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Molecular basis for SNX-BAR-mediated assembly of distinct endosomal sorting tubules.

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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.)

1st Editorial Decision 15 June 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see, the referees all consider the study as interesting and important and would support its publication here after appropriate revision. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript that addresses the points put forward by the referees in an adequate manner and to their satisfaction. In particular, it will be important to include in vivo (cell culture) data along the lines suggested by referee 2 to strengthen the functional and physiological aspect of the study and to address the mechanistic points raised by the other two referees. Please do not hesitate to get back to us at any time in case you would like to discuss any aspect of the revision further.

I should add that it is EMBO Journal policy to allow only a single round of revision and that acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the

conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript by van Weering et al. describes a global analysis of the entire SNX-BAR protein family, which includes proteins that are involved in generating and organizing the tubular endosomal network. By comparing the tubulation activity of all twelve human SNX-BAR proteins, the authors demonstrate that only a subset of proteins facilitates vesicle-to-tubule transitions, while others only bind to vesicles without deforming them. A new crystal structure of the BAR domain of SNX1 and structure-based modeling reveals further heterogeneity within the SNX-BAR family. The structural analysis provides a rationale for the distinct homo- and heterodimerization preferences, which the authors observed in SNX-BAR family-wide pull-down experiments. They also identify an amphipathic helix as a conserved feature of SNX-BAR proteins. Finally, they establish the importance of the BAR domain tip region as another determining factor that distinguishes tubulation-active vs inactive SNX-BAR proteins.

Overall, this is a well-written, comprehensive manuscript, providing valuable insight into the molecular mechanisms that act in concert to generate a compartmentalized endosomal network. It demonstrates the importance of three conserved features within the SNX-BAR family: 1. Dimerization of the BAR domains; 2. Membrane interactions involving an amphipathic helix; and 3. The tip region of the BAR domain dimer. It is also an interesting study with regard to the evolution of SNX-BAR proteins. Altogether, it will be of great interest to the field.

There are several points the authors may want to consider:
1. It is clear from the data that the distal tip region of the BAR dimer is an important feature for the tubulation activity of a subset of SNX-BAR domain proteins. In particular, the authors show that the tip of the tubulating SNX1, introduced into an otherwise tubulation-incompetent SNX-BAR protein, confers tubulation activity. I am just not convinced that the authors have demonstrated unambiguously that the positive effect of the tips is due to lattice contacts or tip-to-tip interactions. The experimental evidence and analysis is probably the weakest point of the manuscript, although I acknowledge the complexity of proofing this point beyond doubt (given the cooperativity that is built into this system). Yet, an alternative worth discussing is the more trivial explanation that introducing two positively charged residues at the tips may increase membrane affinity, especially at these critical locations on the BAR domain dimer. While it is an elegant solution for the generation of distinct membrane compartments or tubules, I am not sure that one has to infer tip-to-tip interactions as the underlying mechanism. I suggest a less biased discussion regarding this point.

2. Is it sufficient to mutate two Snx5 tip residues to lysines (rather than swapping out the entire tip region)?

3. Figure 7D: How does the SNX1-K442/445A mutant behave in liposome pelleting assays?

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heterodimers? Such an analysis may reveal secondary interactions important for high-affinity heterodimerization.

5. There is very little discussion regarding the functional roles of the PX domain, which is present in the SNX-BAR proteins. Is it possible that a difference in their affinity for certain phosphatidylinositol phosphate head groups could account for homomeric (lattice) interactions, as part of a conincidence detection mechanism and the generation of distinct endosomal tubules?

6. Given the nature of the work, the reader would benefit from a final table summarizing the results. Such a table could list the isoform-specific membrane deformation potential, dimerization preferences, domain makeup of the SNX-BAR proteins, and the sequence of the amphipathic helix.

7. Is it possible that the non-tubulating SNX-BAR proteins are autoinhibited, rather than inactive? A functional comparison between full-length and PX-BAR domains would address this question.

8. The authors mention differences between the SNX1-BAR domain structure and the previously published SNX9-PX-BAR domain structures (Pylypenko et al., EMBO J, 2007). Illustrating a side-by-side comparison would be a valuable addition to Figure 4.

9. The authors mention the possibility of conformational changes within the BAR domain dimer (in the results section dealing with the SNX1 crystal structure) and tip-to-tip interactions of SNX-BAR proteins (in the discussion). Both aspects have been described for SNX9 previously (Wang et al., Structure, 2008), and the author could consider discussing these independent results in the present context.

10. Could the authors comment on the poor correlation of hydrophobic extent of the amphipathic helix and tubulation activity/tube diameter?

11. What would happen when liposomes were presented with two tubulation capable SNX-BAR proteins with distinct curvature prefences?

12. As a final comment, on the philosophical side, considering the hetero-dimerization of SNX-BAR proteins (especially of tubulation-active and inactive isoforms), could the authors discuss a functional consequence for the cell?

Referee #2 (Remarks to the Author):

Van Weering et al. Molecular insight into the SNX-BAR-mediated formation of distinct endosomal sorting tubules.

In this manuscript the authors report an extensive study of the mechanics of tubules formation by Sorting nexin proteins using in-vitro assays. This work builds on and extends what has been published previously that Sorting nexins of the Snx-BAR family can mediate tubule formation from endosomes and other organelles.
The approach of conducting the experiments using an in-vitro system of liposome binding/tubulation is both a strength and a weakness. Clearly in order to study the ability of individual Sorting nexin proteins to mediate tubule formation, an in vitro approach is appropriate. However, this approach and the conclusions that can be drawn from it, is also limited by virtue of the fact that in-vivo, tubule formation involves a multitude of factors including key roles for the cytoskeleton and possibly other proteins such as clathrin.
Given the extensive and detailed nature of the study by Van Weering et al., it would be wrong to suggest that the study is too preliminary but I do think that some additional data that explores the requirements of tubule formation in vivo would strengthen the study and broaden its appeal.

Major points:

1. Is there any evidence that Snx1 operates as a homo dimer in vivo? Much of the in-vitro evidence presented involves use of a single sorting nexin protein added to liposomes. The conclusions drawn from this approach are open to question however as, in vivo, Snx1 associates with Snx5 or Snx6.

The yeast homolog of Snx1 is Vps5p that functions as an obligate heterodimer with Vps17p. I think the authors should exercise caution and restraint when interpreting their data regarding the tubulation observed when single sorting nexin proteins are tested in their assay.
2. A set of experiments that the authors could perform that would exploit the in-vitro system fully is to test the effect of different splice variants of sorting nexin proteins on tubulation. For example, Snx1 is alternatively spliced to generate three variants, could the authors test these different variants in the tubulation assay to explore whether the different variants confer different biological properties?
3. Could the authors test some of the mutations they made in the amphipathic helices or tip-loop residues in an in vivo situation? The authors have experience of imaging tubule formation using GFP-tagged constructs. Whilst I accept there are limitations to how much can be learned in a relatively 'dirty' whole-cell system, extending the observations made through the in-vitro assays to the cell would add much to the biological relevance of the study.
4. How do the observed liposome-derived tubules for Snx9, Snx18 and Snx33 compare to the in-vivo situation? Clearly Snx1-tubules formed in-vitro are a good fit for the in-vivo tubules observed, but does this hold true for the other sorting nexins investigated?
5. It looks to me that Snx8 is somehow better at binding to liposomes that many other Snx proteins tested. Is this real? Is there an explanation for this observation?
6. The model at the end suggests that tubules generated by different snx proteins can form from the same endosome. Can this be tested in vitro using, for example, recombinant Snx1 and Snx4 simultaneously to determine if the model is valid. It would be necessary in such an assay to label the tubules formed with antibodies and this may be a limiting factor but it should be possible to express the recombinant proteins with epitope tags to aid detection.
7. A minor point really but I think the title is a little strange. I'm not sure what a "molecular insight" is. I would suggest that a change in the wording that reflects the findings is appropriate; for example, "In vitro analysis of SNX-BAR assembly reveals insights into the molecular mechanisms of distinct endosomal tubule formation."

Referee #3 (Remarks to the Author):

The manuscript by Van Weering et al evaluates systematically the membrane binding and remodeling activities of the mammalian SNX-BAR proteins. Their contribution clarifies the nature of the hetero- and homo-dimers formed within the family--with implications for understanding sorting specificity within the endosomal system. Moreover, their observations are consistent with a large number of reports that focus on three mechanisms at work in membrane re-modeling: 1) the specificity of BAR domain dimer-interfaces and the resulting degree of overall curvature generated by dimerization; 2) the insertion of amphipathic helices into one leaflet to buckle the membrane; and 3) the propagation of local curvature over longer length scales by higher-order oligomer formation between neighboring BAR domains. The work reported is a useful step forward, although I have two major comments:

1) Several membrane-binding proteins that tubulate liposomes in vitro will also vesiculate liposomes. The balance between stable tubules versus small vesicles depends on a few factors, including lipid-to-protein ratio, lipid species within the model membranes, temperature and the melting point of the abundant lipid species used, and finally the mechanical forces involved in depositing onto charged TEM grids and subsequent dessication and staining. To my eye, the negative stain TEM for all the Snxs (including the non-tubulating Snx5, Snx7, Snx30 and Snx5-AA) suggest some degree of small vesicle formation. It seems critical to me to evaluate this for this paper. There are many ways of doing so, including 1) reverse sedimentation centrifugation, 2) light scattering, 3) cryo-TEM, 4) evaluating the lipid content of the supernatant versus the pellet of a sedimentation assay (See Boucrot et al Cell 2012 for example).

2) While I agree with their overall view of the importance of higher-order oligomer/coat formation, this manuscript does not report a compelling loss-of-function mutant for coat formation. The two tip lysines that were mutated may be involved in electrostatic attraction to the membrane rather than coat formation (though even the double mutant does not appear to have a l-o-f phenotype). The chimera that confers tubulation competence to Snx5 is notable, but the chimera involves a large region (41 amino acids). As with F-BAR and N-BAR proteins that appear to form helical coats, the authors could notably strengthen their claim for the sorting nexins if they identified key residues that

mediate specific inter-subunit interactions and show that mutants have phenotypes in vitro and in living cells.

Minor comments:
1) Fig 6C, some of the TEM panels are too "red", suggesting an unusual color balance.

2) I believe there is a spelling error on Page 13, the last fifth, "from" should be form.

1st Revision - authors' response 05 September 2012

Referee comments are quoted in italic.
Referee #1 (Remarks to the Author):

The manuscript by van Weering et al. describes a global analysis of the entire SNX-BAR protein family, which includes proteins that are involved in generating and organizing the tubular endosomal network. By comparing the tubulation activity of all twelve human SNX-BAR proteins, the authors demonstrate that only a subset of proteins facilitates vesicle-to-tubule transitions, while others only bind to vesicles without deforming them. A new crystal structure of the BAR domain of SNX1 and structure-based modeling reveals further heterogeneity within the SNX-BAR family. The structural analysis provides a rationale for the distinct homo- and heterodimerization preferences, which the authors observed in SNX-BAR family-wide pull-down experiments. They also identify an amphipathic helix as a conserved feature of SNX-BAR proteins. Finally, they establish the importance of the BAR domain tip region as another determining factor that distinguishes tubulation-active vs inactive SNX-BAR proteins. Overall, this is a well-written, comprehensive manuscript, providing valuable insight into the molecular mechanisms that act in concert to generate a compartmentalized endosomal network. It demonstrates the importance of three conserved features within the SNX-BAR family: 1. Dimerization of the BAR domains; 2. Membrane interactions involving an amphipathic helix; and 3. The tip region of the BAR domain dimer. It is also an interesting study with regard to the evolution of SNX-BAR proteins. Altogether, it will be of great interest to the field.

There are several points the authors may want to consider:
1. It is clear from the data that the distal tip region of the BAR dimer is an important feature for the tubulation activity of a subset of SNX-BAR domain proteins. In particular, the authors show that the tip of the tubulating SNX1, introduced into an otherwise tubulation-incompetent SNX-BAR protein, confers tubulation activity. I am just not convinced that the authors have demonstrated unambiguously that the positive effect of the tips is due to lattice contacts or tip-to-tip interactions. The experimental evidence and analysis is probably the weakest point of the manuscript, although I acknowledge the complexity of proofing this point beyond doubt (given the cooperativity that is built into this system). Yet, an alternative worth discussing is the more trivial explanation that introducing two positively charged residues at the tips may increase membrane affinity, especially at these critical locations on the BAR domain dimer. While it is an elegant solution for the generation of distinct membrane compartments or tubules, I am not sure that one has to infer tip-to-tip interactions as the underlying mechanism. I suggest a less biased discussion regarding this point.

We have altered the discussion to reflect the point raised by the reviewer. Indeed, we cannot exclude that the tip-loop region of SNX1 in the SNX5-BAR domain regulates interactions - other then the tip-to-tip contacts - to further stabilize the membrane tubule. It is however unlikely that K442 or K445 are responsible for this effect, as we show that these residues are dispensable for the membrane remodeling

capacity of SNX1 (Figure 7B, and see also comment below). Furthermore, the liposome sedimentation assays do not indicate a difference in membrane association between SNX5-WT and SNX5-1TIP, suggesting that a protein-protein interaction(s) is/are responsible for the gain-of-function of this SNX5 mutant rather than a general increase in membrane association.

2. Is it sufficient to mutate two Snx5 tip residues to lysines (rather than swapping out the entire tip region)?

To clarify the difference between SNX5-WT and SNX5-1TIP mutant, we have included the amino-acid alignment of the tip-loop region of these proteins and the charge distribution of the SNX5-WT and SNX5-1TIP in a new supplementary figure (Supplementary Figure S7). These show that the predicted tip-loop regions of SNX5 have very similar charge distribution to that observed with SNX1. More specifically, the tip-loop region contains similar positioned lysines as SNX1 (SNX5 K332/ K334), indicating that the presence of lysines in the tip-loop region is apparently not different between these SNX-BARs.

3. Figure 7D: How does the SNX1-K442/445A mutant behave in liposome pelleting assays?

We have included the SNX1-K442/445A sedimentation data in a revised Figure 7D, confirming that the mutant associates with liposomes to a similar extent as wild-type.

4. The SNX1 crystal structure and SNX5 homology model highlight the importance of charged residues in the BAR domain dimer interface, which destabilize SNX1 homodimers in favor of the formation of SNX1-SNX5 complexes. Have the authors attempted the modeling of SNX1-SNX5 heterodimers? Such an analysis may reveal secondary interactions important for high-affinity heterodimerization.

The sequence homology among the BAR domains of SNX-BAR proteins is low (less then 20%), which makes molecular modeling very challenging. For this reason, we only included data from the SNX5 homology model that we could experimentally verify in immuno-precipitation and/or membrane remodeling assays. We have however fitted the SNX1 crystal structure and the SNX5 homology model to construct a SNX1:SNX5 heterodimer model and, apart from the charged SNX1 R337 and SNX5 E280/E383 residues, no obvious residues stand out as contributing to the specific SNX1:SNX5 dimerization. However, we prefer not to include this "mixed" SNX1:SNX5 dimer model because it could be misleading due to the uncertainties outlined above.

5. There is very little discussion regarding the functional roles of the PX domain, which is present in the SNX-BAR proteins. Is it possible that a difference in their affinity for certain phosphatidylinositol phosphate head groups could account for homomeric (lattice) interactions, as part of a conincidence detection mechanism and the generation of distinct endosomal tubules?

Phosphoinositide binding to the PX domain is crucial for the recruitment of SNX-BAR proteins to different membrane domains of the endocytic network. For example, SNX9 associates with PtdIns(4,5)P_2 and is mainly localized to endocytic pits on the plasma membrane (Yarar et al., (2008) Traffic 9, 133-146; Nunez et al., (2011) Traffic 12, 1868-1878), while SNX1 has affinity for PtdIns(3)P (and PtdIns(3,5)P_2) and is associated with early and early-to-late transition endosomes (Cozier et al., (2002) J Biol Chem 277, 48730-48736; Mari et al., (2008) Traffic 9, 380-393; van Weering et al., (2012) Traffic 13, 94-107). For these proteins, the formation of tubules on spatially separated compartments will therefore be determined, in part, by their distinct phosphoinositide-binding profiles. However for other SNX-BARs, for example the PtdIns(3)P-binding SNX4 and SNX8, co-localisation is observed with SNX1 on PtdIns(3)P-enriched endosomes (Traer et al., (2007) Nat Cell Biol 9, 1370-1380; van Weering et al., (2012) Traffic 13, 94-107). From these compartments live cell imaging has revealed the formation of distinct tubules emanating from the same endosomal vacuole, for instance distinct SNX1- and SNX4-

decorated tubules (Traer et al., (2007) Nat Cell Biol 9, 1370-1380). In these cases, differences in phosphoinositide-binding would appear insufficient to explain the formation of such distinct tubules (Note: the Folch bovine brain lipid extracts used in the present study contains a number of phosphoinositides including PtdIns(4,5)P_2). Finally, it is worth noting that there is no correlation between phosphoinositide binding profiles and the ability of SNX-BARs to drive tubulation: for example SNX4 (forms tubules) and SNX7 (does not form tubules) each associate with PtdIns(3)P (Xu et al., (2001) Nat Cell Biol 3, 658-666; Traer et al., (2007) Nat Cell Biol 9, 1370-1380). Also, by selective mutation of the BAR domain of the non-tubulating SNX5 we have engineered a SNX5 mutant capable of remodeling membranes; in this instance, the SNX5 PX domain was common to both wild-type and mutant proteins, again consistent with variation in phosphoinositide-binding not being a major determinant in lattice formation.

6. Given the nature of the work, the reader would benefit from a final table summarizing the results. Such a table could list the isoform-specific membrane deformation potential, dimerization preferences, domain makeup of the SNX-BAR proteins, and the sequence of the amphipathic helix.

We have included an overview of the data presented in new Table III and an outline of the domain structure of the human SNX-BAR proteins in Supplementary Figure 3A.

7. Is it possible that the non-tubulating SNX-BAR proteins are autoinhibited, rather than inactive? A functional comparison between full-length and PX-BAR domains would address this question.

The non-tubulating SNX5 is composed almost exclusively of PX and BAR domains. Of the 404 amino acids encoding for the SNX-BAR, the PX domain stretches from residue 32-169 and the BAR domain from 179-393, leaving 31 N-terminal and 10 C-terminal residues outside these annotated domains (Supplementary Figure 3A). Since these residues are also present in the SNX5AA-1TIP mutant that can remodel liposomes into tubules (Fig. 7C), we have no evidence that these residues are inhibiting SNX5-WT to form membrane tubules.

8. The authors mention differences between the SNX1-BAR domain structure and the previously published SNX9-PX-BAR domain structures (Pylypenko et al., EMBO J, 2007). Illustrating a side-by-side comparison would be a valuable addition to Figure 4.

We have included the side view of the SNX9-BAR dimer in Figure 4B to illustrate the comparison to the SNX1-BAR dimer.

9. The authors mention the possibility of conformational changes within the BAR domain dimer (in the results section dealing with the SNX1 crystal structure) and tip-to-tip interactions of SNX-BAR proteins (in the discussion). Both aspects have been described for SNX9 previously (Wang et al., Structure, 2008), and the author could consider discussing these independent results in the present context.

We have highlighted this in the discussion.

10. Could the authors comment on the poor correlation of hydrophobic extent of the amphipathic helix and tubulation activity/tube diameter?

The amphipathic helix, the PX domain and the BAR-domain all contribute to the membrane remodeling as shown in the present study and by others (*e.g.* Carlton et al (2004) Curr Biol 14, 1791-1800; Pylypenko et al., (2007) EMBO J 26, 4788-4800). It is clear that a functional amphipathic helix is essential for membrane remodeling, but tubule diameter will most likely be determined by the geometry of the SNX-BAR oligomer. Such regulation of tubule diameter has been suggested for F-BAR domains (Frost et al., (2008) Cell 132, 807-817). We have added this comment in the discussion.

11. What would happen when liposomes were presented with two tubulation capable SNX-BAR proteins with distinct curvature prefences?

We have included new experimental data of preincubating SNX9 (forming ~60 nm tubules) with SNX33 (forming ~20 nm tubules) before adding the protein mixture to the liposomes. We observed two completely separated populations of tubules of ~ 20 nm and ~60 nm, respectively. These results suggest that SNX9 and SNX33 form two distinct sets of tubules *in vitro,* and do not mix identity. We have included these results in new Figure 7E and F.

12. As a final comment, on the philosophical side, considering the hetero-dimerization of SNX-BAR proteins (especially of tubulation-active and inactive isoforms), could the authors discuss a functional consequence for the cell?

Previous work from our lab has shown that the SNX1- and SNX2-retromer complexes carry different cargo (Bujny et al. 2007 J Cell Sci 120 pp2010-21). Hence, the expansion from one retromer containing the VPS5-homodimer in *T. brucei* to 6 possible SNX-BAR heterodimer variants (SNX1 or SNX2, with SNX5, SNX6 or SNX32) of retromer in mammalian cells could provide a mechanism for differential regulation of selective cargo trafficking through the retromer pathway. We have included this point in the discussion.

Referee #2 (Remarks to the Author):

Van Weering et al. Molecular insight into the SNX-BAR-mediated formation of distinct endosomal sorting tubules.

In this manuscript the authors report an extensive study of the mechanics of tubules formation by Sorting nexin proteins using in-vitro assays. This work builds on and extends what has been published previously that Sorting nexins of the Snx-BAR family can mediate tubule formation from endosomes and other organelles. The approach of conducting the experiments using an in-vitro system of liposome binding/tubulation is both a strength and a weakness. Clearly in order to study the ability of individual Sorting nexin proteins to mediate tubule formation, an in vitro approach is appropriate. However, this approach and the conclusions that can be drawn from it, is also limited by virtue of the fact that in-vivo, tubule formation involves a multitude of factors including key roles for the cytoskeleton and possibly other proteins such as clathrin. Given the extensive and detailed nature of the study by Van Weering et al., it would be wrong to suggest that the study is too preliminary but I do think that some additional data that explores the requirements of tubule formation in vivo would strengthen the study and broaden its appeal.

Major points:

1. Is there any evidence that Snx1 operates as a homo dimer in vivo? Much of the in-vitro evidence presented involves use of a single sorting nexin protein added to liposomes. The conclusions drawn from this approach are open to question however as, in vivo, Snx1 associates with Snx5 or Snx6. The yeast homolog of Snx1 is Vps5p that functions as an obligate heterodimer with Vps17p. I think the authors should exercise caution and restraint when interpreting their data regarding the tubulation observed when single sorting nexin proteins are tested in their assay.

The reviewer is correct in that *in vivo* SNX1 forms preferential dimers with SNX5 or SNX6 rather then forming homodimers (Wassmer et al., (2007) J Cell Sci 120, 45-54; Wassmer et al., (2009) Dev Cell 17, 110-122) and that, by taking an *in vitro* approach, our experimental conditions are not identical to the

remodeling of endosomal membranes occurring in the living cell. That said, SNX5- and SNX30-homodimers still cannot remodel membranes *in vitro*, and overexpression of SNX5 in HeLa cells does not produce excessive endosomal tubulation as is observed for SNX1 (new Supplementary Figure S6), suggesting that SNX5 indeed lacks the intrinsic ability to remodel membranes. We have included this comment in the discussion.

2. A set of experiments that the authors could perform that would exploit the in-vitro system fully is to test the effect of different splice variants of sorting nexin proteins on tubulation. For example, Snx1 is alternatively spliced to generate three variants, could the authors test these different variants in the tubulation assay to explore whether the different variants confer different biological properties?

The experiments described in this study have been performed with the SNX1A variant. Compared to SNX1A, the SNX1B splice variant lacks residues 91-155, which is in the region leading up to the annotated PX domain. Splice variant SNX1D contains 25 extra residues at the C-terminus compared to SNX1A. Both splice variants contain a BAR domain that is identical to SNX1A, including the amphipathic helix region, the charged arginine in the BAR-dimer interface and the identical tip-loop region. Given that these splice variants all contain the PX-BAR unit, the basic function of membrane association and remodeling is predicted to be similar to the SNX1A isoform. Differences in N- and C-terminal residues may well prove to be functionally important with regard to protein:protein interactions, but addressing this is beyond the scope of the current study.

3. Could the authors test some of the mutations they made in the amphipathic helices or tip-loop residues in an in vivo situation? The authors have experience of imaging tubule formation using GFP-tagged constructs. Whilst I accept there are limitations to how much can be learned in a relatively 'dirty' whole-cell system, extending the observations made through the in-vitro assays to the cell would add much to the biological relevance of the study.

Previously published work on the amphipathic helix of SNX9 has established that mutants targeting the helix (e.g. F208A/F211A) retain liposome association but fail to induce tubulation (Pylypenko et al., (2007) EMBO J 26, 4788-4800). Moreover, while the F208A/F211A mutant is unable to induce tubulation when expressed in cells, it also has a reduced ability to associate with membranes (see Fig. 5 in Pylypenko et al., (2007)). Consistent with this, when the amphipathic helix mutants of SNX8 is expressed in HeLa cells as a GFP-fusion protein, association to endosomes is retained but one no longer observes the extensive membrane tubulation that is readily apparent with the wild-type protein (Supplementary Figure S6). Similar to SNX9, SNX5-dAH and SNX1-dAH expression did not induce GFP-decorated tubules but interpretation of these data was compromised by the predominant cytosolic distribution of these mutants. Together these results indicate that *in vivo* the AH is vital for the membrane tubulating properties of SNX8, and for some SNX-BARs this helix also contributes to membrane association. These new data are included and discussed in the revised manuscript.

4. How do the observed liposome-derived tubules for Snx9, Snx18 and Snx33 compare to the in-vivo situation? Clearly Snx1-tubules formed in-vitro are a good fit for the in-vivo tubules observed, but does this hold true for the other sorting nexins investigated?

SNX9, SNX18 and SNX33 form the SH3-SNX-BAR subfamily, and have been suggested to play roles in endocytosis (Lundmark and Carlsson, (2009) J Cell Sci 122, 5-11), and most recently the progression and completion of mitosis (Ma and Chircip (2012) J Cell Sci PMID:22718350). Endocytosis proceeds through a variety of stages that differ in molecular composition and level of membrane curvature. SNX9 begins to be recruited to clathrin-coated pits just prior to scission, peaking approximately 12 seconds after scission (Taylor et al., (2011) PLoS Biol 9, e1000604). The timed recruitment of SNX9, mediated through the ability to sense the high membrane curvature formed during these late-stages of clathrin-mediated endocytosis, is argued to regulate further membrane remodeling and co-ordinate dynamin

activity with a localized burst of actin polymerization that is necessary for efficient scission of clathrin-coated pits. In line with this, SNX9 is enriched in purified fraction of clathrin-coated vesicles of 50-100 nm diameter (Hirst et al., (2003) Mol Biol Cell 14, 625-641), and is associated with EpsF induced tubules of ~50 nm diameter (Alto et al (2007) J Cell Biol 178, 1265-1278 – EpsF is an effector of enteropathogenic *Escherichia coli*). The geometry of these structures correlates well with the tubules formed from liposomes by SNX9 *in vitro* (Figure 2C). Using similar *in vitro* liposome assays, Willenborg and colleagues (Willenborg et al., (2011) J Cell Biol 195, 71-86) have documented that SNX18 forms tubules with comparable diameters to those observed in our study, but as no immunoEM data is available for SNX18 (or SNX33), a correlation of *in vitro* structures with the corresponding tubular/vesicular structures observed in fixed cell is not possible.

5. It looks to me that Snx8 is somehow better at binding to liposomes that many other Snx proteins tested. Is this real? Is there an explanation for this observation?

From the liposome sedimentation assays we do not find a clear difference in membrane association of SNX8 compared to other SNX-BARs. The example shown in Figure 6E is in that sense not representative, we therefore replaced the example coomassie gel with another example.

6. The model at the end suggests that tubules generated by different snx proteins can form from the same endosome. Can this be tested in vitro using, for example, recombinant Snx1 and Snx4 simultaneously to determine if the model is valid. It would be necessary in such an assay to label the tubules formed with antibodies and this may be a limiting factor but it should be possible to express the recombinant proteins with epitope tags to aid detection.

We have tested various methods to address this question:

- Immuno-gold labeling of liposomes incubated with SNX1 and SNX8 proved difficult due to the high amount of protein absorbed by the carbon film on the EM grid, resulting in high levels of background gold label in the absence of liposomes.
- Visualization of different tubules by fluorescence microscopy, labeling recombinant SNX-BAR proteins with fluorescent dyes (Alexa labeling kits, 488 and 594 nm (Molecular Probes)), resulted in the SNX-BAR proteins precipitated upon labeling, rendering them non-functional in membrane remodeling assays.
- Exploiting the largest difference in tubule diameter within the SNX-BAR family, SNX9- and SNX33-decorated tubules (~60 nm and ~20 nm diameter, respectively), to allow morphological recognition of tubule-identity was however successful. When a pre-incubated mix of these proteins was exposed to liposomes, two clearly distinct populations of tubules were observed of ~20 and ~60 nm diameter (data included in revised Figure 7E, F). These data indicate that distinct SNX-BAR-decorated tubules are formed simultaneously *in vitro*, without mixing of their molecular identity.

7. A minor point really but I think the title is a little strange. I'm not sure what a "molecular insight" is. I would suggest that a change in the wording that reflects the findings is appropriate; for example, "In vitro analysis of SNX-BAR assembly reveals insights into the molecular mechanisms of distinct endosomal tubule formation."

We have changed the title to *"In vitro* SNX-BAR assembly reveals molecular details of distinct endosomal tubule formation".

Referee #3 (Remarks to the Author):

The manuscript by Van Weering et al evaluates systematically the membrane binding and remodeling

activities of the mammalian SNX-BAR proteins. Their contribution clarifies the nature of the hetero- and homo-dimers formed within the family--with implications for understanding sorting specificity within the endosomal system. Moreover, their observations are consistent with a large number of reports that focus on three mechanisms at work in membrane re-modeling: 1) the specificity of BAR domain dimer-interfaces and the resulting degree of overall curvature generated by dimerization; 2) the insertion of amphipathic helices into one leaflet to buckle the membrane; and 3) the propagation of local curvature over longer length scales by higher-order oligomer formation between neighboring BAR domains. The work reported is a useful step forward, although I have two major comments:

1) Several membrane-binding proteins that tubulate liposomes in vitro will also vesiculate liposomes. The balance between stable tubules versus small vesicles depends on a few factors, including lipid-to-protein ratio, lipid species within the model membranes, temperature and the melting point of the abundant lipid species used, and finally the mechanical forces involved in depositing onto charged TEM grids and subsequent dessication and staining. To my eye, the negative stain TEM for all the Snxs (including the non-tubulating Snx5, Snx7, Snx30 and Snx5-AA) suggest some degree of small vesicle formation. It seems critical to me to evaluate this for this paper. There are many ways of doing so, including 1) reverse sedimentation centrifugation, 2) light scattering, 3) cryo-TEM, 4) evaluating the lipid content of the supernatant versus the pellet of a sedimentation assay (See Boucrot et al Cell 2012 for example).

We tested the vesiculation of liposomes by SNX1 (forms tubules) and SNX5 (does not form tubules) in our liposome preparation according to the suggested sedimentation assay (Boucrot et al., 2012). We did not observe a difference in the amount of small vesicles observed in the supernatant after spinning down the liposomes incubated with SNX1, SNX5, or buffer control, suggesting that these small vesicles are present in the liposome preparation independent of the SNX-BAR added to the mix. Most likely they are formed upon sonication of the rehydrated lipid-cake generating a suspension of lipid structures of many shapes and sizes. On extruding this suspension through 200 nm cut-off filters, these vesicles are smaller then the filter pore and hence remain in the liposome preparation. We included these new data, and a discussion of this alternative hypothesis on the absence of tubules after SNX5 incubation, in the manuscript and new supplementary Figure S2.

2) While I agree with their overall view of the importance of higher-order oligomer/coat formation, this manuscript does not report a compelling loss-of-function mutant for coat formation. The two tip lysines that were mutated may be involved in electrostatic attraction to the membrane rather than coat formation (though even the double mutant does not appear to have a l-o-f phenotype). The chimera that confers tubulation competence to Snx5 is notable, but the chimera involves a large region (41 amino acids). As with F-BAR and N-BAR proteins that appear to form helical coats, the authors could notably strengthen their claim for the sorting nexins if they identified key residues that mediate specific inter-subunit interactions and show that mutants have phenotypes in vitro and in living cells.

While we agree with the reviewer's comments, identifying key residues for specific inter-subunit interactions would constitute a massive undertaking. This statement is based on the following:

- The papers to which the reviewer refers (Frost et al., (2008) Cell 132, 807-817; Min et al., (2012) Cell 149, 137-145) have based their mutagenic strategies on interactions inferred from cryo-EM structures. Without an equivalent SNX-BAR structure identifying equivalent residues would require an extensive, essentially 'blind' mutagenic screen.
- This is further compounded by the fact that the SNX-BARs helical coats are predicted to be inherently different to those of F-BAR and N-BAR since the BAR domain of SNX-BARs lies adjacent to the PX domain (see structure of the SNX9 PX-BAR unit (Pylypenko et al., (2007) EMBO J 26, 4788-4800)). The necessity to accommodate the PX domain will almost certainly mean that inter-subunit interactions are distinct to those described for the F-BAR and N-BAR. Using the published N-BAR and F-BAR inter-subunit interactions as a guide to describe the

SNX-BAR interactions is therefore unlikely to succeed.
It is for these reasons that in the present study we chose, as the reviewer acknowledges, to focus our attention on the tip-loop interactions. Overall, we therefore respectively conclude that without a cryoEM structure identifying precise residues necessary for inter-subunit interactions is way beyond the scope of the present study.

Minor comments:
1) Fig 6C, some of the TEM panels are too "red", suggesting an unusual color balance.

We corrected the colour balance or CMYK conversion of these grey scale images.

2) I believe there is a spelling error on Page 13, the last fifth, "from" should be form.

We have corrected this spelling mistake.

2nd Editorial Decision 13 September 2012

Thank you for sending us your revised manuscript. Referee 2 has now seen it again, and you will be pleased to learn that in his/her view you have addressed all criticisms in a satisfactory manner and that the paper will therefore be publishable in The EMBO Journal. Still, he/she puts forward a few minor points (please see below) that you may wish to consider addressing in an amended version of the manuscript.

Furthermore, there are a number of editorial issues that need further attention:

* Please include a conflict of interest statement into the main body of the manuscript text after the author contribution section.

* Please fuse the supplementary figures together with the supplementary figure legends to one supplementary material file and remove the supplementary figure legends from the main manuscript text file.

* You may still wish to simplify the title. One suggestion would be: 'Molecular basis for SNX-BAR-mediated formation of distinct (specific?) endosomal sorting tubules'

* We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Thank you very much for your kind cooperation at this late stage and for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #2 (Remarks to the Author):

The revised version of the manuscript by van Weering et al. has addressed most of the comments I raised in my initial review. I have only three fairly minor suggestions:

1. Could the authors suggest or speculate as to why Snx33 produces such thin tubules when compared to Snx9 and Snx18. What is it about Snx33 that causes this Snx protein to behave so differently? The differences may illuminate the process of tubule formation or may yield insights into the geometry of sorting nexin oligomerization and therefore merits greater discussion than the manuscript currently provides.
2. The paper describing the characterization of VPS5 and VPS17 in yeast (PMID: 12181349) should probably be added to the list of citations.
3. There is a typo in the legend for Figure 1c, "Homo sampiens"

2nd Revision - authors' response 21 September 2012

Many thanks for accepting our revised manuscript "*Molecular basis for SNX-BAR-mediated assembly of distinct endosomal sorting tubules*" for publication in the *EMBO Journal*. We are happy that you and the reviewers are satisfied with the corrections we have made to the manuscript. Based on the final suggestions we have made the following changes to the work:

- We changed the title to "*Molecular basis for SNX-BAR-mediated assembly of distinct endosomal sorting tubules".*
- We spotted an error in figure 7D, where SNX1-K442A gel was annotated with both SNX1-K442A and SNX1-K445A. We replaced the SNX1-K445A gel for the correct corresponding experiment.
- We added a conflict of interest statement.
- We moved the supplementary figure legends to the supplementary figures.
- We included the original uncropped blot/gel scans of the images used in the figures as source data.
- We included the PDB code for the SNX1 homodimer structure.
- We made the small adjustments suggested by referee 2 as stated below.

With these adjustments, we hope that the new manuscript is now suitable for publication.

Referee comments are quoted in italic.
Referee #2 (Remarks to the Author):

The revised version of the manuscript by van Weering et al. has addressed most of the comments I raised in my initial review. I have only three fairly minor suggestions:

1. Could the authors suggest or speculate as to why Snx33 produces such thin tubules when compared to Snx9 and Snx18. What is it about Snx33 that causes this Snx protein to behave so differently? The differences may illuminate the process of tubule formation or may yield insights into the geometry of sorting nexin oligomerization and therefore merits greater discussion than the manuscript currently provides.

We do not know the reason why SNX33-induced tubules are so much thinner compared to SNX9 or

SNX18-decorated tubules. Potentially, the SNX33-BAR dimer structure is more curved then the dimer of SNX9, or the SNX33 oligomer might organized differently that stabilized high membrane curvature. We have put forward these two possible explanations for the difference in tubule diameter in the results section that discusses these results.

2. The paper describing the characterization of VPS5 and VPS17 in yeast (PMID: 12181349) should probably be added to the list of citations.

We included this citation.

3. There is a typo in the legend for Figure 1c, "Homo sampiens"

We corrected this typo.

