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Triggered Ca^{2+} influx is required for extended-synaptotagmin 1-induced ER-plasma membrane tethering

Alice Lü, Beichen Xie, Pietro De Camilli and Olof Idevall-Hagren

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Editor: Andrea Leibfried

1st Editorial Decision 17 April 2015

Thank you for submitting your manuscript entitled 'Triggered Ca^{2+} influx is required for extended-synaptotagmin 1-induced ER-plasma membrane tethering'. I have now received reports of all three referees, which are enclosed below.

As you will see, while the referees consider that your study deepens our knowledge on the regulation of E-Syt1, they also raise several concerns and think that some of your conclusions are not sufficiently supported by the data provided. I won't list all concerns here, as all reports are very constructive and clear. Essentially, more insight and controls regarding the calcium-mediated regulation are required (see referee #1, point 1-3; referee #2, 3rd paragraph; see also referee #3), and the potential interdependence of SOCE related junctions and E-Syt1 mediated junctions should be addressed (see referee #1 and #2).

Given the constructive comments provided by the referees, I would like to invite you to provide me with a revised version of your manuscript, should you be able to substantiate your work along the lines suggested by the referees. The referees also note that further insight into the physiological function of E-Syt1-mediated tethers would be desirable (see reports from referee #1 and #3), and we agree with this view. I am certain that adding more functional insight will make your paper an outstanding one. Please let me know in case you want to discuss the necessary revisions further, and also if you foresee a problem in addressing all comments.

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

REFEREE COMMENTS

Referee #1:

The study by Igvall-Hagren et al. expands on previous studies from Dr. De Camilli's group regarding the plasma membrane recruitment of E-Syt proteins (1, 2, and 3). While the study elucidates some important aspects of E-Syt1 recruitment, such as the co-operativity in response to increases in [Ca2+]I and possible tuning of the [ca2+] sensitivity via heteromerization between the proteins, this important aspect has not been fully explored. Further, several major properties of the protein, including its physiological role and how that relates to the functions examined in the study, have not been clarified. My major concerns are as follows:

1. The data shown in the paper do not conclusively establish whether the [Ca2+] measurements carried out using the TIRF mode in permeabilized cells actually represent cytosolic [Ca2+]. What is the effect of SERCA inhibition in these experiments? These controls are required. Further, and more important, what are the changes in [Ca2+]i (measured using TIRF) in depolarized cells and BHQ stimulated cells in non-permeabilized state (i.e. experiments shown in Figs 4 and 5).
2. The authors refer to the Ca2+-add- back assay for SOCE as physiological, but actually it is not. In depolarization conditions where VGCC are activated, one can envision a large bolus of Ca2+ entry in cells such as pancreatic islets or neuronal cells since all channels are activated at the same time. On the other hand in the case of SOCE, there is low possibility of such sudden bolus of Ca2+ influx into cells. SOCE experiments should be carried out in the continued presence of Ca2+. What happens to E-Syt1, is it even recruited to the ER-PM junctions under these conditions? Further, physiologically SOCE is activated by very low agonist concentrations where [Ca2+]i oscillates and under these conditions, few Orai1-STIM1 channels are assembled.
3. The buffering experiments do not conclusively demonstrate whether local or global [Ca2+]i increase is required for translocation of E-Syt1 to the PM regions. The local [Ca2+]i increase via SOCE is detected by NFAT and this assay should be measured as a control to confirm buffering of local [Ca2+]i via SOCE channels, such as Orai1.
4. It is puzzling to see the loss of STIM1 from the ER-PM junctions following add back of Ca2+. It appears that in Figure 4, cells are incubated briefly with BHQ, which is then washed off, when Ca2+ is readded. So basically readdition of Ca2+ will allow the ER store to be refilled, which might be why STIM1 moves away from the periphery? As stores are filled SOCE is gradually inactivated and E-Syt translocates out of the junctions? It is not clear whether this is what the authors want to imply? A better approach would be to show the effect of 2-APB or BTP2 which inhibits SOCE without losing STIM1 puncta?
5. Similarly, E-Syt KD effect on SOCE needs to be assessed by adding Ca2+ back in the continued presence of BHQ or agonist, which will measure true SOCE. In the experiments shown in Fig 4K and Fig EV6, BHQ was removed thus allowing stores to refill, and the SOCE to inactivate.
6. It would add considerably to the importance of this study, if recruitment of E-Syt1 to the same site as STIM1 is shown to be physiological. Does the possible lipid transfer via ESyt1 have any consequence on SOCE or in the function/secretion in islets?
7. Finally it is unclear whether the authors mean to imply that there is competition between STIM1 and E-Syt for the same junctions? So, in the continued presence of STIM1 in puncta E-Syt1 cannot be recruited?

Referee #2:

The authors describe here the interaction of the extended synaptotagmin 1 (E-Syt1) with the plasma membrane. This molecule participates in the formation of endoplasmic reticulum-plasma membrane contacts, in a calcium-dependent fashion. The authors follow here on excellent previous work, which they have published on the subject recently (Giordano et al., Cell, 2013), and in which they have already introduced many aspects of E-Syt biology.
In this manuscript, the authors show that the binding (recruitment) of E-Syt1 to the plasma membrane requires relatively high calcium levels, which can be derived from store-operated calcium entry (SOCE), or from the response to depolarization of excitable cells. On its own, the release of calcium from intracellular stores is not sufficient to recruit E-Syt1 to the plasma membrane. In

addition, the authors show that the C2 domains of E-Syt1 behave in a similar fashion to those of synaptotagmin 1, a molecule that is well known from studies of synaptic transmission. This work refines our knowledge on the regulation of E-Syt1, although it does not change the current knowledge in a fundamental fashion.
The manuscript is solid, and the experiments are generally well performed. The assay based on cellular permeabilization with alpha-toxin, which enables exchanges of cellular ions, but not proteins, is elegant and interesting.
One issue that is still open, though, is whether the amount of calcium that enters the cytosol is the only difference between the effects of SOCE and of calcium release from intracellular stores. Could the location of the calcium channels also play a role, with calcium entry through plasma membrane stores playing a more important role than calcium release from internal stores? One experiment to test this would be to repeat the imaging study from Figure 4A-C, using different calcium levels in the extracellular solution, lower than the 3mM used in the figure. The authors' interpretation is that SOCE would have no effect when it results in the same calcium entry as the BHQ stimulus. This should be tested directly.
Minor comments:
- The title of the legend to Figure 1 is somewhat misleading. The expansion of ER-PM contacts can be interpreted from the data presented in this figure, but is not directly measured in the figure.
- The figure legend of Figure 4A-C seems to mix the panels B and C.

Referee #3:

This paper by Idevall-Hagren and colleagues is a direct follow up of the authors' previous work (Giordano et al., 2013). The authors show the contributions of the different C2 domains of E-Syt1 to calcium- and PI(4,5)P2-dependent/independent recruitment of E-Syt1 to the plasma membrane. In addition, the study further substantiates that homo- and hetero-dimer formation of E-Syt1 and E-Syt2 affects plasma membrane recruitment. Importantly, the results demonstrate that the recruitment of E-Syt1 to ER-plasma membrane contact sites occurs at calcium concentrations in the low micromolar range. These calcium concentrations can be reached by the influx of extracellular calcium via store operated calcium entry or by the membrane depolarization of excitable cells. Briefly, E-Syt1-recruitment to the plasma membrane in the low micromolar calcium range requires the cooperative presence of both the C2C (calcium-sensing) and C2E (calcium-independent PI(4,5)P2 binding), membrane anchoring and likely involves E-Syt1/E-Syt2 dimer formation. Membrane recruitment results in an expansion of ER-PM contact sites.

Overall, the paper represents a very systematic study, deciphering the role of various E-Syt1 domains in calcium-dependent formation and extension of ER-PM tether sites. Most of the experiments are technically convincing, employing both semi-intact and intact cells and physiological stimuli (glucose) to trigger exocytosis (in MIN6 cells). Interestingly, glucose stimulation, which triggers insulin secretion in MIN6 cells also favors the formation of extended ER-PM tethers. Although the paper does not provide any functional roles for the extended ER-PM contact sites, the data are of general interest for a broad readership and are of relevance for several research areas.

The following issues should be addressed to improve the manuscript:

Do the requirements for the calcium-dependent recruitment of E-Syt1 to ER-PM contact sites differ from the requirements for ER-PM contact site expansion? For example, does the calcium-dependent expansion of the ER-PM contact sites require the formation of E-Syt-1/E-Syt2 heterodimers (SMP domain mutant in a E-Syt1-knock down)? Does the tandem C2A-C2B domain of synaptotagmin 1, when appended to the ER-anchor of E-Syt1 also result in an ER-PM contact site expansion?

Minor points:
Add scale bars to the figure panels showing microscopic images.
Please show the expression levels of the different constructs compared to endogenous E-Syts to provide some information to which degree the exogenously expressed proteins from self homo-oligomers or heterodimers with the endogenous E-syt proteins. (Western blot analyses will also ensure that the various truncation constructs are not further degraded in the cells.)

Figure 1E: In the figure legend, briefly mention that the different gray levels mark distinct C2 domains with functional homologies.
Figure 1D: For consistency (and in analogy to Figure 1H), please label the y-axis in addition to "Norm. fluor" with Fluo-4.
Figure 2B: At 200 µM Ca2+, the distribution of mCh-E-Syt1-SMP-C2ABCDE construct appears spotty. (This also applies to the mChSyt1-C2AB construct). Do these constructs aggregate at this high Ca2+ concentration, which would lower the total concentration available for recruitment to the plasma membrane. Alternatively, these constructs could bind intracellular membranes. Please provide an explanation.

1st Revision - authors' response 26 May 2015

Response to the review of our manuscript "Triggered Ca^{2+} influx is required for extended-synaptotagmin 1-induced ER-plasma membrane tethering".

We thank the three referees for their constructive comments that have helped us improve our manuscript. We have addressed their suggestions with a considerable body of new experimentation and text additions/modifications as discussed below. The following data are new:
- **Figure 1F** (showing Ca2+-induced expansion of ER-PM contacts in permeabilized cells)
- **Figure 4H-J** (showing that inhibition of SOCE prevents E-Syt1 PM binding)
- **Figure EV1A-C** (Western blot detection of truncation constructs)
- **Figure EV6D-G** (showing that reversible and irreversible blockage of the SERCA pump has the same effect on E-Syt PM binding)
- **Figure EV6H-J** (showing that recruitment of E-Syt1 to ER-PM contacts is STIM1 independent)
- **Figure EV7D** (showing that E-Syt1 KD is without effect on thapsigargin-induced SOCE)

An additional experiment is shown in this letter for the reviewers.

Reviewers' text is in *italics* and our responses in regular text

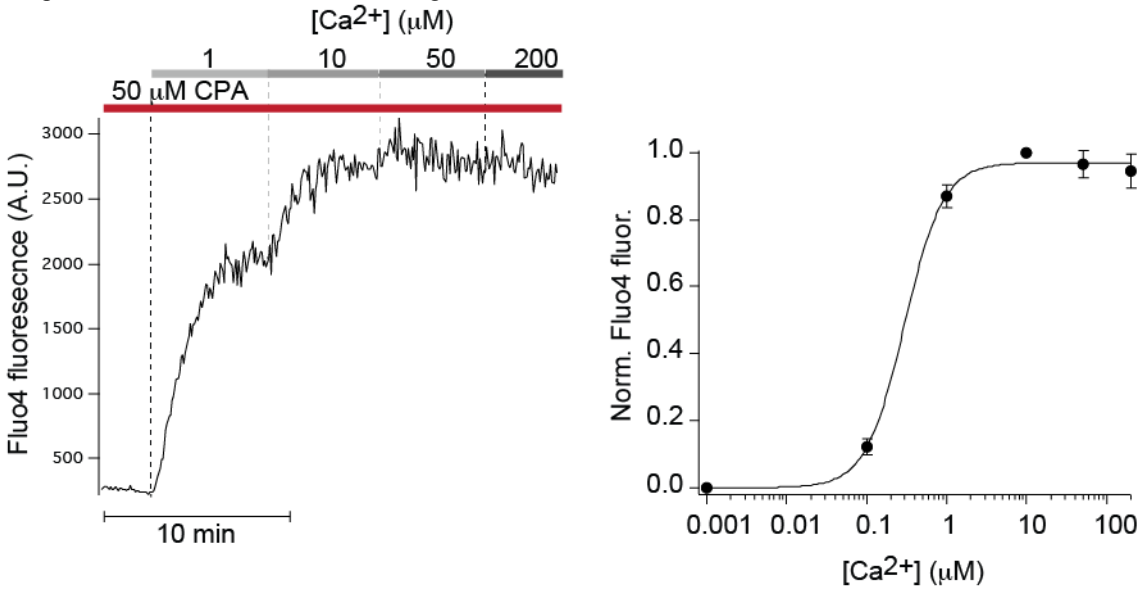
Referee #1:

The study by Idevall-Hagren et al. expands on previous studies from Dr. De Camilli's group regarding the plasma membrane recruitment of E-Syt proteins (1, 2, and 3). While the study elucidates some important aspects of E-Syt1 recruitment, such as the co-operativity in response to increases in [Ca2+]I and possible tuning of the [ca2+] sensitivity via heteromerization between the proteins, this important aspect has not been fully explored. Further, several major properties of the protein, including its physiological role and how that relates to the functions examined in the study, have not been clarified. My major concerns are as follows:

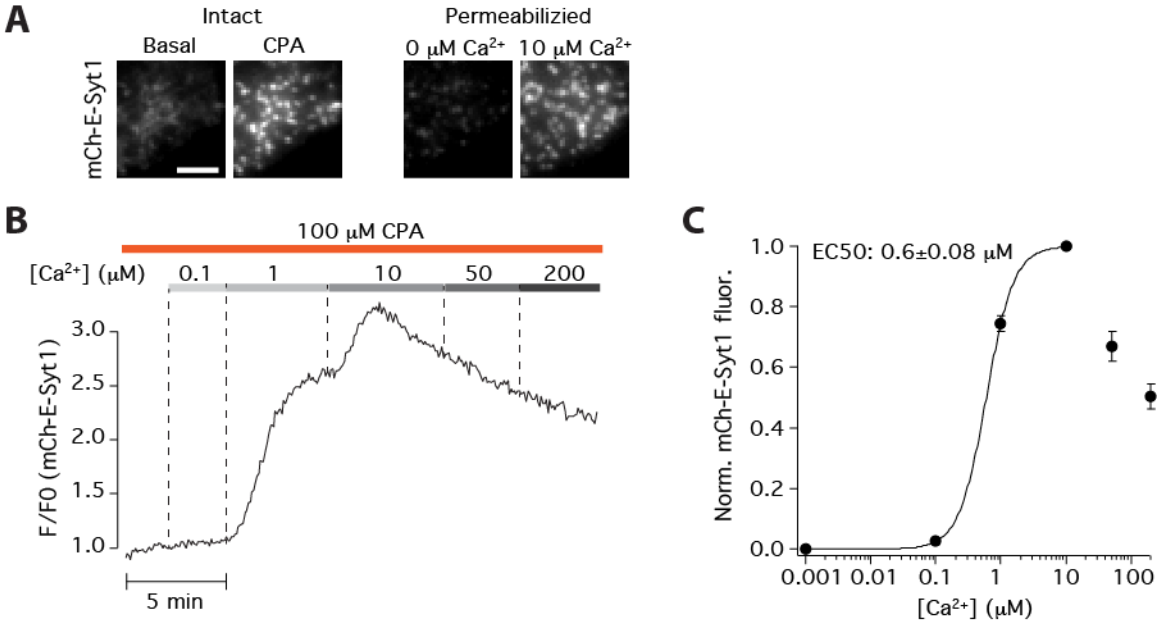
1. The data shown in the paper do not conclusively establish whether the [Ca2+] measurements carried out using the TIRF mode in permeabilized cells actually represent cytosolic [Ca2+]. What is the effect of SERCA inhibition in these experiments? These controls are required. Further, and more important, what are the changes in [Ca2+]i (measured using TIRF) in depolarized cells and BHQ stimulated cells in non-permeabilized state (i.e. experiments shown in Figs 4 and 5).

Reply: Regarding the first point:
We are not sure we understand the question. Since the cells are permeabilized, the Ca^{2+} concentration outside and inside the cell should be very similar and this is confirmed by Fluo-4-based estimations. The impact of SERCA inhibition on Ca^{2+} in the cytosolic space under these conditions is expected to be irrelevant. The reviewer may refer to a potential impact of lumenal ER depletion. We show elsewhere that lumenal ER depletion does not have a relevant impact on the dynamics of E-Syt1 binding to the plasma membrane (see e.g. Figure 4A).
In any case, to further prove this point, we have now performed an additional experiment, which we show here for the reviewer. We prefer not to include the data as the experiment is complex and we

deem it unnecessary in view of the data already shown, but we will show it if the reviewer deems it necessary. We have expressed mCherry-tagged full-length E-Syt1 in HeLa cells (loaded with the Ca^{2+} indicator Fluo-4) and activated SOCE by the addition of 100 mM CPA (a SERCA pump inhibitor) in the presence of 1.3 mM Ca^{2+} before cell permeabilization. This resulted in pronounced E-Syt1 plasma membrane binding, as expected due to the SOCE-dependent rise of cytosolic Ca^{2+}. We subsequently replaced the buffer surrounding the cells with intracellular-like buffer containing 100 uM CPA and permeabilized the cells with alpha-toxin. Step increases in the buffer Ca^{2+} concentration resulted in dose-dependent increases in the cytosolic Ca^{2+} concentration (similar to what is shown in Figure 1 in the absence of CPA). We find that in the presence of CPA, the dose-response curve for E-Syt1 plasma membrane binding is only slightly left-shifted (from approximately 1.8 to 0.6 uM Ca2+). We believe this is due to the STIM1-mediated expansion of ER-PM contacts that occur under these conditions, which may facilitate encounters of E-Syt1 with the plasma membrane, as it diffuse along the ER membrane.



Fluo-4 fluorescence recorded by TIRF microscopy in a permeabilized HeLa cell exposed to the SERCA inhibitor CPA and the indicated Ca^{2+}-buffers. To the right is shown the dose-response curve for Fluo-4 in the presence of CPA (means±SEM, 16 cells from 3 experiments).



mCherry-E-Syt1 fluorescence recorded by TIRF microscopy in intact and permeabilized HeLa cells exposed to the SERCA inhibitor CPA and the indicated Ca^{2+}-buffers. Increasing the cytosolic Ca^{2+} concentration in the presence of the SERCA inhibitor CPA still results in E-Syt1 plasma membrane binding. To the right is shown the dose-response curve for mCherry-E-Syt1 PM-binding in the presence of CPA (means±SEM, 16 cells from 3 experiments).

Regarding the second point:
The key message of experiments of Fig. 4 and 5 is to relate/validate with experiments in living cells the results obtained with permeabilization experiments. Fig. 4 confirms the relatively low Ca^{2+} affinity of E-Syt1 (the elevation of cytosolic Ca^{2+} induced by SERCA inhibition is not sufficient to induce E-Syt1 accumulation at the plasma membrane), while Fig. 5 demonstrates that in spite of this low affinity, manipulations that mimic physiological stimuli do induce E-Syt1 accumulation at the plasma membrane. Accurate determination of sub-plasma membrane Ca^{2+} concentrations using TIRF microscopy is not straightforward. The ratiometric indicator typically used to measure the exact Ca^{2+} concentration (Fura-2) is of high affinity and not well suited for this purpose. Additionally, use of such an indicator on a TIRF microscope would require UV-lasers and UV-compatible optical mirrors and objectives. Importantly, this ratiometric Ca^{2+} indicator is soluble and readily diffuse in and out of the evanescent field, complicating accurate measurements. We show in Figures 4 and 5 that both SOCE- and VDCE-induced E-Syt1 PM-binding is blocked by the fast Ca^{2+} chelator BAPTA, but to a lesser extent by the slow chelator EGTA, speaking in favor of E-Syt1 sensing sub-PM Ca^{2+}.

2. The authors refer to the Ca2+-add- back assay for SOCE as physiological, but actually it is not. In depolarization conditions where VGCC are activated, one can envision a large bolus of Ca2+ entry in cells such as pancreatic islets or neuronal cells since all channels are activated at the same time. On the other hand in the case of SOCE, there is low possibility of such sudden bolus of Ca2+ influx into cells. SOCE experiments should be carried out in the continued presence of Ca2+. What happens to E-Syt1, is it even recruited to the ER-PM junctions under these conditions? Further, physiologically SOCE is activated by very low agonist concentrations where [Ca2+]i oscillates and under these conditions, few Orai1-STIM1 channels are assembled.

Reply: We already show in Figure EV6 that release of Ca^{2+} from the ER by activation of Muscarinic receptors cause E-Syt1 PM-binding only when coupled to SOCE. We have also shown this (response of E-Syt1 recruitment to SOCE in intact cells) in our previous paper Giordano et al. (PMID:23791178, Fig 5B of that paper), which we now quote in connection with this point. To complement SOCE based experiments we have now added one additional panel to Figure EV6 (D-G) where we show that addition of 1 uM thapsigargin to irreversibly block the SERCA pump results

in release of Ca^{2+} from the ER, but does not cause E-Syt1 PM binding unless extracellular Ca^{2+} is present at concentrations approaching, or above, 1 mM.

3. The buffering experiments do not conclusively demonstrate whether local or global [Ca2+]i increase is required for translocation of E-Syt1 to the PM regions. The local [Ca2+]i increase via SOCE is detected by NFAT and this assay should be measured as a control to confirm buffering of local [Ca2+]i via SOCE channels, such as Orai1.

Reply: Our experiments with BAPTA versus EGTA strongly favor the predominance of local changes. As the binding of E-Syt1 occurs at the plasma membrane, clearly what counts is the local Ca^{2+} concentration. Our experiments shows that E-Syt1 accumulates at pre-existing ER-PM membrane contact sites (see below) indicating that E-Syt1 does not actively translocate to the plasma membrane but becomes trapped there as it diffuses through the ER membrane, when Ca^{2+} is sufficiently high.

4. It is puzzling to see the loss of STIM1 from the ER-PM junctions following add back of Ca2+. It appears that in Figure 4, cells are incubated briefly with BHQ, which is then washed off, when Ca2+ is readded. So basically readdition of Ca2+ will allow the ER store to be refilled, which might be why STIM1 moves away from the periphery? As stores are filled SOCE is gradually inactivated and E-Syt translocates out of the junctions? It is not clear whether this is what the authors want to imply? A better approach would be to show the effect of 2-APB or BTP2 which inhibits SOCE without losing STIM1 puncta?

Reply: We believe that it is just like the referee writes, that under the conditions we use, Ca^{2+} add-back causes SOCE which recruit E-Syt1 to pre-existing STIM1 puncta. STIM1 dissociates from the ER-PM contacts at the same time as E-Syt1 gets recruited due to the Ca^{2+}-induced refilling of the ER. The requirement of SOCE (and the resulting elevation of cytosolic Ca^{2+}) for E-Syt1 PM-binding is demonstrated by the inability of BHQ to cause E-Syt1 PM-binding after STIM1 knockdown (see Figure 4). To further support the claim that E-Syt1-induced ER-PM contact expansion can occur in the presence of STIM1, we now show that photolysis of caged Ca^{2+} (no STIM1 puncta) and subsequent addition of thapsigargin (which cause STIM1 puncta formation) results in E-Syt1 translocation to the same ER-PM contact sites (Figure EV6, panels H,J).

Moreover, we now also shown in new Figure 4H-I that blockage of Orai-channels by the lanthanide Gd^{3+} (1 mM), which does not affect STIM1-Orai1 contacts, reverses SOCE and results in dissociation of E-Syt1 from the plasma membrane. Together, these observations show that although both STIM1 and E-Syt1 are found at the same ER-PM contacts, the localization of these two proteins to these sites are independently regulated.

5. Similarly, E-Syt KD effect on SOCE needs to be assessed by adding Ca2+ back in the continued presence of BHQ or agonist, which will measure true SOCE. In the experiments shown in Fig 4K and Fig EV6, BHQ was removed thus allowing stores to refill, and the SOCE to inactivate.

Reply: The question asked here is whether the KD of the E-Syts affects SOCE under conditions in which the SERCA pump remains inhibited. We have shown that this is not the case in our previous publication (Giordano et al. PMID:23791178; Fig 7I of that paper). In that paper we had reported that the knockdown of all three isoforms of the E-Syts does not impact the onset of SOCE induced by the addition of the irreversible SERCA blocker thapsigargin. We now show that there is no difference in the effect of E-Syt1 knockdown on SOCE induced by either reversible (Figure 4) or irreversible (Figure EV7 panel D) inhibition of the SERCA pump.

6. It would add considerably to the importance of this study, if recruitment of E-Syt1 to the same site as STIM1 is shown to be physiological. Does the possible lipid transfer via ESyt1 have any consequence on SOCE or in the function/secretion in islets?

Reply: An impact of lipid transfer by the E-Syts on SOCE is an interesting possibility. However,

exploring such a possibility is beyond the purpose of this study. Concerning the issue of the recruitment of the E-Syts to the same sites as STIM1, we find this not surprising as it will be easier for an ER protein with plasma membrane binding properties to accumulate where a contact is already formed.

7. Finally it is unclear whether the authors mean to imply that there is competition between STIM1 and E-Syt for the same junctions? So, in the continued presence of STIM1 in puncta E-Syt1 cannot be recruited?

Reply: We do not think that there is competition. We have found that contacts may undergo major expansion upon overexpression of either STIM1 or E-Syts. E-Syts bind $PI(4,5)P_2$ which is available in large excess in the plasma membrane. See also the reply to point 4 above.

Referee #2:

The authors describe here the interaction of the extended synaptotagmin 1 (E-Syt1) with the plasma membrane. This molecule participates in the formation of endoplasmic reticulum-plasma membrane contacts, in a calcium-dependent fashion. The authors follow here on excellent previous work, which they have published on the subject recently (Giordano et al., Cell, 2013), and in which they have already introduced many aspects of E-Syt biology.
In this manuscript, the authors show that the binding (recruitment) of E-Syt1 to the plasma membrane requires relatively high calcium levels, which can be derived from store-operated calcium entry (SOCE), or from the response to depolarization of excitable cells. On its own, the release of calcium from intracellular stores is not sufficient to recruit E-Syt1 to the plasma membrane. In addition, the authors show that the C2 domains of E-Syt1 behave in a similar fashion to those of synaptotagmin 1, a molecule that is well known from studies of synaptic transmission. This work refines our knowledge on the regulation of E-Syt1, although it does not change the current knowledge in a fundamental fashion.
The manuscript is solid, and the experiments are generally well performed. The assay based on cellular permeabilization with alpha-toxin, which enables exchanges of cellular ions, but not proteins, is elegant and interesting.

We thank the reviewer for these positive comments.

One issue that is still open, though, is whether the amount of calcium that enters the cytosol is the only difference between the effects of SOCE and of calcium release from intracellular stores. Could the location of the calcium channels also play a role, with calcium entry through plasma membrane stores playing a more important role than calcium release from internal stores? One experiment to test this would be to repeat the imaging study from Figure 4A-C, using different calcium levels in the extracellular solution, lower than the 3mM used in the figure. The authors' interpretation is that SOCE would have no effect when it results in the same calcium entry as the BHQ stimulus. This should be tested directly.

Reply: This is an excellent suggestion, which we have now addressed in Figure EV6 (panels D-G)(see also above). We coexpressed the Ca^{2+} indicator GCaMP5G and mCherry-E-Syt1 in HeLa cells and treated them with 1 uM thapsigargin (which is then present throughout the experiment) in the absence of extracellular Ca^{2+}. Subsequent addition of lower than physiological extracellular Ca^{2+} (0.2 or 0.5 mM) causes increases in cytosolic Ca^{2+} similar to those achieved after store depletion, and does not result in E-Syt1 binding to the plasma membrane. Further increase of the extracellular Ca^{2+} concentration (>1 mM) cause higher elevations in the cytosolic Ca^{2+} concentration and dose-dependent E-Syt1 PM-binding (similar to what we observed in the permeabilized cell assay). These experiments show that it is the Ca^{2+} concentration, and not the source of Ca^{2+}, that is important for E-Syt1 PM-binding.

Minor comments:
- *The title of the legend to Figure 1 is somewhat misleading. The expansion of ER-PM contacts can*

be interpreted from the data presented in this figure, but is not directly measured in the figure.

Reply: We have now added a new panel in Figure 1F in which we show expansion of ER-PM contacts by Ca^{2+} as measured by the fluorescence increase in the TIRF microscopy field of the luminal ER marker ER-oxGFP in cells co-expressing un-tagged E-Syt1. As this is a "passive" marker that simply reflects increased apposition of the ER to the plasma membrane, the increase of its fluorescence in the TIRF field reflects an expansion of the ER-PM contacts. We have also made changes to the figure legends to clarify this point.

- The figure legend of Figure 4A-C seems to mix the panels B and C.

Reply: This has now been corrected.

Referee #3:

This paper by Idevall-Hagren and colleagues is a direct follow up of the authors' previous work (Giordano et al., 2013). The authors show the contributions of the different C2 domains of E-Syt1 to calcium- and PI(4,5)P2-dependent/independent recruitment of E-Syt1 to the plasma membrane. In addition, the study further substantiates that homo- and hetero-dimer formation of E-Syt1 and E-Syt2 affects plasma membrane recruitment. Importantly, the results demonstrate that the recruitment of E-Syt1 to ER-plasma membrane contact sites occurs at calcium concentrations in the low micromolar range. These calcium concentrations can be reached by the influx of extracellular calcium via store operated calcium entry or by the membrane depolarization of excitable cells. Briefly, E-Syt1-recruitment to the plasma membrane in the low micromolar calcium range requires the cooperative presence of both the C2C (calcium-sensing) and C2E (calcium-independent PI(4,5)P2 binding), membrane anchoring and likely involves E-Syt1/E-Syt2 dimer formation. Membrane recruitment results in an expansion of ER-PM contact sites.

Overall, the paper represents a very systematic study, deciphering the role of various E-Syt1 domains in calcium-dependent formation and extension of ER-PM tether sites. Most of the experiments are technically convincing, employing both semi-intact and intact cells and physiological stimuli (glucose) to trigger exocytosis (in MIN6 cells). Interestingly, glucose stimulation, which triggers insulin secretion in MIN6 cells also favors the formation of extended ER-PM tethers. Although the paper does not provide any functional roles for the extended ER-PM contact sites, the data are of general interest for a broad readership and are of relevance for several research areas.

We thank the reviewer for these positive comments

The following issues should be addressed to improve the manuscript:

Do the requirements for the calcium-dependent recruitment of E-Syt1 to ER-PM contact sites differ from the requirements for ER-PM contact site expansion? For example, does the calcium-dependent expansion of the ER-PM contact sites require the formation of E-Syt-1/E-Syt2 heterodimers (SMP domain mutant in a E-Syt1-knock down)? Does the tandem C2A-C2B domain of synaptotagmin 1, when appended to the ER-anchor of E-Syt1 also result in an ER-PM contact site expansion?

Reply: We have shown in our previous publication (Giordano et al. PMID:23791178) that overexpression of the E-Syts results in exaggerated ER-PM contact formation. All constructs that are anchored to the ER and bind the plasma membrane expand ER-PM contact sites, irrespective of the presence of the SMP domain or of dimerization. For example, in the present study we show (in Figure 3 and EV3) that E-Syt1 lacking the SMP domain can still bind the PM and expand ER-PM contacts in response to increases in the cytosolic Ca^{2+} concentration.

Minor points:
Add scale bars to the figure panels showing microscopic images.

Reply: This has now been done.

Please show the expression levels of the different constructs compared to endogenous E-Syts to provide some information to which degree the exogenously expressed proteins from self homo-oligomers or heterodimers with the endogenous E-syt proteins. (Western blot analyses will also ensure that the various truncation constructs are not further degraded in the cells.)

Reply: We now show Western blot analysis of the expression of most mCherry-tagged proteins used in the study (detected using a mCherry-antibody) (New Figure EV1A,B). We also show the relative expression-level of some E-Syt1-constructs compared to endogenous E-Syt1 (detected with an E-Syt1-antibody) (New Figure EV1C). The level of over-expressed protein, under these conditions, was modest compared to the endogenous protein levels, although it is possible that antibody detection of the fusion proteins is hampered by the mCherry-tag (a fusion protein lacking the hydrophobic stretch and SMP domain is not detected by the E-Syt1 antibody, but clearly detected by the mCherry antibody).

Figure 1E: In the figure legend, briefly mention that the different gray levels mark distinct C2 domains with functional homologies.

Reply: The following sentence has been added to the figure legend: “C2 domains with greater structural similarity to each other are indicated by the same shade of gray.”

Figure 1D: For consistency (and in analogy to Figure 1H), please label the y-axis in addition to "Norm. fluor" with Fluo-4.

Reply: This has now been corrected.

Figure 2B: At 200 µM Ca2+, the distribution of mCh-E-Syt1-SMP-C2ABCDE construct appears spotty. (This also applies to the mChSyt1-C2AB construct). Do these constructs aggregate at this high Ca2+ concentration, which would lower the total concentration available for recruitment to the plasma membrane. Alternatively, these constructs could bind intracellular membranes. Please provide an explanation.

Reply: We observe this “clustering” at high (>200 uM) Ca^{2+} concentratoins for several of the C2-domain constructs used (both E-Syt1 and Syt1 derived), however not for all (see Figure EV1). We performed colocalization studies in permeabilized HeLa cells (see below) and find little colocalization between these protein aggregates and intracellular membranes of endosomes (Rab5) or ER (luminal marker). We believe these clusters are cytosolic aggregates of the overexpressed proteins, possibly formed by electrostatic interactions between Ca^{2+} and the protein domains. Since the clustering only occurs at supra-physiological Ca^{2+} concentrations we do not believe that this is of importance for the function of the E-Syts. We have now mentioned this in the figure legend.

2nd Editorial Decision 16 June 2015

Thank you for submitting your revised manuscript for our consideration.
Your manuscript has now been seen once more by the original referees (see comments below), and I am happy to inform you that they are broadly in favor of publication, pending satisfactory minor revision.

I would therefore like to ask you to address referee #3's minor comments and to provide a final version of your manuscript incorporating the suggested text changes.

A few editorial points need to be taken care of at this stage:

- please check whether all figure files are of adequate resolution and quality for production, and upload improved versions if necessary.
- please suggest (in a cover letter) a one-sentence summary 'blurb' of your paper, as well as 2-5 one-sentence 'bullet points', containing brief factual statements that summarize key aspects of the paper; this will form the basis for an editor-drafted 'synopsis' accompanying the online version of the article. Please see the latest research articles on our website (emboj.embopress.org) for examples - I am happy to offer further guidance on this if necessary.
- as you might know, we encourage our authors to provide original source data (uncropped/-processed electrophoretic blots, excel files etc.) for the main figures of your manuscript. If you would like to add source data, we would welcome one PDF-file per figure for this information. These will be linked online as supplementary "Source Data" files.

I am therefore formally returning the manuscript to you for a final round of minor revision. Once we should have received the revised version, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

REFEREE COMMENTS

Referee #1:

With the revisions, the authors have addressed all the concerns in the previous review and the paper can now be accepted for publication.

Referee #2:

The authors have addressed all of my comments, and I am now happy to suggest the publication of the manuscript.

Referee #3:

Overall, in their revised manuscript, the authors have addressed my previous concerns.

However, several minor formal issues still need attention.
Legend to Figure 3: Delete "All images have been inverted to show fluorescence in black". (This figure does not contain any "fluorescence" images.)
Please check if all abbreviations such as "BHQ, CPA, ..." are explained/written out somewhere.
In several cases, the figure numbers mentioned in the text seem to be incorrect. Here are a few examples:
Page 5, 3rd paragraph: In the context of E-Syt dimers, the authors mention several times Fig 3I. This figure panel does not exist.
Page 5, 4th paragraph: Concerning mCherry-E-Syt2, please refer to EV5 not EV4.
Figure EV5: The panel numbers mentioned in the figure legend seem to be incorrect.
Figure EV7: Figure panel D is mentioned as C in the legend.

2nd Revision - authors' response 22 June 2015

Reviewers' text is in *italics* and our responses in regular text

Referee #1:
With the revisions, the authors have addressed all the concerns in the previous review and the paper can now be accepted for publication.

Referee #2:
The authors have addressed all of my comments, and I am now happy to suggest the publication of the manuscript.

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Overall, in their revised manuscript, the authors have addressed my previous concerns.
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Legend to Figure 3: Delete "All images have been inverted to show fluorescence in black". (This figure does not contain any "fluorescence" images.)
Please check if all abbreviations such as "BHQ, CPA, ..." are explained/written out somewhere.

Response: the sentence indicated has been deleted**.** All frequently used abbreviations (e.g. SMP, TIRF, SERCA, BHQ, PLC, CPA) are now written out when they are first mentioned in the text.

In several cases, the figure numbers mentioned in the text seem to be incorrect. Here are a few examples:
Page 5, 3rd paragraph: In the context of E-Syt dimers, the authors mention several times Fig 3I. This figure panel does not exist.
Page 5, 4th paragraph: Concerning mCherry-E-Syt2, please refer to EV5 not EV4.
Figure EV5: The panel numbers mentioned in the figure legend seem to be incorrect.
Figure EV7: Figure panel D is mentioned as C in the legend.

Response: We thank the reviewer for bringing this to our attention. We have now corrected the above-mentioned errors as well as similar errors relating to Figures 1, 3, 4 and EV5.

Comment: In the final version of the manuscript we have replaced the immunoblot in Figure EV1C, which shows the level of overexpression of selected mCherry-tagged E-Syt1 deletion constructs. In the previous version of the manuscript, the blot showed modest expression of the mCherry-tagged proteins compared to endogenous E-Syt1. The reason for this was that an alternative transfection method was used for these experiments. In the new blot. cells were transfected exactly as in all the imaging experiments, and the blot shows that the expression levels of the mCherry-tagged proteins are equal to or higher than that of endogenous E-Syt1.

