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An activated Q-SNARE/SM protein complex as a possible intermediate in SNARE assembly

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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.)

1st Editorial Decision 18 January 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see, the referees appreciate your data. However, they think that several points need to be addressed. Importantly,

- rate constants for t- and v-SNARE association need to be shown (referee #1, point 1)
- the (non-)existence of N-to-C SNARE zippering and the different observations on the role of t-SNAREs and Munc18 in SNARE assembly and membrane fusion must be discussed (referee #1, point 2-4; referee #2, point 2) as well as some other observations (referee #2, point 1 and 3-5)
- data on the behavior of the ternary complex in reconstituted fusion (liposome fusion assay using stx1/SNAP-25/Munc18 and VAMP2 liposomes) should be added and it would be also good to add some in vivo evidence (sequential immunoprecipitation of stx1, SNAP-25 with Munc18 from tissues or cells) (referee #3)

REFEREE REPORTS

Referee #1:

This is an interesting manuscript that demonstrated the potential role of the Munc18-1-bound t-SNARE complex as a receptor on the plasma membrane for v-SNARE assembly. Despite intensive

research in the past two decades, it remains unclear how the three synaptic SNARE proteins assemble into a four-helix bundle and drive membrane fusion. Jakhanwal et al. formed a ternary receptor complex between Munc18-1 and the binary t-SNARE complex and tested its association with the v-SNARE and its possible disassembly by NSF. They found that the receptor complex greatly accelerated the association between t- and v-SNAREs and resisted disassembly by NSF. These observations suggest that Munc18-1 can target the t-SNARE complex to accelerate SNARE assembly. The manuscript is well written and the primary conclusion on the enhanced SNARE assembly is convincing. The use of purified Munc18-1-t-SNARE complex and the TMD-containing syntaxin molecule is novel. However, the work is rather descriptive and lacks required numbers to back up the major conclusions. In addition, the proposed mechanism of the Munc18-1-enhanced SNARE assembly is not so convincing. Overall, the manuscript is publishable after careful revision to address the following major concerns:

1. The rate constants for t- and v-SNARE association under different experimental conditions (with Munc18-1, without Munc18-1, with Vc or Vn peptides) should be calculated and compared. The kinetic experiments in this work are generally well conducted. But data are not well analyzed in commensurate with the data quality. As a result, descriptions of the experimental results are relatively vague or misleading. For example, "The ternary complex binds synaptobrevin with fast kinetics, resulting in the almost instantaneous formation of a fully zippered SNARE complex" (lines 34-35); "binding of synaptobrevin to the ternary syntaxin1:SNAP24:Munc18-1 complex was almost instantaneous, comparable to the ΔN complex, and much faster than the binary syntaxin 1:SNAP25 complex (lines 186-187). Such descriptions give readers an impression that Munc18-1 mediates unprecedentedly fast SNARE assembly, which may not be true. For example, Fasshauer and co-workers showed that the ΔN-complex mediates fast SNARE assembly with a high bimolecular rate constant of 5×105 /s/M. Although normalized fluorescence (F/F0) at 100 s or 600 s are shown in Figs. 3 and EV2, such normalized fluorescence is not equivalent to the binding rate constant. In fact, the normalized fluorescence also depends on the affinity. In lines 215-216, the authors stated that "none of the tested truncated fragments were able to bind to the ternary syntaxin:SNAP25:munc18-1 complex". But in Fig. 3A, syb1-65 appears to bind as fast as Syb1-96, but with lower binding affinity. In conclusion, the binding rate constants are required to better compare the t- and v-SNARE binding kinetics. A table may be added to list all the binding rate constants.

2. In the abstract (lines 37-38) and lines 220-222, the authors argued for a cooperative binding mechanism between the Munc18-1-t-SNARE complex and the v-SNARE, which was considered to contradict an N-to-C SNARE zippering mechanism. However, this conclusion is not supported by the experimental data shown in the manuscript. Replacing the full cytoplasmic synaptobrevin with Vn and Vc peptides, the authors did not observe enhanced t- and v-SNARE association mediated by Munc18-1, indicating that a full synaptobrevin sequences is required for the Munc18-1-mediated SNARE assembly. However, the observation does not necessarily contradict N-to-C SNARE zippering. For example, in the template model proposed by Baker et al.1, Qa- and R-SNAREs are stabilized in a half-zippered state through interactions between SM proteins and the whole R-SNAREs. The authors showed that mutations in the R-SNARE C-terminus abolishes membrane fusion. Thus, the observations in this work can equally be explained by an N-to-C SNARE zippering mechanism and cooperative binding between Munc18-1-t-SNARE complex and synaptobrevin does not rule out N-to-C SNARE zippering. Finally, many studies have demonstrated that SNAREs assemble cooperatively in the absence of membranes but in an N-to-C direction in the presence of membranes due to membrane opposing force and topological orientation of SNAREs2-4.

3. The role of t-SNAREs as a potential target for Munc18-1 in SNARE assembly has extensively been studied in the past two decades and recently been revisited. In particular, Shen et al. did not observe any enhanced membrane fusion when pre-incubating Munc18-1 with membrane-anchored t-SNAREs5. In contrast, Ma et al. observed that Munc18-1 promotes SNARE assembly via a t-SNARE intermediate, indicating that Munc18-1 catalyzes t-SNARE association to enhance SNARE zippering6. Based on the similarity between Munc18-1 and Vc-peptides, these authors further suggest that Munc18-1 activates t-SNAREs to accelerate SNARE assembly and membrane fusion7. These closely related work should be discussed to broaden the impact of this work.

4. The authors made an important observation that the purified t-SNARE-Munc18-1 complex is meta-stable, with a lifetime of one to two hours. The binding affinity between t-SNAREs and Munc18-1 has been measured8. It is interesting to check how the t-SNARE-Munc18-1 complex

characterized in this work differs from the previous complexes and what the most stable t-SNARE-Munc18-1 complex is. This will help clarify the different observations on the role of t-SNAREs and Munc18-1 in SNARE assembly and membrane fusion.

Some minor questions or comments:

1. To test the association between the Munc18-1-t-SNARE ternary complex and synaptobrevin, the authors labeled a fluorophore on S28C of synaptobrevin. Based on the crystal structure of the ternary SNARE complex, this position appears to be disordered. Is S28C the best position for the fluorescence anisotropy measurement?

2. The sentence in lines 215-216 is not accurate.

3. Figure 4B is described before Fig. 4A.

4. In "For comparison, we used the same labeled proteins to prepare a standard syntaxin1:SNAP25 complex (see cartoon to the left of Figure 5B)" in lines 271-272. Is the 2:1 t-SNARE complex referred to here?

5. The kinetic traces in Fig. 2B vary significantly among different sub-figures (red traces). Please comment on these variations and show the standard deviations in the measured binding rate constants.

References:

1. Baker, R.W., Jeffrey, P.D., Zick, M., Phillips, B.P., Wickner, W.T. & Hughson, F.M. A direct role for the Sec1/Munc18-family protein Vps33 as a template for SNARE assembly. Science 349, 1111-1114 (2015).
2. Kyoung, M., Srivastava, A., Zhang, Y.X., Diao, J.J., Vrljic, M., Grob, P., Nogales, E., Chu, S. & Brunger, A.T. In vitro system capable of differentiating fast Ca2+-triggered content mixing from lipid exchange for mechanistic studies of neurotransmitter release. Proc. Natl. Acad. Sci. U.S.A. 108, E304-E313 (2011).
3. Gao, Y., Zorman, S., Gundersen, G., Xi, Z.Q., Ma, L., Sirinakis, G., Rothman, J.E. & Zhang, Y.L. Single reconstituted neuronal SNARE complexes zipper in three distinct stages. Science 337, 1340-1343 (2012).
4. Shin, J., Lou, X.C., Kweon, D.H. & Shin, Y.K. Multiple conformations of a single SNAREpin between two nanodisc membranes reveal diverse pre-fusion states. Biochem. J. 459, 95-102 (2014).
5. Shen, J.S., Tareste, D.C., Paumet, F., Rothman, J.E. & Melia, T.J. Selective activation of cognate SNAREpins by Sec1/Munc18 proteins. Cell 128, 183-195 (2007).
6. Ma, L., Rebane, A.A., Yang, G., Xi, Z., Kang, Y., Gao, Y. & Zhang, Y.L. Munc18-1-regulated stage-wise SNARE assembly underlying synaptic exocytosis. eLIFE 4, e09580 (2016).
7. Zhang, X.M., Rebane, A.A., Ma, L., Li, F., Jiao, J., Qu, H., Pincet, F., Rothman, J.E. & Zhang, Y.L. Stability, folding dynamics, and long-range conformational transition of the synaptic t-SNARE complex. Proc. Natl. Acad. Sci. U.S.A. 113, E8031-E8040 (2016).
8. Zhang, Y. et al. Munc18a does not alter fusion rates mediated by neuronal snares, synaptotagmin, and complexin. J Biol Chem 290, 10518-34 (2015).

Referee #2:

The study by Jakhanwal et al. addresses the existence and composition of SNARE intermediates to elucidate the sequence of events involved in SM-protein guided SNARE assembly. Using advanced biochemical assays, the authors provide evidence for a complex containing t-SNAREs and Munc18-1 that acts as an acceptor complex for synaptobrevin/VAMP and facilitates subsequent full SNARE assembly.

This study addresses an important question, adding to the long-standing debate on the sequence of events during SNARE assembly. New insights in the exact conformation of SNARE intermediates, as provided in this study, present an important step forward. Especially the evidence that synaptobrevin/VAMP enters faster into the Syx1-SNAP25-Munc18-1 intermediate as compared to t-

SNAREs alone, is important and helps to explain the currently unexplained facilitatory effect of Munc18-1 on SNARE assembly. The finding that the Syx1-SNAP25-Munc18-1 intermediate is NSF-aSNAP resistant is also important and novel. The methodology used in this study is state of the art, using full length proteins whenever possible and independent (albeit similar) techniques to validate the conclusions (fluorescence anisotropy and FRET).

On the other hand, the main conclusions in this manuscript are not always consistent with previous data or point in different directions without sufficient discussion on such issues, and some conclusions appear too strong for the available data. In general, the discussion section is heavily focused on the central binding cleft of Munc18-1 and would benefit from more complete interpretation of all findings.

Major issues:
1) The fact that in this study Syb did not bind monomer Munc18-1 (EV3B) is surprising, as this binding is a replicated observation: Xu et al., 2010 using the 1-96 fragment and Parisotto et al. 2016 using the full-length Syb/VAMP2. It is in the interest of the field to clearly mention this difference and hear the opinion of the authors on this issue.

2) Figure 3a-b: the Syb(1-65) fragment does have some binding affinity for the Syx-SNAP25-M18 complex, especially in Fig3b. The authors should discuss that. The conclusion drawn from this figure that SNARE zippering occurs cooperative and bi-directionally seems too strong, based on the data presented and goes against a large body of evidence supporting N- to C- terminal zippering, also in intact systems. The difference in binding of the Syb1-65 fragment in the Syx:SNAP25:M18 complex compared to the deltaN-complex might be pre-structuring of the helix, also discussed in Li et al., 2016 (PNAS), which may be supported in vivo by additional factors, not present in the reduced systems used in the current study. The paper would benefit from a balanced discussion on this topic.

3) An intriguing finding in this manuscript is the NSF-aSNAP resistance of the Syx:SNAP25:M18 complex. However, this is not addressed at all in the discussion section. Could the authors discuss this, especially in the light of the Ma et al., 2013 paper, which proposes such a disassembly resistant pathway for Munc18-1-SNARE formation?

4) The comparison between Syx:SN25 2:1 complexes and Syx:SNAP25:M18-1 complexes seems not entirely fair. Differences in Syb association could be caused by a different stoichiometry, and the main function of Munc18-1 at this point might be to change to a 1:1 acceptor complex more than changing the composition of this complex. The authors touch upon this point, but do not really address this issue.

5) By focusing on the cleft as the decisive catalytic region of SM-proteins, the authors bypass recent evidence for structural modifications in domain 3a (helix 12 extension) being essential for SNARE assembly and synaptic vesicle priming. Again, it is in the interest of the field to clearly mention this difference and hear the opinion of the authors on this issue.

Minor issues:
- Figure 1 and 2 have the same message. It might be better to combine the two. Figure 2e could move to the supplemental data. Or it should be explained better what we learn from this figure?
- Line 153: very fast relative to what?
- In line 158 the authors mention an interesting observation concerning an assay with syntaxin(1-262), but do not show the data. Would be good if they did, since it makes the interesting point of the necessity of Syx1a transmembrane domain for Synaptobrevin binding to the Syx1:SNAP25:M18 complex.
- Figure EV2b: both groups seem to have reached their max anisotropy at 100ms (at least judging from the quantification). Quantification at an earlier time point might have led to the conclusion that binding of synaptobrevin to the ternary complex, while much faster than the binary complex, still be slower compared to the (non-physiological) deltaN-complex.
- For clarification, it would be good if the cartoons of figure 2a were used to indicate which complex was used in subsequent figures, like in figure 3.
- Line 33: (typo) authors refer to Figure 4b, not 4c
- Figure order. Subfigures are not always discussed in logical order in the text (e.g. Fig 4b precedes

4a)
- Figure 5: Include in the figure legends that red arrows are used to indicate when chemicals were added to the mixture, while black arrows indicate the condition. This is not immediately clear now.
- Line 298-300: do the cross-linking experiments in figure 4 not suggest that Munc18-1 binds to the Habc domain of Syntaxin1, instead of its far N-peptide?
- Line 326: unformatted reference (10)

Referee #3:

In this article, using recombinant proteins, the authors identify a complex between syntaxin 1, SNAP-25, and Munc18-1. They further show that this complex functions as a receptor for synaptobrevin and is resistant to disassembly by NSF.

The experiments are well carried out and controlled. The identification of the interaction regions by cross-linking and MS is particularly smart and powerful. Overall, the novels finding presented herein have very important functional implications for our understanding of the molecular principles of membrane fusion and neuronal secretion.

However, in its present form, this article falls short of a convincing conclusion because of the lack of data on the behavior of this ternary complex in reconstituted fusion in vitro and the lack of a demonstration of its occurence in cells or tissue. With the addition of these complementary data, this article would bring a strong and clear demonstration.

1st Revision - authors' response 02 March 2017

Referee #1:
This is an interesting manuscript that demonstrated the potential role of the Munc18-1-bound t-SNARE complex as a receptor on the plasma membrane for v-SNARE assembly. Despite intensive research in the past two decades, it remains unclear how the three synaptic SNARE proteins assemble into a four-helix bundle and drive membrane fusion. Jakhanwal et al. formed a ternary receptor complex between Munc18-1 and the binary t-SNARE complex and tested its association with the v-SNARE and its possible disassembly by NSF. They found that the receptor complex greatly accelerated the association between t- and v-SNAREs and resisted disassembly by NSF. These observations suggest that Munc18-1 can target the t-SNARE complex to accelerate SNARE assembly. The manuscript is well written and the primary conclusion on the enhanced SNARE assembly is convincing. The use of purified Munc18-1-t-SNARE complex and the TMD-containing syntaxin molecule is novel.

We thank the reviewer for the positive assessment of our contribution.

However, the work is rather descriptive and lacks required numbers to back up the major conclusions. In addition, the proposed mechanism of the Munc18-1-enhanced SNARE assembly is not so convincing. Overall, the manuscript is publishable after careful revision to address the following major concerns:

1. The rate constants for t- and v-SNARE association under different experimental conditions (with Munc18-1, without Munc18-1, with Vc or Vn peptides) should be calculated and compared. The kinetic experiments in this work are generally well conducted. But data are not well analyzed in commensurate with the data quality. As a result, descriptions of the experimental results are relatively vague or misleading. For example, "The ternary complex binds synaptobrevin with fast kinetics, resulting in the almost instantaneous formation of a fully zippered SNARE complex" (lines 34-35); "binding of synaptobrevin to the ternary syntaxin1:SNAP24:Munc18-1 complex was almost instantaneous, comparable to the ΔN complex, and much faster than the binary syntaxin 1:SNAP25 complex (lines 186-187). Such descriptions give readers an impression that Munc18-1 mediates unprecedentedly fast SNARE assembly, which may not be true. For example, Fasshauer and co-workers showed that the ΔN-complex mediates fast SNARE assembly with a high bimolecular rate constant of 5×105 /s/M. Although normalized fluorescence (F/F0) at 100 s or 600 s are shown in Figs. 3 and EV2, such normalized fluorescence is not equivalent to the binding rate constant. In

fact, the normalized fluorescence also depends on the affinity. In lines 215-216, the authors stated that "none of the tested truncated fragments were able to bind to the ternary syntaxin:SNAP25:munc18-1 complex". But in Fig. 3A, syb1-65 appears to bind as fast as Syb1-96, but with lower binding affinity. In conclusion, the binding rate constants are required to better compare the t- and v-SNARE binding kinetics. A table may be added to list all the binding rate constants.

We agree that experimentally determined rate constants would strengthen the argument. However, due to the instability of the ternary acceptor complex, it is not possible to accurately determine its concentration. For the information of the referee, we have re-analyzed our data to calculate apparent rate-constants for the acceptor complex with Munc18-1, without Munc18-1, and for the synaptobrevin fragments. Fitting was performed using a double-exponential fit, using non-linear regression (note that fitting was not possible for some of the traces, *see below*). The observed rate constants (K_1obs) and the half-times of the reactions are indicated in the table. These values are only an approximation (very probably represent an underestimation), and we have therefore decided not to publish these numbers. We have revised the interpretation of these results to clarify this point. Also, we would like to thank the reviewer for alerting us to the binding behavior of Syb 1-65 that was not correctly described in the text earlier– this has been changed.

Table 1. Observed rate-constants for the binding of the synaptobrevin fragments to the syntaxin1:SNAP25:Munc18-1 complex, syntaxin:SNAP25 (2:1) complex and the ΔN-complex. The fitting for the syntaxin1:SNAP25:Munc18-1 complex and the ΔN-complex was obtained using a double-exponential fit using traces from the FRET experiments and for the 2:1 complex using anisotropy experiment.

Acceptor complex	Fragment	K_1obs (s^{-1})	t_1 (s)	Half-time $t_{1/2}$ (s)
Syntaxin1:SNAP25:Munc18-1 complex	Syb1-96	0,46 ± 0,02	2,16 ± 0,10	1,499
	Syb1-65	0,19 ± 0,042	5,2252± 1,16	3,621
	Syb1-52	n/a	n/a	n/a
	Syb49-96	n/a	n/a	n/a
ΔN-Complex	Syb1-96	0,30 ± 0,014	3,24 ± 0,15	2,24
	Syb1-65	0,071± 0,013	13,97 ± 2,56	9,681
	Syb1-52	0,078±0,0052	12,74± 0,84	8,83
	Syb49-96	n/a	n/a	n/a
Syntaxin:SNAP25 (2:1) complex	Syb 1-96	0,0531 ± 0,0051	18,82 ± 1,83	13,05

2. In the abstract (lines 37-38) and lines 220-222, the authors argued for a cooperative binding mechanism between the Munc18-1-t-SNARE complex and the v-SNARE, which was considered to contradict an N-to-C SNARE zippering mechanism. However, this conclusion is not supported by the experimental data shown in the manuscript. Replacing the full cytoplasmic synaptobrevin with Vn and Vc peptides, the authors did not observe enhanced t- and v-SNARE association mediated by

Munc18-1, indicating that a full synaptobrevin sequences is required for the Munc18-1-mediated SNARE assembly. However, the observation does not necessarily contradict N-to-C SNARE zippering. For example, in the template model proposed by Baker et al.1, Qa- and R-SNAREs are stabilized in a half-zippered state through interactions between SM proteins and the whole R-SNAREs. The authors showed that mutations in the R-SNARE C-terminus abolishes membrane fusion. Thus, the observations in this work can equally be explained by an N-to-C SNARE zippering mechanism and cooperative binding between Munc18-1-t-SNARE complex and synaptobrevin does not rule out N-to-C SNARE zippering. Finally, many studies have demonstrated that SNAREs assemble cooperatively in the absence of membranes but in an N-to-C direction in the presence of membranes due to membrane opposing force and topological orientation of SNAREs2-4.
We agree, and we have changed the text during revision to clarify this point and to avoid any misunderstanding (*see lines 198-204*). We did not intend to exclude N-C terminal zippering (an idea that indeed was put forward and promoted many years ago by our laboratory) but rather highlight the fact that N-terminal nucleation of the SNARE complex does require the C-terminal part of synaptobrevin. The referee is correct in stating that the mechanism proposed by Baker et al. may accommodate our findings although there are certain concerns. First, we would like to remind the referee that Baker et al. were unable to show simultaneous binding of the Qa- and the R-SNAREs to Vps33, which, in our opinion, renders the model somewhat tenuous. Moreover, when comparing the structures, it is evident that syntaxin1 needs to be open in the syntaxin1a:SNAP25:Munc18-1 complex in order to provide access to the R-SNARE-binding site on Munc18-1. Second, our work shows that SNAP-25 is bound before binding of synaptobrevin, which is different from the mechanism proposed by Baker according to which the Qb/Qc SNAREs only bind after the Qa/R-SNARE/SM-protein complex has formed (see also the review by Baker and Hughson).

3. The role of t-SNAREs as a potential target for Munc18-1 in SNARE assembly has extensively been studied in the past two decades and recently been revisited. In particular, Shen et al. did not observe any enhanced membrane fusion when pre-incubating Munc18-1 with membrane-anchored t-SNAREs5. In contrast, Ma et al. observed that Munc18-1 promotes SNARE assembly via a t-SNARE intermediate, indicating that Munc18-1 catalyzes t-SNARE association to enhance SNARE zippering6. Based on the similarity between Munc18-1 and Vc-peptides, these authors further suggest that Munc18-1 activates t-SNAREs to accelerate SNARE assembly and membrane fusion7. These closely related work should be discussed to broaden the impact of this work.

Possibly the reviewer overlooked that one of these papers was mentioned both in the introduction and in the discussion. We have changed the text to better emphasize these previously published findings (*see lines 287-293 and 305-307*). Also, see our reply below to Referee# 3 with regard to fusion.

4. *The authors made an important observation that the purified t-SNARE-Munc18-1 complex is meta-stable, with a lifetime of one to two hours. The binding affinity between t-SNAREs and Munc18-1 has been measured. It is interesting to check how the t-SNARE-Munc18-1 complex characterized in this work differs from the previous complexes and what the most stable t-SNARE-Munc18-1 complex is. This will help clarify the different observations on the role of t-SNAREs and Munc18-1 in SNARE assembly and membrane fusion.*
This is not a trivial issue since stability depends on the experimental conditions, which in our opinion largely explains the discrepancies between published studies. However, there are some major conclusions that can be drawn: (1) The affinity of Munc18-1 for syntaxin1 appears to be much lower when syntaxin1 contains its transmembrane domain (Syx1 -288) as compared to its cytoplasmic variant (Syx1-262) (Lewis et al., 2001). Affinities of Munc18-1 for the t-SNARE complex (syntaxin:SNAP25 complex) and the ternary SNARE complex, however, have only reliably been determined for complexes lacking the transmembrane domain of syntaxin (see e.g. (Zhang *et al*, 2015). (2) In exocytosis-competent lawns of plasma membranes, i.e an experimental *in-vitro* system close to the natural state, addition of synaptobrevin can effectively drive syntaxin1 bound to Munc18-1 into SNARE-complexes (Zilly *et al*, 2006). Here, however, it was not possible to discern whether the syntaxin1:Munc18-1 complex contained SNAP-25. In conclusion, we still lack quantitative data determining which fraction of syntaxin1 in an exocytosis-competent membrane is (i) bound to Munc18-1 alone, (ii) bound to Munc18-1 together with SNAP-25, (iii) bound to SNAP-25 in a binary complex in 1:1 or 2:1 stoichiometry, or (iv)forms homooligomers.

Some minor questions or comments:

1. To test the association between the Munc18-1-t-SNARE ternary complex and synaptobrevin, the authors labeled a fluorophore on S28C of synaptobrevin. Based on the crystal structure of the ternary SNARE complex, this position appears to be disordered. Is S28C the best position for the fluorescence anisotropy measurement?
As correctly pointed out by the reviewer, the region surrounding S28C on synaptobrevin is disordered. It is, however, a good choice for monitoring SNARE-assembly because it lies slightly upstream of the SNARE-core-complex, i.e. it becomes conformationally constrained during assembly but does not interfere with the zippering process. This labeling position was used in several previous studies (Pobbati *et al*, 2006; Winter *et al*, 2009; Walter *et al*, 2010), and we generally consider it as a highly reliable reporter in both anisotropy and FRET experiments for monitoring SNARE-complex assembly.
2. The sentence in lines 215-216 is not accurate.
3. Figure 4B is described before Fig. 4A.
These issues have been addressed.

4. In "For comparison, we used the same labeled proteins to prepare a standard syntaxin1:SNAP25 complex (see cartoon to the left of Figure 5B)" in lines 271-272. Is the 2:1 t-SNARE complex referred to here?
Yes, the reference goes to the 2:1 t-SNARE complex. We have clarified this issue.

5. The kinetic traces in Fig. 2B vary significantly among different sub-figures (red traces). Please comment on these variations and show the standard deviations in the measured binding rate constants.
Some differences do exist in the kinetic traces presented in Figure 2. These differences mainly result from the heterogeneity of the preparation and the inability to accurately assess the concentration of the complex at any given time-point (see above). This is clearly indicated in the quantification of these experiments in FigureEV2. The error bars in FigureEV2 represent the range of values that were recorded from three independent experiments.

References:

1. Baker, R.W., Jeffrey, P.D., Zick, M., Phillips, B.P., Wickner, W.T. & Hughson, F.M. A direct role for the Sec1/Munc18-family protein Vps33 as a template for SNARE assembly. Science 349, 1111-1114 (2015).
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3. Gao, Y., Zorman, S., Gundersen, G., Xi, Z.Q., Ma, L., Sirinakis, G., Rothman, J.E. & Zhang, Y.L. Single reconstituted neuronal SNARE complexes zipper in three distinct stages. Science 337, 1340-1343 (2012).
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5. Shen, J.S., Tareste, D.C., Paumet, F., Rothman, J.E. & Melia, T.J. Selective activation of cognate SNAREpins by Sec1/Munc18 proteins. Cell 128, 183-195 (2007).
6. Ma, L., Rebane, A.A., Yang, G., Xi, Z., Kang, Y., Gao, Y. & Zhang, Y.L. Munc18-1-regulated stage-wise SNARE assembly underlying synaptic exocytosis. eLIFE 4, e09580 (2016).
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The study by Jakhanwal et al. addresses the existence and composition of SNARE intermediates to elucidate the sequence of events involved in SM-protein guided SNARE assembly. Using advanced biochemical assays, the authors provide evidence for a complex containing t-SNAREs and Munc18-1 that acts as an acceptor complex for synaptobrevin/VAMP and facilitates subsequent full SNARE assembly.

This study addresses an important question, adding to the long-standing debate on the sequence of events during SNARE assembly. New insights in the exact conformation of SNARE intermediates, as provided in this study, present an important step forward. Especially the evidence that synaptobrevin/VAMP enters faster into the Syx1-SNAP25-Munc18-1 intermediate as compared to t-SNAREs alone, is important and helps to explain the currently unexplained facilitatory effect of Munc18-1 on SNARE assembly. The finding that the Syx1-SNAP25-Munc18-1 intermediate is NSF-aSNAP resistant is also important and novel. The methodology used in this study is state of the art, using full length proteins whenever possible and independent (albeit similar) techniques to validate the conclusions (fluorescence anisotropy and FRET).

We thank the reviewer for the overall positive assessment of our work.

On the other hand, the main conclusions in this manuscript are not always consistent with previous data or point in different directions without sufficient discussion on such issues, and some conclusions appear too strong for the available data. In general, the discussion section is heavily focused on the central binding cleft of Munc18-1 and would benefit from more complete interpretation of all findings.

Major issues:
1) The fact that in this study Syb did not bind monomer Munc18-1 (EV3B) is surprising, as this binding is a replicated observation: Xu et al., 2010 using the 1-96 fragment and Parisotto et al. 2016 using the full-length Syb/VAMP2. It is in the interest of the field to clearly mention this difference and hear the opinion of the authors on this issue.
We appreciate the reviewer's comment on this issue. The discrepancy is most probably simply due to the low affinity of this interaction, i.e. it can only be measured when excess amounts of synaptobrevin are present: Xu et al. used synaptobrevin in 2-fold excess over Munc18 whereas Parisotto et al used up to 25-fold excess of synaptobrevin). In our experiments (Figure EV3B), we used 2-fold more Munc18-1 than synaptobrevin. These concentration differences could probably account for the differences for the binding data in the earlier reports.

2) Figure 3a-b: the Syb(1-65) fragment does have some binding affinity for the Syx-SNAP25-M18 complex, especially in Fig3b. The authors should discuss that. The conclusion drawn from this figure that SNARE zippering occurs cooperative and bi-directionally seems too strong, based on the data presented and goes against a large body of evidence supporting N- to C- terminal zippering, also in intact systems. The difference in binding of the Syb1-65 fragment in the Syx:SNAP25:M18 complex compared to the deltaN-complex might be pre-structuring of the helix, also discussed in Li et al., 2016 (PNAS), which may be supported in vivo by additional factors, not present in the reduced systems used in the current study. The paper would benefit from a balanced discussion on this topic.

We appreciate the reviewer's viewpoint and we are thankful for the suggestions. This issue was also pointed by Referee#1 (see our reply above). We have revised the interpretation of the data with the synaptobrevin fragments to arrive at a more balanced discussion.

3) An intriguing finding in this manuscript is the NSF-aSNAP resistance of the Syx:SNAP25:M18 complex. However, this is not addressed at all in the discussion section. Could the authors discuss this, especially in the light of the Ma et al., 2013 paper, which proposes such a disassembly resistant pathway for Munc18-1-SNARE formation?

As requested, we have revised the discussion to highlight this finding *(see lines 268-271,332-334).*

4) The comparison between Syx:SN25 2:1 complexes and Syx:SNAP25:M18-1 complexes seems not entirely fair. Differences in Syb association could be caused by a different stoichiometry, and the main function of Munc18-1 at this point might be to change to a 1:1 acceptor complex more than changing the composition of this complex. The authors touch upon this point, but do not really address this issue.

The point of using Syx:SN25 (2:1 complexes) was to show (as a control) that the Syx:SN25:M18 complexes are much faster acceptors as compared to the Syx:SN25 (2:1) complexes. As has been correctly pointed out by the reviewer, the fast binding of synaptobrevin to the Syx:SN25:M18

complex probably results from the ability of Munc18-1 to prevent the formation of the (2:1) Syx:SN25 complexes. We have changed the text in the discussion to clarify this issue *(see lines 287-289).*

5) By focusing on the cleft as the decisive catalytic region of SM-proteins, the authors bypass recent evidence for structural modifications in domain 3a (helix 12 extension) being essential for SNARE assembly and synaptic vesicle priming. Again, it is in the interest of the field to clearly mention this difference and hear the opinion of the authors on this issue.
This issue has been addressed in the discussion section.

Minor issues:
- Figure 1 and 2 have the same message. It might be better to combine the two. Figure 2e could move to the supplemental data. Or it should be explained better what we learn from this figure?
The messages from Figure 1 and Figure 2 are related, but the conclusions are quite different. Figure 1 shows that a syntaxin1:SNAP25:Munc18-1 ternary complex can be purified *in-vitro* which is competent for binding to synaptobrevin and the architecture of this complex as obtained from MS/MS points out that SNAP25a interacts with the 'cleft' region of Munc18-1, which in the closed syntaxin-Munc18-1 structure is occupied by syntaxin1a. Figure 2 shows the comparison of the ternary syntaxin1:SNAP25:Munc18-1 complex with the previously characterized acceptor complexes, namely the syntaxin1:SNAP25 (2:1) and the C-terminally stabilized ΔN-complex. This comparison is important to show that the ternary syntaxin1:SNAP25:Munc18-1 complex binds synaptobrevin with kinetics comparable to the previously characterized but artificial ΔN-complex whereas the syntaxin/SNAP-25 complex binds with an at least 6-fold slower kinetics. Figure2e shows a dose-dependent response of the ternary syntaxin1:SNAP25:Munc18-1 complex in the FRET experiments.

- Line 153: very fast relative to what?
This issue has been addressed.

- In line 158 the authors mention an interesting observation concerning an assay with syntaxin(1-262), but do not show the data. Would be good if they did, since it makes the interesting point of the necessity of Syx1a transmembrane domain for Synaptobrevin binding to the Syx1:SNAP25:M18 complex.

We thank the reviewer for the suggestion. We have now added this figure in the Expanded View (EV3C).

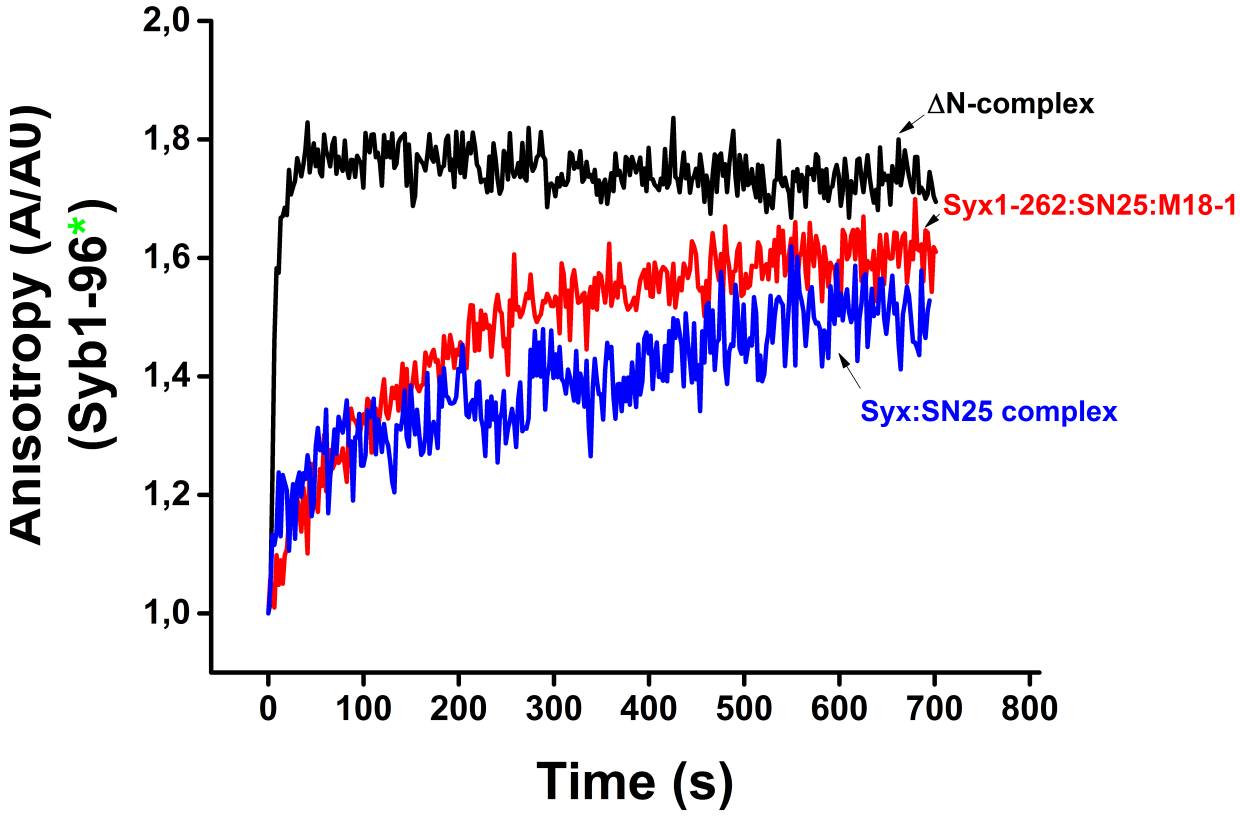


Figure EV3:

(C) Binding of synaptobrevin to the syntaxin1 (1-262):SNAP25:Munc18-1 complex (red curve) was slower as compared to the ΔN-complex (black curve) and resembled binding to the binary syntaxin1a:SNAP25 (2:1) complex (blue curve). Precise time-point measurements using this complex were, however, not performed.

- Figure EV2b: both groups seem to have reached their max anisotropy at 100ms (at least judging from the quantification). Quantification at an earlier time point might have led to the conclusion that binding of synaptobrevin to the ternary complex, while much faster than the binary complex, still be slower compared to the (non-physiological) deltaN-complex.

We thank the reviewer for the suggestion. We have now performed the quantification at 50s instead of 100s (Figure EV2 A, B).

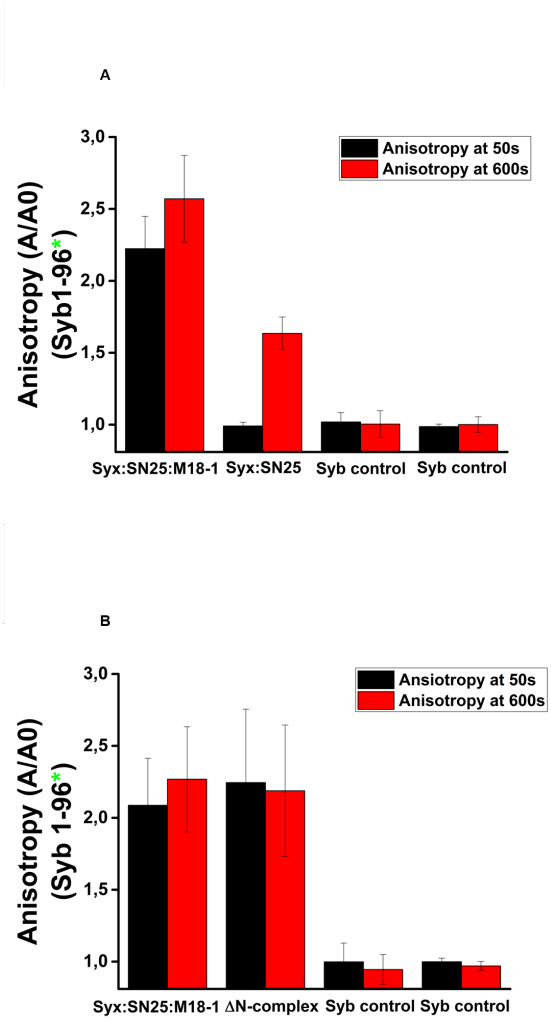


Figure EV2. (A) Quantification of synaptobrevin binding to the syntaxin1:SNAP25 complex and syntaxin1:SNAP25:Munc18-1 complex as measured by fluorescence anisotropy, and
(B) Quantification of synaptobrevin binding to the ΔN-complex and the syntaxin1: SNAP25:Munc18-1 complex. Error bars in both (A) and (B) indicate the range of values (*n=3)*

- For clarification, it would be good if the cartoons of figure 2a were used to indicate which complex was used in subsequent figures, like in figure 3
Cartoons have now been added for Figure 3 as well.

- Line 33: (typo) authors refer to Figure 4b, not 4c
- Figure order. Subfigures are not always discussed in logical order in the text (e.g. Fig 4b precedes 4a)
These issues have been addressed.

- Figure 5: Include in the figure legends that red arrows are used to indicate when chemicals were added to the mixture, while black arrows indicate the condition. This is not immediately clear now.
The explanation has now been added to the figure legend.

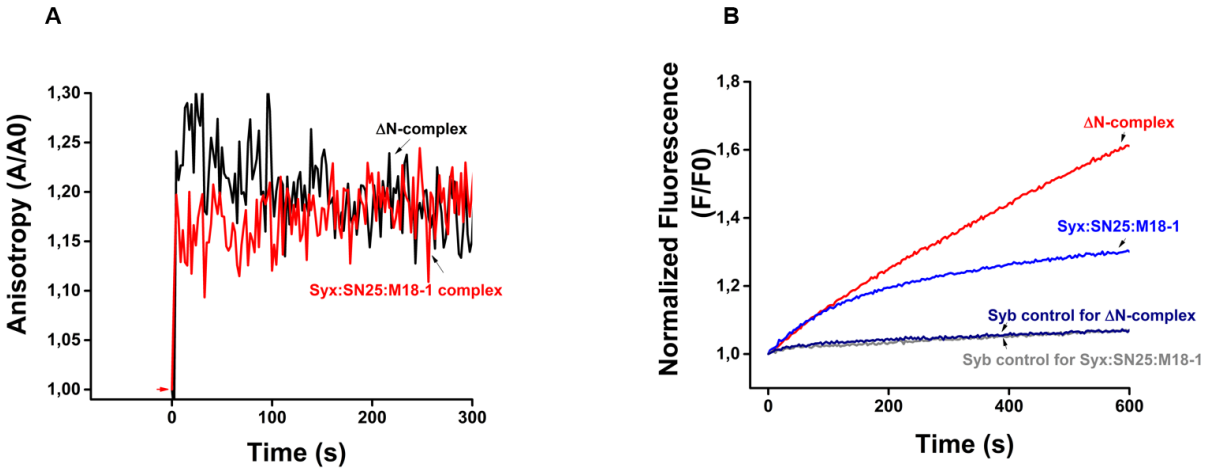
- Line 298-300: do the cross-linking experiments in figure 4 not suggest that Munc18-1 binds to the Habc domain of Syntaxin1, instead of its far N-peptide?
Yes, the cross-links in Figure 4 indicate that Munc18-1 binds to the Habc domain of syntaxin1. We thank the reviewer for pointing out this error in the text. In lines 298-300, we intended to write the N-terminal domain and not the N-peptide.

- Line 326: unformatted reference (10)
This issue has been addressed.

Referee #3:
In this article, using recombinant proteins, the authors identify a complex between syntaxin 1, SNAP-25, and Munc18-1. They further show that this complex functions as a receptor for synaptobrevin and is resistant to disassembly by NSF.
The experiments are well carried out and controlled. The identification of the interaction regions by cross-linking and MS is particularly smart and powerful. Overall, the novels finding presented herein have very important functional implications for our understanding of the molecular principles of membrane fusion and neuronal secretion.
We are very thankful to the reviewer for the positive response.

However, in its present form, this article falls short of a convincing conclusion because of the lack of data on the behavior of this ternary complex in reconstituted fusion in vitro and the lack of a demonstration of its occurrence in cells or tissue. With the addition of these complementary data, this article would bring a strong and clear demonstration.
We agree with the reviewer that it is the ultimate goal of all of this in-vitro work to reconstitute the full sequence of regulated exocytosis in-vitro using purified component and to show that intermediate steps identified in-vitro also occur in an intact cell. The reason why we, unfortunately, have not yet achieved this point (here with respect to the complex characterized in this manuscript) are as follows:

1. In-vitro fusion: The problem is that these multiprotein/membrane systems are highly complex, and many – in hindsight – erroneous conclusions were published in the past because the parameter space of these experiments is very difficult to control. The effects of accessory proteins on the fusion kinetics in such experiments are all over the place in the published literature, and the number of "molecular models" in the field (frequently contradicting each other) is still increasing.
Yes, we have carried out the requested experiments (in fact, we have been working on them since quite a while) but we decided not to include the data into the manuscript since we need more data and controls before we understand what is going on:
Briefly, we have incorporated the ternary syntaxin1:SNAP25:Munc18-1 complex into liposomes and compared it with our reference system, the reconstituted ΔN-complex. When adding synaptobrevin either in solution (soluble fragment) or in liposomes, we made the following observations (see figures below):
1. Binding of soluble synaptobrevin to both complexes is very fast, confirming that both of them serve as highly active acceptor complexes.
2. Fusion, however, is slower when using the syntaxin1:SNAP25:Munc18-1 complex than when using the ΔN-complex. The reasons for this discrepancy are not clear and presently under investigation. It is conceivable that the relative orientation of the proteins on the membrane is different. For these reasons, we are presently investigating whether using palmitoylated SNAP-25 may influence the kinetics (as recently suggested by the Tamm laboratory). Also, it is conceivable that due to its unstable nature, the complex decomposes over time, a notion supported by the observation that the initial rates are more similar between the two complexes than the later parts of the reaction. Note that additional proteins such as Munc 13 have previously been invoked in stabilization of intermediates. Moreover, both synaptotagmin and complexin have effects on the fusion rate that –depending on the system –can be quite dramatic.
For these reasons, we hope the referee agrees that it is premature to publish such data. We hope the reviewer also agrees that our biochemical characterization of this novel reactive intermediate complex is sufficiently comprehensive and novel to justify publication. In fact, we believe that only if these sub-complexes are better understood, it is possible to piece the pathway together and to find out what exactly CATCHR-proteins and the calcium regulators are doing to these intermediates.



Synaptobrevin-binding activity of the syntaxin1:SNAP25:Munc18-1 complex on liposomes and its fusion behavior with liposomes containing full-length synaptobrevin. (A) Binding of fluorescently-labeled synaptobrevin to syntaxin1:SNAP25:Munc18-1 complex reconstituted in liposomes (red curve) proceeded with similar kinetics as binding to the reconstituted ΔN-complex (black curve). (B) Kinetics of fusion between liposomes containing the ΔN-complex (red curve), and the syntaxin1:SNAP25:Munc18-1 complex (blue curve) with liposomes containing full-length synaptobrevin. In both the cases, fusion was inhibited when the acceptor liposomes were pre-incubated with the cytoplasmic fragment of synaptobrevin (dark blue and grey curves).

Concerning the presence of the syntaxin1:SNAP25:Munc18-1 complex in intact cells or membranes: So far, there is only indirect evidence, and we do not see how to change this since it is well known that SNAREs and accessory proteins rapidly re-arrange themselves upon detergent solubilisation. The best support is derived from analysis of microdomains using super-resolution microscopy in which syntaxin1, SNAP25, and Munc18-1 were seen to be colocalized on the neuronal plasma membrane (Pertsinidis *et al*, 2013). Moreover, ternary interactions were also shown by single-molecule experiments (Weninger *et al*, 2008) and also recently using electron paramagnetic resonance (Dawidowski & Cafiso, 2016).

2nd Revision - authors' response 23 March 2017

- The abstract has been altered according to the suggestion of Referee#3

3rd Editorial Decision 24 March 2017

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Most of the figures show exemplary traces (except of Fig. 3 where traces averaged from three independent experiments are shown). Quantification was performed using three independent experiments, with the bars showing the range of values as required(See legends of Figure 2,3 and EV2). Note that our conclusions are validated by several independent experimental approaches.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	not applicable
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	not applicable
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	not applicable
For animal studies, include a statement about randomization even if no randomization was used.	not applicable
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	not applicable
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5. For every figure, are statistical tests justified as appropriate?	No statistical tests were carried out
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	not applicable
Is there an estimate of variation within each group of data?	not applicable
Is the variance similar between the groups that are being statistically compared?	not applicable

C- Reagents

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The following antibodies were used for the study: SNAP25a, 71.1 (Synaptic Systems), Synaptobrevin, 69.1 (Synaptic Systems), Munc18-1 (polyclonal antibody from Struppi), NSF, 83.6 (Synaptic Systems).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	not applicable

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9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	not applicable
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	not applicable

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	not applicable
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	not applicable
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14. Report any restrictions on the availability (and/or on the use) of human data or samples.	not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	not applicable
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	not applicable

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The tables containing the cross-linking information is included in the expanded view.
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21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	This has been done
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