EMBO reports

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Vesicle uncoating regulated by SH3-SH3 domain-mediated complex formation between endophilin and intersectin at synapses

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

Editor: Barbara Pauly

1st Editorial Decision 05 August 2014

Thank you very much for the submission of your research manuscript to our editorial office and for your patience while we were waiting to hear back from the referees. We have now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I will only repeat the main points here. You will see that while referees 2 and 3 are in general more positive about the study and only raise minor issues, reviewer 1 raised several more substantial concerns about it. Upon further discussion with the other two referees, we came to the conclusion that these issues would need to be addressed before we can offer publication of your study in EMBO reports. Specifically, referee 1 points out that due to the subtle phenotype of the intersectin knockout, the physiological relevance of the endophilin/intersectin interaction remains somewhat unclear. In order to provide stronger proof for the relevance of this interaction, referee 1 suggests verifying that the mutations in endophilin that disrupt the interaction with intersectin do not interfere with endophilin's other functions. Referee 1 also recommends testing the recruitment of endophilin in mice that lack intersectin and in a situation

in which the endophilin/intersectin interaction does not occur due to mutations in one of the partners. This reviewer also highlights some other, minor, points that would require your attention, but since the detailed reports are pasted below I would rather not go into the details of them here.

Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

In this article the Haucke lab reports a novel interaction between the SH3 domain of the scaffolding protein intersectin 1 and the SH3 domain of endophilin A1, and they propose that this interaction contributes to the recruitment of endophilin A1 to specific sites of the plasma membrane where clathrin-mediated endocytosis occurs. The authors have performed different experiments to characterize this interaction in fine detail, using co-immunoprecipitations, pull-down experiments, isothermal titration calorimetry and NMR spectroscopy. These biochemical data are of good quality, but the physiological relevance based on the phenotypes shown is not sufficiently supported and I do not think the findings are of the significance expected for publication in embo reports.

My major concern is that the phenotype of intersectin 1 KO mice is extremely subtle, showing a some more coated vesicles that account for about 2% of the uncoated vesicles observed in synapses compared to 0.5% in controls (figure 1H). The authors write that "This phenotype is similar, though less severe than that observed in cortical synapses from endophilin A1-3 TKO mice [... ". Yet in those endophilin triple KO mice about 25-30% of synaptic vesicle retain their clathrin coat (Milosevic et al. Neuron 2011), suggesting that the contribution of intersectin 1 in endophilin A1 recruitment is at best very small. In fact, interfering with the endophilin A1 - intersectin 1 interaction also has only very mild effect in the giant lamprey synapse (figure S2).

The main argument provided in support of a functional role of the interaction between intersectin 1 and endophilin A is that a mutant form of endophilin A1 that can no longer bind intersectin 1 is not able to rescue vesicle uncoating in neurons from endophilin A1-3 TKO mice, in contrast to wild-type endophilin A1 (figure 4E). Given the importance of this result for the conclusions of the study I think the authors should verify that these mutations in endophilin A1 (E329K and S366K) do not impair its other functions, including the binding to partners with a proline-rich motif (dynamin1, synj1 and/or VGLUT1) via the SH3 domain and to membranes via its N-BAR domain. Without such controls I do not think that the lack of rescue can be unambiguously attributed to defective binding to intersectin 1.

The authors propose that binding to intersectin 1 contributes to endophilin recruitment. In addition to testing this model by looking at clathrin clustering as a read-out for endophilin function at the plasma membrane, they could have looked directly at the distribution of endophilin A in neurons from wild-type compared to intersectin 1 KO mice. Also the effect of mutations in endophilin A1 or in intersectin 1 that disrupt their interaction should have been tested.

minor comments:
* In figure 2A it isn't clear what the control immunoprecipitates correspond to. This should be described in the figure legend.
* The SH3B domain of intersectin 1 and the W949, Y965E variant do show different migration profiles in SDS-PAGE (figure 4A). Similar to my above comment about endophilin A1 E329K, S366K I would therefore suggest the authors to verify that this mutant protein is not misfolded.
* In figure 4C the authors should include an additional panel showing the anti-GST signal.
* In the text figure 4E is described before figure 4D, so I would suggest inverting these two panels

in the figure.

Referee #2:

The manuscript by Pechstein and colleagues reports a novel interaction between ITSN1 and endophilin. Of interest, the interaction is mediated by an SH3 domain to SH3 domain interface, and structural studies nicely define this non-canonical interaction. Functional rescue studies reveal that the interaction is required to support, in part, the role of endophilin in the uncoating of CCVs.

Overall a very interesting and well crafted study. A few issues related to the biochemistry experiments in figure 2 need to be addressed.

-Fig. 2A is of low quality. One should see ITSN1 and endophilin in the input lane. The Hsc70 control is weak. Presumably the dashed line indicates the gel has been cut but this is not indicated. This experiment needs to be repeated.

-Fig. 2C. There is a distinct mobility difference between the input and the bound for His6-endoA1. The band in lane 3 could in principle be an aggregation of the GST fusion protein. Running fusion protein alone or performing anti-His blots could clarify. This issue needs to be clarified.

Referee #3:

EMBO Reports manuscript EMBOR-2014-39260V1 by Pechstein and colleagues. In this manuscript, the authors report the accumulation of clathrin-coated vesicles in neurons from mice in which the intersectin 1 gene was genetically inactivated. The authors then show that intersectin 1 binds via its SH3 domain to a site of the SH3 domain of endophilin A1 that is different from the site via which endophilin A1 interacts with the proline-rich motifs of its classical binding partners, such as dynamin. The structural basis of this interaction is clarified, and its functional importance demonstrated in the uncoating of clathrin from endocytic vesicles.

This is a very comprehensive manuscript that defines a novel interaction between key proteins of the secretory apparatus in neurons, defines the structural basis for this interaction, and then documents the functional consequences of interfering with the newly defined interaction. The subject as such is of interest to a general readership in molecular and cellular biology, and the experiments are well performed and well described. The writing is straightforward and pleasant to read. I could not identify any major deficiency in the work as it was presented to me, and would recommend it to the community.

1st Revision - authors' response 06 November 2014

Response to referees:
(reviewers' comments are given in italics, our response to each point is given below).

We would like to thank all three referees for their careful reading of our Ms and for their supportive and constructive comments that have greatly helped in improving our study and tailoring the paper for the readership of *EMBO Reports*.

Referee #1:
In this article the Haucke lab reports a novel interaction between the SH3 domain of the scaffolding protein intersectin 1 and the SH3 domain of endophilin A1, and they propose that this interaction contributes to the recruitment of endophilin A1 to specific sites of the plasma membrane where clathrin-mediated endocytosis occurs. The authors have performed different experiments to characterize this interaction in fine detail, using coimmunoprecipitations, pull-down experiments, isothermal titration calorimetry and NMR spectroscopy. These biochemical data are of good quality, but the physiological relevance

based on the phenotypes shown is not sufficiently supported and I do not think the findings are of the significance expected for publication in embo reports.

Response:
We thank the referee for his/ her positive comments on our Ms and the quality of the data contained therein. We respectfully disagree, however, with the notion that the physiological relevance of our findings is insufficiently supported.

As explained in more detail below, we provide evidence for the compensation of intersectin 1 loss-of-function by the structurally and functionally highly similar sister protein intersectin 2. We also include a Figure for referee 1, which shows that double KO mice lacking both intersectin 1 and 2 are born well below Mendelian ratios and suffer from severly reduced postnatal viability and growth (Figure 1 for referee 1). We hope that the referee will agree that these exciting results warrant a careful analysis of the phenotype of intersectin 1/2 DKO mice that in our opinion goes well beyond the scope of the present Ms. Yet, in any case these data explain the comparably weak phenotype of intersectin 1 KO mice when compared to endophilin A1-3 triple KOs.

Following the suggestion of the referee we also provide additional functional insights into the role of intersectin-endophilin complex formation described in our paper: Specifically, we have conducted an analysis of the localization of endophilin A1 in wild-type vs. intersectin 1 KO neurons. Intriguingly, we find that targeting of endophilin A1 to sites of clathrin/ AP-2 mediated SV reformation is impaired in the absence of intersectin 1 (**new Figure 4E**). We consider this finding particularly important in light of new data from McMahon and colleagues regarding a key role of endophilin in clathrin-*in*dependent endocytosis that will appear in *Nature* later this month as our results explain how endophilin, which itself does not interact with clathrin coat proteins, is recruited to clathrincoated pits. Together with the observed partial failure of mutant intersectin-binding defective endophilin A1 to fully rescue clathrin/ AP-2 accumulation at synapses of endophilin A1-3 triple KO mice these data provide further support for our conclusion that complex formation of endophilin A with intersectin regulates vesicle uncoating at synapses - a still poorly understood process.

Lastly, we would like to reiterate here the overall significance and high general interest of our findings:

(1) We identify a molecular mechanism for targeting endophilin to sites of clathrin-mediated SV reformation that critically depends on intersectin 1 and explains the functional concurrence of the two proteins.

This key finding almost certainly will be met with great interest in view of the new data from McMahon and colleagues regarding a key role of endophilin in clathrinindependent endocytosis that will appear in *Nature* later this month alluded to above.

(2) Our data identify intersectin 1 as a novel regulator of vesicle uncoating at synapses *in vivo*, a finding of medical interest given the fact that intersectin 1 is overexpressed in Down syndrome.

(3) The association of endophilin with intersectin represents one of less than a handful of examples where SH3-SH3 domain interactions between proteins underlie complex formation. The observed “backside” binding of endophilin SH3 allows for independent binding of proline-rich motifs to the canonical site thereby assigning a non-cooperative adaptor function to this domain.
We hope that the referee will agree that the insights provided in our paper are well within the scope of *EMBO Reports*.

My major concern is that the phenotype of intersectin 1 KO mice is extremely subtle, showing a some more coated vesicles that account for about 2% of the uncoated vesicles observed in synapses compared to 0.5% in controls (figure 1H). The authors write that

"This phenotype is similar, though less severe than that observed in cortical synapses from endophilin A1-3 TKO mice [... ". Yet in those endophilin triple KO mice about 25-30% of synaptic vesicle retain their clathrin coat (Milosevic et al. Neuron 2011), suggesting that the contribution of intersectin 1 in endophilin A1 recruitment is at best very small. In fact, interfering with the endophilin A1 - intersectin 1 interaction also has only very mild effect in the giant lamprey synapse (figure S2).

Response:
We provide evidence for the compensation of intersectin 1 loss-of-function by the structurally and functionally highly similar sister protein intersectin 2. In the **new Figures 2C and 2D** we demonstrate that the SH3 domain of endophilin A1 specifically binds to intersectin 2 much the same way as intersectin 1. These data together with prior observations indicate that intersectin 2 is able to functionally compensate for intersectin 1 loss. Consistent with this possibility, we observe strong about 2-fold upregulation of intersectin 2 levels in brains or hippocampi derived from intersectin 1 KO mice. These new data are shown in the new **Figure S4D**.

As said above, we have included a figure for the referee, which shows that double KO mice lacking both intersectin 1 and 2 are born well below Mendelian ratios and suffer from severly reduced postnatal viability and growth (**Figure 1 for referee 1**). These data explain the comparably weak phenotype of intersectin 1 KO mice when compared to endophilin A1-3 triple KO mice.

We nonetheless agree with the referee that one might not expect an exact phenocopy of the strong phenotypes seen in endophilin A1-3 triple KOs in mice lacking intersectin 1 only. We have therefore somewhat downtoned our corresponding statements in the text, though we note that all phenotypic changes described in both lamprey and mouse synapses are highly statistically significant and, hence, are consistent with the physiological relevance of intersectin-endophilin complex formation *in vivo*.

The main argument provided in support of a functional role of the interaction between intersectin 1 and endophilin A is that a mutant form of endophilin A1 that can no longer bind intersectin 1 is not able to rescue vesicle uncoating in neurons from endophilin A1-3 TKO mice, in contrast to wild-type endophilin A1 (figure 4E). Given the importance of this result for the conclusions of the study I think the authors should verify that these mutations in endophilin A1 (E329K and S366K) do not impair its other functions, including the binding to partners with a proline-rich motif (dynamin1, synj1 and/or VGLUT1) via the SH3 domain and to membranes via its N-BAR domain. Without such controls I do not think that the lack of rescue can be unambiguously attributed to defective binding to intersectin 1.

Response:
We show in **new revised Figures 4B and C** that mutant endophilin A1 E329K,S336K avidly binds to all other known endophilin A1 binding partners such as dynamin 1, synaptojanin 1, and VGLUT1. These data are further supported by our NMR analyses, which clearly demonstrate the presence of two independent binding sites on endophilin A1-SH3 for VGLUT1 and intersectin 1 (see **Figure 3**).

We have also carried out fractionation experiments to analyze the ability of mutant endophilin to associate with membranes. As demonstrated in the **new Figure 4D** mutant endophilin A1 E329K,S336K partitions indistinguishably between cytosolic/ soluble and membrane fractions when compared to wild-type endophilin A1.

These results clearly exclude the possibility that the functional deficits observed in rescue experiments using endophilin A1-3 triple knockout neurons expressing mutant endophilin A1 are due to impaired association of the mutant protein with other known interactors including dynamin, synaptojanin, and VGLUT1, or with membrane lipids and, thus, confirm the validity of our conclusions.

The authors propose that binding to intersectin 1 contributes to endophilin recruitment. In

addition to testing this model by looking at clathrin clustering as a read-out for endophilin function at the plasma membrane, they could have looked directly at the distribution of endophilin A in neurons from wild-type compared to intersectin 1 KO mice.

Response:
We are grateful to the referee for this suggestion that we have gladly followed. We have conducted an analysis of the localization of endophilin A1 in wild-type vs. intersectin 1 KO neurons. Intriguingly, we find that targeting of endophilin A1 to sites of clathrin/ AP-2 mediated SV reformation is impaired in the absence of intersectin 1 (**new Figure 4E**). We consider this finding particularly important in light of new data from McMahon and colleagues regarding a key role of endophilin in clathrin-*in*dependent endocytosis that will appear in *Nature* later this month as our results explain how endophilin, which itself does not interact with clathrin coat proteins, is recruited to clathrin-coated pits.

Also the effect of mutations in endophilin A1 or in intersectin 1 that disrupt their interaction should have been tested.

Response:
This is precisely what we have done in the experiment shown in revised Figure 4F,G, where we have carried out rescue experiments in endophilin A1-3 triple KO neurons using either wild-type or intersectin-binding defective mutant endophilin A1. Our data clearly demonstrate that re-expression of intersectin-binding defective mutant endophilin A1 (E329K, S366K) (Figure 4F) only resulted in a minor rescue reflected in significantly elevated clathrin puncta size compared to either wild-type neurons or TKO neurons expressing wild-type endophilin A1. The inability of mutant endophilin A1 to fully rescue clathrin accumulation in endophilin TKO neurons was not due to protein instability as WT and mutant endophilin A1 were expressed to the same levels (Figure 4G). We agree with referee 1 that these data indeed strongly support an important physiological function for endophilin-intersectin complex formation in vesicle uncoating *in vivo*.

minor comments:
** In figure 2A it isn't clear what the control immunoprecipitates correspond to. This should be described in the figure legend.*

Response:
We have repeated the experiment that is now shown the revised Figure 2A. The control immunoprecipitation was done using non-immune rabbit IgG as described in the revised legend.

** The SH3B domain of intersectin 1 and the W949, Y965E variant do show different migration profiles in SDS-PAGE (figure 4A). Similar to my above comment about endophilin A1 E329K, S366K I would therefore suggest the authors to verify that this mutant protein is not misfolded.*

Response:
As there are no other known interaction partners for the SH3B of intersectin 1, we have opted to eliminate Figure 4A from the revised Ms, which was simply meant to confirm our NMR analysis.

** In figure 4C the authors should include an additional panel showing the anti-GST signal.*

Response:
In the revised Figure 4B we have now included the Ponceau S stain of the nitrocellulose membrane, which clearly shows that equal amounts of GST-fused endophilin A1 WT and mutants were used for the experiment.

** In the text figure 4E is described before figure 4D, so I would suggest inverting these two panels in the figure.*

Response:

We are thankful to the referee and have modified the text accordingly.

Referee #2:
The manuscript by Pechstein and colleagues reports a novel interaction between ITSN1 and endophilin. Of interest, the interaction is mediated by an SH3 domain to SH3 domain interface, and structural studies nicely define this non-canonical interaction. Functional rescue studies reveal that the interaction is required to support, in part, the role of endophilin in the uncoating of CCVs.

Overall a very interesting and well crafted study. A few issues related to the biochemistry experiments in figure 2 need to be addressed.

Response: We thank the referee for highlighting the importance and quality of our study and for his/ her enthusiastic recommendation to publish our Ms in *EMBO Reports*.

-Fig. 2A is of low quality. One should see ITSN1 and endophilin in the input lane. The Hsc70 control is weak. Presumably the dashed line indicates the gel has been cut but this is not indicated. This experiment needs to be repeated.

Response:
We have repeated the experiment that is now shown the revised Figure 2A: Both the input and the Hsc70 signal are now clearly visible.

-Fig. 2C. There is a distinct mobility difference between the input and the bound for His6-endoA1. The band in lane 3 could in principle be an aggregation of the GST fusion protein. Running fusion protein alone or performing anti-His blots could clarify. This issue needs to be clarified.

Response:
The referee is correct in that purified endophilin A1 migrates slightly differently in the input and the GST-SH3B lanes. This is due to the fact that the total protein amount in these lanes is different and frequently observed in our experience. We have modified the figure now to reveal the entire gel.

Referee #3:
EMBO Reports manuscript EMBOR-2014-39260V1 by Pechstein and colleagues. In this manuscript, the authors report the accumulation of clathrin-coated vesicles in neurons from mice in which the intersectin 1 gene was genetically inactivated. The authors then show that intersectin 1 binds via its SH3 domain to a site of the SH3 domain of endophilin A1 that is different from the site via which endophilin A1 interacts with the proline-rich motifs of its classical binding partners, such as dynamin. The structural basis of this interaction is clarified, and its functional importance demonstrated in the uncoating of clathrin from endocytic vesicles.

This is a very comprehensive manuscript that defines a novel interaction between key proteins of the secretory apparatus in neurons, defines the structural basis for this interaction, and then documents the functional consequences of interfering with the newly defined interaction. The subject as such is of interest to a general readership in molecular and cellular biology, and the experiments are well performed and well described. The writing is straightforward and pleasant to read. I could not identify any major deficiency in the work as it was presented to me, and would recommend it to the community.

Response: We thank the referee for highlighting the importance and quality of our study and for his/ her enthusiastic recommendation to publish our Ms in *EMBO Reports*. No further action was necessary.

Figure 1 for referee1:

Data not shown.

2nd Editorial Decision 25 November 2014

Many thanks for your patience while the referees assessed the revised version of your manuscript. I am happy to tell you that the two reviewers now support publication of your study after some minor issues (as indicated in the reports below) have been addressed. I am therefore making an 'accept-in-principle' decision, which basically means that I will be happy to officially accept your manuscript for publication once you have addressed the minor concern of referee 1.

Once you have made these minor revisions, please submit the final version of your study through our website.

REFEREE REPORTS:

Referee #1:

The authors have provided additional experimental results to address all my concerns about the original manuscript and I am largely satisfied with the new figures. I would only suggest that they include images corresponding to the new figure 4G in supplemental figure 4. A clear description of the way clathrin clustering was quantified should also be included in the material and methods, as the text refers both to "puncta intensity" and "puncta size" while the bar graph in figure 4E shows normalized intensity.

Regarding the text itself I think the authors now adopted the right tone to discuss their results, and that the new data they present regarding the likely overlapping roles of intersectin 1 and 2 may well explain the relatively mild phenotypes caused by intersectin 1 KO. Finally, I also agree that their findings deserve more attention in the context of new data from McMahon and colleagues that will soon be published in Nature. I would thus recommend the revised manuscript for publication.

Referee #2:

My two minor issues with the manuscript have been addressed in this revised version, which is now suitable for publication.

2nd Revision - authors' response 28 November 2014

We were delighted to hear that our manuscript has been accepted in principle for publication in *EMBO Reports*. We have addressed the remaining minor concern of referee 1 by including images corresponding to the new figure 4G into supplemental figure 4. We have also added a more detailed description of the method used to quantify clathrin clustering in the extended materials and methods

section. The wording in the results section of the main manuscript has been changed to more precisely describe that clathrin intensity was quantified rather than puncta size.

We very much hope that this final revised version of our manuscript is now officially acceptable for publication in *EMBO Reports* and look forward to hearing from you.

3rd Editorial Decision 01 December 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

