This is an unavoidable artifact of the mathematical reconstruction algorithm. To address this issue quantitatively, we averaged single frames of centered LDCVs positive for Synaptobrevin-2. If there were an enrichment of vti1a on vesicles, one would expect that the signal in the vti1a channel should be enriched (together with the Synaptobrevin-2 signal) on the vesicles compared to the background. If, however, vti1a were not enriched on the LDCVs and the "speckled" signal was merely "noise" from the SIM reconstruction, the prediction would be that "speckles" should average out and the intensity in the vti1a channel should not be increased together with Synaptobrevin-2, but should rather be a flat distribution. The latter is exactly the case, as can be seen from Fig. 3C. Furthermore, the intensity of the vti1a signal in the wildtype (green line in Fig. 3C) is similar to the level in the knockout (Fig. 3D), suggesting that all the signal in the vti1a channel seen in Fig. 3C is unspecific. Based on this we think we can firmly conclude that vti1a is not enriched on synaptobrevin-2 positive LDCVs. We have tried to improve the description of the rationale for these experiments in the text.
We have now also done a co-staining between vti1a and Chromogranin-B, because it might be argued that all LDCVs should have chromogranin B, whereas the possibility exists (although unlikely) that some LDCVs might not have synaptobrevin-2. These stainings are shown in EFig. 1E, however also in these stainings we do not see colocalization between the LDCV marker and vti1a. We cannot rule out that vti1a might be present on other vesicular structures, which given the suggested involvement of vti1a in endosome fusion might be endosomes. However, given the scarcity of these vesicular-like structures in WT animals (Fig. 3A), and the occasional presence of similar stained structures in the vti1a null (Fig. 3B), this has not been investigated any further.

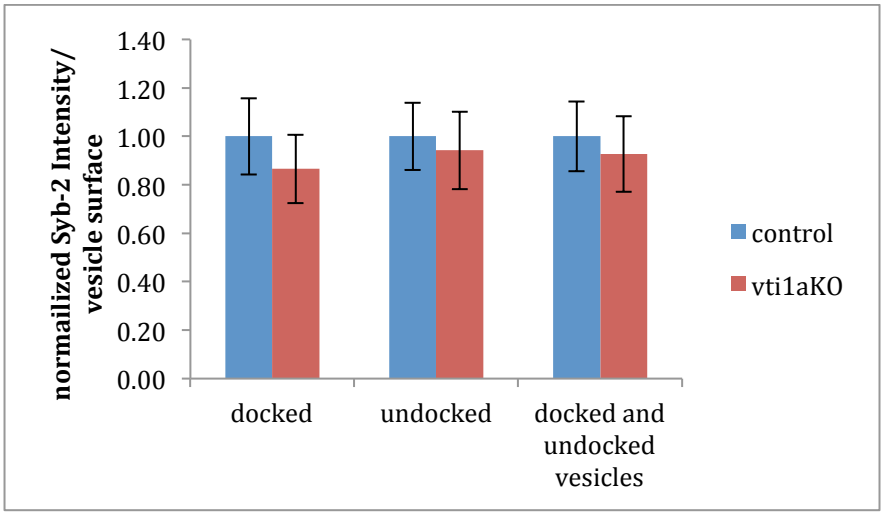
Surprisingly, the vti1a null also results in reduced sodium and calcium currents, which may be explained by trafficking defects of these ion channels or other components. Did the authors further investigate this finding?

At the time of submission we did not have any further information regarding this observation. Both reviewer 2 and reviewer 3 asked us to investigate this further and we therefore focused on the Ca2+ currents, since only these were significantly reduced. We hypothesized that the reduction seen could either be due to (1.) a change in Ca2+-channel activation, (2.) a decrease in the single channel conductance or (3.) a reduction in Ca2+-channel number. Only the latter hypothesis would be consistent with a trafficking defect of the channels themselves, whereas the two first hypotheses would be consistent with impaired trafficking of a component related to channel function.
We investigated this issue by two independent approaches. In the first approach we studied the properties of the Ca2+ currents in more detail using electrophysiology. Measuring the Ca2+ currents in response to increasing depolarization pulses, we could plot the fraction of open Ca2+ channels by investigating the tail currents (EFig. 2A). The activation of Ca2+ channels follows a sigmoid curve which can be fitted with a Boltzmann function to calculate the activation threshold of the Ca2+-channels. We found that wildtype and vti1a nulls had overlapping activation curves and indistinguishable activation thresholds, ruling out hypothesis 1. Next we wanted to address whether the single channel conductance or the number of channels was changed. To do this, we performed non-stationary fluctuation analysis on the activation of the Ca2+-currents (Fenwick et al, 1982). Fitting the variance-mean relationship with parabolas allowed us to estimate the single channel conductance and the number of channels. We found that single channel currents were not significantly different between vti1a nulls and controls but we observed a trend towards even larger currents in the knockout. In contrast, we found that the number of channels was significantly lower in vti1a nulls (EFig. 2B). To verify this finding by independent means, we performed live staining (on ice to prevent endocytosis) with an antibody recognizing the extracellular part of the α2δ4 subunit of all Ca2+-channel subtypes expressed in chromaffin cells. Quantitative confocal analysis revealed a highly significant reduction in the Ca2+-channel levels, in line with our electrophysiological analysis. These data are now shown as an additional panel (D) in Figure 4.

Based on our analysis we conclude that the reduction in Ca2+ currents in vti1a nulls is due to a reduction in the number of Ca2+ channels on the cell's surface, consistent with a trafficking defect. We thank the reviewers for encouraging us to investigate this phenomenon in more detail. We think these findings are important and we have therefore extended their discussion in the text and included this information in the abstract.

The authors observe a reduction of LDCV diameter and a reduced syb-2 staining. Are these reductions directly proportional or does it result in a change of surface density of syb-2 on the vesicle.

This is a very good point. We have addressed this question by calculating the per-area fluorescence intensity of the synaptobrevin-2 staining from the diameters determined from the EM micrographs assuming perfectly spherical vesicles. The reviewer is right; the decrease in synaptobrevin-2 intensities on vti1a null LDCVs scales with the reduction of surface area, demonstrated by near-identical per-area fluorescence intensities (area of the Gaussian AGauss from the fit in Figure 3 C and D divided by the calculated surface area of a vesicle Aves=π*d2 with d being the diameter of LDCVs determined in Fig. 6. The ratio= AGauss/Aves was further normalized to the control condition and the error was calculated using Gauss' formula for error propagation):



We have now added a statement in the results section to point this out. We think this is a quite interesting phenomenon and we thank the reviewer for this insightful remark.

Compared to the vti1a knock out, the double vti1a/vti1b double knock out shows rather a minute reduction in the amperometric current (compare figures 4A and 7Ai). The authors should comment on this result.

It is correct that the amperometric currents in these two particular datasets look different. However, these differences are due to different experimental settings. Amperometric measurements in uncaging experiments suffer from a light-induced artifact due to the photoelectric effect on the carbon fiber. The double knockout dataset is the only dataset that has been recorded with carbon fibers with a diameter of 10 µm. In these fibers the artifact is lager and therefore the difference between vti1a null and control cells seems smaller. We now point out in Extended Methods that these data were obtained with a different type of amperometric fiber.

The authors' argument that a long-term expression (2 days) of vti1a, required to rescue regulated exocytosis, suggests an involvement of vti1a in an upstream step in the secretory pathway is unclear and should be further explained. (A direct comparison of the membrane protein vti1a, which has to travel through parts of the secretory path to reach its functional destination and the cytosolic Rab3 proteins might not be appropriate).

The comparison to the Rab3A/B/C/D-knockout was not clear and has been removed. We have rewritten this section of the manuscript to clarify this statement. It is important to note that 8 hours of expression of the integral membrane vesicular proteins synaptobrevin-2 and synaptotagmin-1 are

sufficient for complete rescue of the respective knockouts, whereas vti1a requires long-term expression. As explained above (see answer to reviewer #1), we have now suggested a tentative model to account for these findings (new Fig. 9D). This model also summarizes previous work from Sharon Tooze's laboratory on syntaxin-6 and immature vesicle fusion and recent work on PICK1 from two groups. We consider the model only tentative at this point, but we hope it can serve to illustrate our point and inspire future work.

Scale bars are missing in figures 6C, 7Bi, 7Biii, and 8A.

We thank the reviewer for pointing out this mistake and have corrected the figures.

Referee #3:

The authors analyze dense-core vesicle release in chromaffin cells lacking the endosomal SNARE vti1a. This molecule has been long thought to be involved in homotypic endosomal fusion, or in fusion of endosomes or endosome-derived vesicles to the Golgi apparatus. Several recent high-profile reports have claimed a role for vti1a in synaptic vesicle exocytosis, an issue which is now highly controversial: several groups claim the involvement of this molecule in neuronal exocytosis, while others deny it.
The current manuscript addresses this issue in detail, in dense-core vesicles. I found the manuscript clear, convincing, and thorough. I suggest its publication, pending correction of a few minor points.

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1. The authors indicate several times that syntaxin 6 is a marker for immature secretory vesicles. This is not a view shared by many investigators. Syntaxin 6 is present in a variety of endosomes and participates in homotypic early endosome fusion, as well as in fusion of endosomes to the trans-Golgi. The authors should make this issue clearer in their manuscript. This issue should be dealt with carefully especially in the first section of Results.

We agree with the reviewer and have corrected the statements in the text dealing with Syntaxin-6. We now write that syntaxin-6 is present in TGN, immature vesicles and endosomes. In the discussion we now mention and discuss two papers (Cao et al, 2013; Holst et al, 2013), which both used syntaxin-6 as a marker for immature vesicles, but we hope the reviewer will agree that we deal carefully with this issue. For instance we mention that there is a need for immuno-EM to understand the nature of the compartment near the TGN harboring syntaxin-6.

2. The following phrase of the abstract is confusing: "CypHer-tagged synaptotagmin-1 antibody loading suggest that normal secretion in chromaffin cells can be maintained without recycling release machinery constituents, suggesting that vti1a participation in recycling does not cause the secretory phenotype". I am now able to understand it, having read the entire manuscript. But I suggest that the authors clarify their meaning, indicating which machinery constituents they refer to.

We have removed this statement from the abstract. Instead, we now mention that long-term expression of vti1a is needed for rescue, as suggested by the reviewer (see below).

3. The authors should add several phrases to the last paragraph of their Introduction, in which they clarify why the references on synaptic vesicle recycling are relevant for a study on chromaffin dense-core vesicles. This will be useful especially for readers from the hippocampal neuron/synaptic vesicle field.

We agree with the reviewer and hope that with our additions to the text our manuscript will be of interest to a larger audience. Given the current controversy regarding the role of vti1a in neurons, we thought we should mention it, but we have been very careful to distinguish between dense-core vesicles in adrenal chromaffin cells and synaptic vesicles throughout the manuscript.

4. The authors seem to largely ignore an extremely interesting issue, the lower size of the calcium currents in vti1a-deficient cells (Fig 3). They should at least discuss this issue in more detail.

To further investigate this point we have now done further experiments using live immunostaining and fluctuation analysis. We have added the results to the abstract and discuss the finding in the Discussion. For details, please see the response above to reviewer #2, who raised a similar point.

5. I find the experiments involving the recycling of CypHer-coupled antibodies convincing. However, I encourage the authors to add a control experiment in which they incubate the cells with antibodies that have been pre-incubated with the antigenic peptide, and which therefore can no longer bind the epitope. Alternatively, they could use antibodies targeting a protein not present in chromaffin cells. This would convince the readers of the specificity of antibody uptake.

We have now done the first experiment that the reviewer suggests: preincubation of the CypHer-coupled antibody with the antigenic peptide. The result is included in EFig. 4B and shows that most of the fluorescence is indeed blocked. The block is not complete, which is in agreement with the information we received from the supplier: even after incubation with the antigenic peptide, the antibody gives a faint band in Western blots.

6. What do the experiments employing pH 5.5 solutions test (Fig. 6D)? The authors suggest that no change in fluorescence is reported, since the antibodies are already in acidified organelles. I can equally suggest that no change in fluorescence is seen, since the antibodies are in intracellular organelles, acidified or not, which are simply not affected by bathing the etracellular membrane of the cell with pH 5.5 buffers. The authors may want to clarify this issue.

Yes, the reviewer is right, the previous interpretation was an over-simplification. We have now toned down the conclusion from this particular part of the experiment.

7. One of the most interesting points of the manuscript is the need for long-term (but not short-term) expression of vti1a for compensating the vti1a-null phenotype. The authors may want to include this finding in their abstract.

Yes, we have now done so.

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2nd Editorial Decision 25 April 2014

I have now received comments (see below) from two of the original referees on your revised manuscript who are both satisfied with the amount of revisions and support publication.
I am still waiting for our routine data check on your manuscript, but wanted to already tell you that your manuscript is in principle accepted for publication in the EMBO Journal. Please see below for important information on how to proceed.
Thank you for contributing to our journal!

Referee #2:
In the comprehensive and thorough revision, the authors have addressed all of my previous concerns in a satisfying manner.

Referee #3:
The authors have replied to all of my comments, and I am now satisfied with the manuscript. I believe it should be published in the EMBO Journal.
