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Direct Interaction of Actin Filaments with F-BAR Protein Pacsin2

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Please note that this manuscript was originally submitted to The EMBO Journal where it was peer-reviewed and revised. It was then transferred to EMBO reports with the original referees' comments attached. (Please see below)

Original decision and referees' comments - The EMBO Journal

Thank you for submitting your manuscript entitled 'Structural Basis for Direct Interaction of Actin Filaments with F-BAR Protein Pacsin2'. I have now received the reports from all referees.

As you can see below, all referees appreciate your model. However, they raise serious technical concerns as well as concerns regarding the data interpretation, and find that the conclusions are at this stage not sufficiently supported by the data presented. They therefore think that the current manuscript is not suitable for publication in The EMBO Journal.

However, given the interest into the topic and the constructive comments provided by the referees, I could offer to consider a revised version should you be able to substantiate your model along the lines suggested by the referees. This clearly demands a lot of work and time, as many of the experiments would have to be repeated and refined, and additional ones would have to be performed as well, with uncertain outcome. I can extend the revision time to 6 months maximum, should that be helpful.
Please note that a revised manuscript will be sent back to the three referees and that I would need strong support from them to consider publication here. Therefore, do consider your options carefully. If you see yourself in a position not to be able to address the concerns raised, then it is in your best interest to seek publication elsewhere at this stage. Please let me know in case you choose this option.

I thank you in any case for the opportunity to consider your work for publication.

REFEREE REPORTS:

Referee #1 (Report):

BAR domain-containing proteins regulate membrane curvature in cells by binding to and polymerizing on membrane surfaces, thus molding them into distinct functional shapes. Such membrane remodeling activity is essential for fundamental processes such as endocytosis and filopodia formation. Structural studies, including crystal structures of BAR domains and cryo-EM maps of membrane-bound BAR domains, have led to detailed understanding of BAR domain properties and functions. The regulation of BAR domain proteins -- especially in terms of the interactions between membranes and the actin cytoskeleton, sites at which BAR domain-containing proteins have been implicated by many lines of investigation -- remains poorly understood. The authors of this manuscript report that the F-BAR protein Pacsin2/Syndapin binds Actin polymers via the same, concave face used to bind membranes. The authors argue that this property may serve to store or sequester Pacsin2 on F-Actin near target membranes in a readily release-able pool. These are interesting observations that may have far-reaching implications for certain BAR domain proteins. However, in its present state this study is not suitable for publication and requires revision as well as additional experiments to warrant consideration by a general interest cell biology journal.

Major Points:

Figure 2:
1) Since the binding data are derived from quantitative western blots, I would like to see one example of a scanned western over the relevant titration range that was used in the analysis. In addition, error bars for the replicates are essential.

2) The use of Endophilin as a control seems less informative than other F-BAR domain-containing proteins, those containing wedge-loops like Pacsin versus those without (like the TOCA family, which are clearly involved in Actin regulatory processes). Endophilin is an interesting point of comparison and may be kept, but the text should clearly explain that Endophilin is an N-BAR domain-containing protein that has not been implicated in actin binding or regulation.

3) The salt at which the authors do the sedimentation experiment should be mentioned clearly. Currently, a range of salts is provided (50mM to 200mM). As the authors might be aware, many proteins, including most of members of the BAR domain superfamily, will pellet at low salt concentrations near 50mM (as suggested by their high fraction of pelleting Endophilin, an observation that indicates this normally soluble domain protein is polymerizing in their solution). I will be particularly interested in the high salt concentration where they no longer see Pacsin2 pelleting with Actin.

I would also like to see a "flotation" or reverse sedimentation experiment testing the idea that there is a competition between Pacsin binding to lipid vesicles versus F-actin. This is an easy experiment and a stringent way to demonstrate competitive lipid binding of Pacsin2 in the presence versus the

absence of F-Actin.

Figure 3:
Overall the experiments shown here seem to be a distraction from the overall message of the paper and the figure seems out of place. It is very well established that Pacsin/Syndapin, like other BAR proteins, binds to membranes containing negatively charged lipid headgroups.

The experimental design, moreover, strikes me as problematic. While other groups have used SPR to study protein-membrane interactions, I have several concerns. First, we don't know what happens to the SUVs attached to the flowcell. Do they remain intact SUVs or do they collapse and fuse into something more like a supported bilayer? If they remain SUVs, then we would not expect a gently curved F-BAR domain like Pacsin to bind efficiently. Several groups have noted that the gently curved F-BARs have a curvature sensitivity in that they bind poorly to sonicated SUVs with a radius of curvature smaller than the intrinsic curvature of the F-BAR domain. Second, Pacsin polymerizes on membrane surfaces and this will lead to strong avidity effects which are not discussed (but which make interpretation of apparent binding constants challenging if not meaningless). Third, the range of protein concentrations used in Figure 3 is reported in the methods but I would like to see the figure annotated with the molar concentrations for the traces. Fourth, the observation of essentially no binding with the wedge loop mutants strikes me as difficult to understand since the concave face retains the generally basic charges that characterize other F-BAR domains and are sufficient for robust membrane binding.

Figure 5 and 7:
The authors state that panel A "it is clear that pacsin2tr is extensively bound to the actin filaments." I am afraid that this is not obvious to me at all from the data presented and it would be helpful if the authors made this claim more explicitly in terms of the data.

For the IHRSR structure, I think the analysis is intriguing but incomplete. The challenge of determining the helical symmetry that describes the bound Pacsin -- if there is a stereotyped symmetry -- is challenging but I believe not insurmountable with more data and further analysis. I would like to see 2D class averages from this dataset in order estimate the fraction of the F-actin that is decorated by Pacsin under these conditions versus undecorated. I would also like to see an asymmetric reconstruction generated, with no symmetry imposed whatsoever, of the decorated filament.

Finally, in past publications these authors have shown how scanning transmission electron microscopy (STEM) should be used in challenging cases like this to determine precise mass-per-unit-length information in order to constrain symmetry assumptions or in this case to complement the biochemical estimates generated by co-sedimentation assays. This would be extremely valuable in this case.

Figure 6:
This figure does not add any value to the paper. The arguments being made rely on comparing a TOCA-type F-BAR domain bound to a ~60+ nm membrane tubule to a wedge-loop "winged" type Pacsin F-BAR bound to an F-actin fiber. The comparison is especially strange regarding panel D, in which the view shown is orthogonal to the view that actually reveals the curvature of the F-BAR polymer. I recommend removal of this figure entirely.

Figure 8:
This is perhaps the most worrisome aspect of the paper. The authors show very convincingly that Pacsin2 has no effect on actin assembly or disassembly - which is hard to reconcile with their model of extensive binding and co-localization in vitro and in living cells. I would like to see the molar ratio of Pacsin2 relative to actin increased in both assays. If there is no effect whatsoever at 5-10x molar excess, then I would remain suspicious of their structural model. It is hard to imagine that a 2-start tip-to-tip polymer of F-BAR domains surrounding the filament, and all of the avidity effects such a model assumes, would have no measureable effects in this bulk assay.

Minor concerns:

1. The manuscript has several typological errors, including inconsistent use of the convex versus

concave descriptions of BAR domain surfaces.

Referee #2 (Report):

I have read the manuscript by Kostan et al which describes the structural basis for a direct interaction between the F-BAR protein Pacsin2 and actin filaments. I think it is an interesting and important topic. The authors want to put forward a model stating that Pacsin2 can shuttle between lipid bilayers and actin filaments by the function of the F-BAR domain, which binds to lipid membranes as well as to actin filaments. I think this would be very exciting, if this model could be substantiated. The main question is off course: do the authors provide enough data to support such a model? In my view, the data do not support such a model, instead it remains an attractive hypothesis.

Does Pacsin2 bind F-actin?
The evidence for a direct interaction rests on three observations:
Pacsin2 colocalizes with F-actin (Fig.1), the full-length Pacsin2 and the F-BAR domain binds F-actin in a cosedimentation assay (Fig.2) and Pacsin2 decorates actin filaments (Fig.5A).
Fig.1 shows that Pacsin2 staining follows the F-actin staining. This does not necessarily imply a direct interaction. Have the authors used controls (i.e. staining with an unrelated IgG, secondary antibody only, etc) to rule out the possibility that the staining is unspecific. It would be nice to compare the staining for Pacsin2 "F-actin binding F-BAR protein" to the staining of a non-actin-binding F-BAR or BAR protein (e.g. endophilin and/or ectopic expression of a non-actin-binding mutant of Pacsin2).
Fig.2B shows that Pacsin2 cosediments with F-actin. The authors clam that Pacsin2 is unique in this regard, since another BAR domain-containing protein (endophilin) does not bind. However, it is not possible to draw general conclusion based on this experiment, in particular since a high fraction of endophilin is found to sediment, suggesting that the protein is not stable (is it at all folded correctly?). The authors need to compare additional BAR domains in order to make general conclusions.
Fig.5A looks like a bad S1 filament decoration experiment. Is it really possible to draw any reliable conclusions from this material? I would like to see a control experiment with BAR domains that do not bind to rule out the possibility that the decoration is an artifact.
The authors have used rabbit skeletal muscle α-actin (not skeletal actin, which they claim on p.19). However, α-actin is quite different from the β-actin/γ-actin which is found in non-muscle cells.

What are the structural evidences for the Pacsin2:F-actin interaction?
The crystal structure of chicken Pacsin2 F-BAR domain appears OK, as far as I can tell but it is not novel enough to warrant publication in EMBO J. Regarding the modeling of the F-actin decoration I am not so convinced. I am surprised that it is possible to use the material in Fig.5A, since it looks a bit dirty to me. In addition, the stoichiometry of the interaction found by cryoEM and cosedimentation do not agree, which I think is a bit strange. The resolution of the reconstruction is rather low so how reliable is it? I also find it a bit strange that Pacsin2 does not have any effect on actin assembly. Some of the authors have claimed previously that Pacsin2 (or Fap52) binds Filamin, is it possible that Filamin is needed for the interaction? The authors do not mention this article at all.

What are the evidences for a shuttling between lipid membranes and actin filaments?
This is really the key issue and the proofs for such a process must be solid. Unfortunately, I do not find any clear evidences for such a process in the current article. The mere fact that the same interaction motif, the F-BAR domain, can bind lipid membranes and F-actin does not imply that they do so in vivo. Does the Pacsin2 F-BAR domain prefer lipids to F-actin. Is it possible to compete out the F-actin binding with phospholipids or vice verse. It is not clear what would be the regulatory mechanism controlling the shuttling, the authors do not provide any data showing that Pacsin2 can in fact be transported from membranes to F-actin. The decoration experiment in Fig.5A is a bit artificial, since, inside cells, the actin filaments are unlikely to be naked, instead they are most likely associated with cross-linking proteins, side-binding proteins, NPFs, formins, etc. and Filamin. As far as I can judge, the shuttling hypothesis is an attractive, yet unproven, hypothesis.

Referee #3 (Report):

In their manuscript Kostan, Salzer, and colleagues report a novel interaction between the F-BAR protein pacsin-2 and actin. Overall the study is very well integrated, drawing data from in vivo, in vitro, and structural experiments. The main findings of the work are: (1) actin and pacsin-2 co-localize in vivo, (2) pacsin-2 engages F-actin through its concave surface, which is also involved in membrane binding, (3) that determinants most important for membrane binding are less important for F-actin binding based on a limited mutational analysis, (4) that the N-BAR protein endophilin does not bind to F-actin, (5) that pacsin-2 can decorate actin filaments in a manner reminiscent of tropomyosin, though using a different set of binding sites, based on a cryoEM reconstruction of the F-actin:pacsin-2 complex, and (6) that pacsin-2 binding does not alter the kinetics of actin fiber assembly, nor does it affect the stability of F-actin. Based on the results, the authors propose a model in which actin filaments can serve as scaffolds that facilitate recruitment of pacsin-2 to relevant membrane locations where they store pacsin-2 when not involved in membrane remodeling. These ideas are very original and address a completely unresolved question: "how do any BAR-protein get targeted to the proper membrane locations?" Thus, the significance and potential impact of this study are very large. However there are a number of conceptual and technical shortcomings that should be addressed before publication.

Significant concerns:

Page 6/Figure 2A: there are a few issues with this - from the co-sedimentation studies the authors determined a 3:1 ratio of actin (monomer):pacsin-2 (dimer). This is inconsistent with the model proposed in Figure 7 as well as the discussion where the authors say that, empirically, a 6:1 ratio is the true answer. This discrepancy needs to be resolved through additional experiments because otherwise one of the data streams (co-sedimentation or structural model) is pointless. Concerning Figure 2: the authors state that each experiment was carried out multiple times. If that is so, the data points should be represented with error bars. In addition, the large scatter observed for the delta1 truncation mutant is worrisome, and the assumption that the binding follows a regular binding curve seems arbitrary. In fact, a sigmoidal curve with a linear tail end seems to fit the data for the delta1 mutant just as well - meaning binding may be cooperative and become non-specific at some point. How would one know? Another questionable aspect is the quantitation of binding data from co-sedimentations that were done under low salt conditions (~50mM). The stated binding constants are not very likely to have much meaning. Fueling the same concern: the binding data shown in the supplemental material (tropomyosin + pacsin-2) were performed at a higher salt concentration (~100mM), where (surprisingly (?)) pacsin-2 binding seems to follow an almost linear (nonspecific?) behavior. The different binding assays should be uniform, including the SPR measurements. Using three different conditions for three different experiments that all try to characterize the same property (ligand binding) is messy, confusing, and almost certainly invalidates quantitative conclusions/comparisons. As is, these data are weak and do not support the author's claims beyond the point of showing that some binding occurs.

Page6/Figure 2B: Testing an N-BAR protein is a great idea and makes the interesting point that not every BAR domain will bind F-actin. That said - an equally, if not more, interesting comparison would have been to use another F-BAR protein to see if discrimination happens among F-BAR domains. Based on the model presented in Figure 7, it seems possible that a significant number of F-BAR domains will engage F-actin, especially F-BAR domains from outside the pacsin subfamily because these domains lack the bends at the tips and hence conform more closely to the conformation that the authors impose on pacsin-2. If this were the case and were shown experimentally, it would greatly broaden the significance of the study because it would provide evidence for a generic and very elegant process that delivers F-BAR domains to membranes. Even if the result were negative, it would still be interesting because it would reveal an intriguing and very specific aspect of the pacsin-2 mechanistic cycle. The finding that N-BAR domains do not bind is interesting but not really all that surprising. Regardless - using endophilin as test case for the N-BAR domains was a poor choice because it spontaneously precipitates under the chosen experimental conditions, which makes the data look quite messy.

Page 7/Figure 3: The membrane binding experiments have a few issues as well. A first concern comes from the fact that curvature limited liposomes were used for the experiments. Behavior of

pacsin-2 on those substrates will be significantly different from binding to substrates that could actually be remodeled. In support off this - observing "off rates" for pacsin-2 suggests that its interaction with the membrane is not related to what actually happens when it forms membrane associated scaffolds, which are very stable (=once assembled, they do not readily come apart = no, or extremely low off rates). Consequently - what do the measured binding affinities mean? Secondly: stabilizing the sensor grids with 0.01M NaOH presumably works by removing excess alkyl baits from the chip surface. However, the same conditions may also result in partial hydrolysis of the lipids, giving rise to lysolipids, which may alter pacsin-2 binding. This would have to be experimentally investigated. Third, the authors say that each titration was done three times - showing the error bars on the "RU vs concentration plots" therefore seems a sensible thing to do when presenting the data. The reason for bringing it up: errors are quite large at 50% of the mean in all but one case. The one exception has an error so large that it makes the average number meaningless. Given these issues, the overall purpose of these experiments is not entirely clear.

Page 7: a crystal structure of pacsin-2 BAR domain is reported, based on previous work by Toro et al. 2004. These efforts were superseded by a murine pacsin-2 structure (PDBID 3LLL from Plomann et al. 2010) with the same space group and a very similar unit cell. Assuming the same packing, the only significant difference of the structure reported here is the higher resolution since mouse and chicken pacsin-2 BAR domains are almost identical. This could be made clearer but regardless, the "hinge" model, with flexibility of the ends of the domains due to Pro144 and Pro220, was previously proposed by Plomann et al. 2010, raising the question what is gained by presenting a "sequel" in this paper - it increases the length, but does not add anything new to it. Maybe this aspect could be moved entirely into the supplemental information.

Page 8/Figure 5: the authors state that "it is clear that pacsin-2tr is extensively bound to actin filaments". How is this clear? The micrograph shown in Fig 5A is not particularly good, and suggests that there is a huge excess of free pacsin-2 that is not bound. A side-by-side comparison of a micrograph showing plain actin filaments with those decorated by pacsin-2 may help to appreciate the author's statement. Concerning the technical details of the reconstruction: what would justify rejection of ~80% of the data? Using ~5,600 out of ~32,000 segments may show what the authors want to see, but it also says very bluntly that the interactions are sporadic at best. This is relevant for the functional model that is presented later because it casts doubts that key aspects of this model hold up under physiological conditions. Concerning the resolution estimate: how was this obtained? Concerning data collection: a wide range of defocus values was used for recording the data (1.9-4.1micron). This begs the question how the image data were treated for the effects of the Contrast Transfer Function (which is not described anywhere). Similarly, the filaments look quite flexible. Were the data corrected for out-of-plane tilt? If so, it should be stated.

Page 8&9/Figure 6: The authors state that the curvature of pacsin-2 filaments on actin is quite similar to that of membrane-bound F-BAR domains. This point is questionable because the overlay shown in Figure 6D demonstrates quite clearly that the actin-bound pacsin-2 filaments do not align with those on the membrane-bound scaffold. In fact, if the section of the pacsin-2 filament that is shown represents a single pacsin-2 dimer, then the interactions are very different from what was observed in the membrane-bound form. For instance, the pacsin-2 filament "skips" an entire "row" of the membrane-bound " CIP4 filament" and forms a 20-degree angle with respect to the line cutting across the membrane tubule. This skew angle is twice of that observed in the narrow membrane tube shown in Frost et al (Fig 5A). Notably, the rigid body fit of F-BAR dimers to that tube appears "strained" and suggests that the structure is approaching the maximum curvature that F-BAR domains of CIP4 can support on bilayers (in fact, the lower end of tube diameters that can be formed (Fig 2B in Frost et al) coincides with the diameter of the narrow tube that is shown in Fig 5A). Doubling this angle implies a local curvature that is much higher than what CIP4 F-BAR domains can support. Thus, it seems that the mode of interaction between pacsin-2 and actin filaments is quite different from what is observed for other F-BAR domains on membranes. Further support for that view comes from EM-images of membrane-bound pacsin1 (Wang et al, 2009, Fig 1), which suggests that the "tilde" shape of pacsins causes them to form "stacked rings" on membranes rather than elongated helical filaments.

Page 9/Figure 7: In this paragraph the authors make a phenomenological argument, leading to the conclusion that a ratio of 6 actin momomers/pacsin-2 dimer is "about right". The authors support this idea with a speculative model, which they present in Figure 7. The concern here is that the

authors ignore the actual molecular shape of pacsin-2 (as shown in the crystal structure) and, for convenience, "straighten" the molecule (presumably to fit their general vision of how this assembly "should" look like). Given that this straightening does not seem to happen in the context of bona-fide membrane-bound scaffolds (at least not those where molecular details were discernable, Wang et al 2009), what makes the authors think that it would happen through the very loose interaction with the actin-filaments? The argument that other F-BAR domains are not twisted and that pacsin-2 may just pivot around the conserved prolines that are the hallmark of this subfamily seems quite arbitrary. Where would the bending energy come from? Have the authors considered that the shape differences between different F-BAR subfamilies may actually be functionally relevant? Furthermore, the authors justify the modeling by stating that they cannot retrieve the proper structure of the actin-bound pacsin-2 because its substoichiometric relation with regard to actin distorts the reconstruction when enforcing the helical symmetry of the actin filament. While it is true that imposing the helical symmetry of actin will average out the details for the pacsin-2 filaments - who says that only the actin symmetry has to be imposed? Given the nature of the sample, why did the authors not segment the raw reconstruction and search separately for the helical symmetries of the two components? Implementing this in IHRSR should be quite possible since the user has to specify a cutoff radius that determines what part of the reconstruction will be used to search for helical symmetry. Done this way, separate reconstructions could be calculated for the actin and pacsin-2 parts before merging them into a composite reconstruction that properly reflects the helical symmetry of each component (similar things have been done in other and far more complicated cases). As is, the authors use IHRSR as a "turnkey" technology, trying to cut corners along the way. This does not seem acceptable. If for some reason a thorough reconstruction proves to be impossible, then the least one can ask for is a significant mutational analysis of the model presented in Figure 7 (just using the wedge mutants as backing argument is insufficient since the authors have no way of telling how these deletion mutants locally distort the structure of the BAR core). In its current state, and keeping in mind the comments made in the previous paragraph, the author's model is a colorful fantasy - and possibly not much more than that.

Page 9/last paragraph: while the motivation for looking at effects of pacsin-2 binding on actin dynamics appears reasonable, a more thorough justification that considers actual cellular concentrations of the various interaction partners may change the perspective on the usefulness of these experiments.

Page 11/Figure 9: the authors speculate that local induction of actin polymerization by pacsin-2 may serve a self-regulating purpose to recruit pacsin-2 that is released from the membrane. At first this seems perfectly reasonable until one does the math. Doing the latter suggests that binding affinities do not really mean much in the context of crowded scaffolds since one of the binding partners is present in large molar excess. Because of that, most of such processes are kinetically controlled. Where affinity comes back into play is in the specifics of the model that the authors propose because in this case the component that "shuttles" (pacsin-2 in this case) will partition between two possible targets. If one were to take the authors data at face value, it would mean that the membrane always wins because the "binding affinities" are about an order of magnitude higher. Given the 6:1 ratio the authors suggest for the actin:pacsin-2 interaction, it should be a fun exercise to calculate whether one could possibly cram enough actin next to the membrane such as to cause a significant relocation of pacsin-2 from the membrane to actin. In other words: this part of the mechanistic model shown in Fig 9 seems unlikely to play a role. In this context it also is worthwhile to point out that the fluorescence microscopy does not really help to address this issue because it lacks the resolution to show that any of the pacsin-2 in the vicinity of membranes is actually bound to actin. Given these unresolved issues, this leaves the "delivery" aspect of the model, which this reviewer thinks is GREAT!

Minor concerns/editorial comments:
Page 3: "tip to tip oligomerization of the F- and N-BAR ...." This is factually incorrect. Both F- and N-BAR scaffolds have (extensive) lateral interactions between BAR-dimers. In F-BAR these interactions are directly between the coiled-coil cores, in N-BAR the lateral interactions involve the H0-helices.
Page 4 (second paragraph): need to insert an "in" into the first sentence
Page 4 (last paragraph): "... whereby the convex site of the F-BAR ...."; should read concave
Page 6: "In order to quest" is not an English phrase
Page 6: "unspecificity" is not an English word

Figure 3 has two sets of curves for Pacsin2-delta1. Is the second one supposed to be Pacsin2-delta2? There also should be error bars for the binding curves.
Figure 5A has no scale bar
Figure 8B has no error bars
Page 13: what do the authors mean by "rigid cast" when talking about N-BAR? These domains form quite flexible scaffolds. Moreover, N-BARs are perfectly capable of accommodating different curvatures (Mim et al 2012), just like F-BARs. Thus, it is unclear what the authors are talking about here. Similarly the discussion about what bends membrane and what senses curvature is very messy. Both F- and N-BARs can do both, and the degree to which each property contributes to function in vivo is very much an unresolved issue.
Page 19: typo, should read "octylglucopyranoside". How much buffer was used to wash out the detergent? Any residual detergent left will change the properties of the next lipid coating of the chip.
Supplementary Info: There is no overlay in Supplementary Figure 1, and the description of what was done is not clear.

Revision - authors' response - The EMBO Journal

Please find attached the resubmitted version of our manuscript entitled

"'Structural Basis for Direct Interaction of Actin Filaments with F-BAR Protein Pacsin2"

As outlined below, we attempted to answer all comments raised by the referees. We hope that the revised manuscript will find your and their approval. Due to new discoveries in the course of our revisions several parts of the manuscript were re-written. All changes made in the manuscript text are highlighted in red.

Referee #1:

1) Figure 2:

Since the binding data are derived from quantitative western blots, I would like to see one example of a scanned western over the relevant titration range that was used in the analysis. In addition, error bars for the replicates are essential.

We repeated some of the experiments and prepared a new Figure 2A showing the mean values and error bars for each data point and give the obtained Kd (which do not differ significantly from the previous values) in the text. We reduced the complexity of the Figure 2 by now showing only the comparison between the pacsin2tr-WT and the wedge loop mutant pacsin2tr-Δ1 (former pacsin2tr-Δ2) where the whole wedge loop is replaced by a glycine residue. New supplementary Figure S4 shows a representative analysis of one data point by quantitative Western blotting. The text was adapted accordingly in the Results section on page 6:

"**However, since pacsin2tr has a similar molecular weight as actin and co-migrates in SDS-PAGE, we used a streptavidin-tagged version of the pacsin2tr and performed quantitative Western blotting to assess the amount of co-sedimentation at various protein concentrations (Supplementary Figure S4). Pacsin2tr was found to bind to F-actin with a dissociation constant of 1.92 ± 0.36 µM (Figure 2A). Since the wedge loop of pacsins was implicated in membrane binding (see below) and membrane tubulating activity, we tested whether this protruding structure is also involved in binding to the actin filaments. We constructed a wedge loop mutant, both in the full length (pacsin2-Δ1) and in the truncated pacsin2 variant (pacsin2tr-Δ1). In the mutant variant residues 121-125 were deleted and replaced by a single glycine so that the deletion results in the removal of the protrusion. The pacsin2tr-Δ1 mutant binds to F-actin with a similar dissociation constant (K_D of 2.73 ± 0.56 µM) as the wild-type**

variant (Figure 2A). This suggests that the wedge loop has no specific role in the interaction between pacsin2 and F-actin."

2) The use of Endophilin as a control seems less informative than other F-BAR domain-containing proteins, those containing wedge-loops like Pacsin versus those without (like the TOCA family, which are clearly involved in Actin regulatory processes). Endophilin is an interesting point of comparison and may be kept, but the text should clearly explain that Endophilin is an N-BAR domain-containing protein that has not been implicated in actin binding or regulation.

We extended our study by testing two F-BAR domain proteins for binding to actin (CIP4 and FCHO). We used both F-actin co-sedimentation assays as well as electron microscopy (negative stain). Both proteins did not associate with F-actin as shown on Supplementary Figure S7. Text was amended on pages 6 and 7:

"In addition, we found that CIP4 and FCHO2, do not bind to F-actin in co-sedimentation and EM studies (Supplementary Results and Supplementary Figures S7). Altogether our results suggest that pacsin2 binds specifically to F-actin, however, binding of BAR domain containing proteins to F-actin is not a common property of this protein superfamily."

Furthermore, we added text into supplementary results on page 2:

"In addition to (N-BAR domain protein) endophilin, we tested binding of other F-BAR domain proteins, namely CIP4, and FCHO2 for binding to the F-actin. Both proteins expressed as truncated variants comprising the BAR domain showed relatively high level of insolubility, making their use for co-sedimentation assay rather limiting. Of these proteins, only FCHO2 seemed to associate with F-actin in the co-sedimentation assay. However, association with F-actin was not confirmed by EM, where the protein was found to form aggregates surrounding naked actin filaments (Supplementary Figures S7A, B). For CIP4, self-precipitation and no specific enrichment in F-actin pellet fraction (similar to endophilin) was observed after incubation with increasing amounts of F-actin in the co-sedimentation assay (Supplementary Figure S7C). In line with this, electron micrographs of negatively stained F-actin incubated with CIP4 showed no decoration actin filaments by CIP4 (Supplementary Figure S7D)."

3) The salt at which the authors do the sedimentation experiment should be mentioned clearly. Currently, a range of salts is provided (50mM to 200mM). As the authors might be aware, many proteins, including most of members of the BAR domain superfamily, will pellet at low salt concentrations near 50mM (as suggested by their high fraction of pelleting Endophilin, an observation that indicates this normally soluble domain protein is polymerizing in their solution). I will be particularly interested in the high salt concentration where they no longer see Pacsin2 pelleting with Actin.

Following the reviewer's suggestion we unified salt concentrations at which individual experiments were done. We used different salt conditions in particular experiments due to better stability and/or solubility of the tested proteins/molecules in these conditions. To clarify effect of the salt on pacsin2 binding to actin, we did co-sedimentation assay with increasing salt concentrations. These experiments showed that there is more than 40 % pacsin2 bound to F-actin at 250 mM KCl when compared to 100 % of binding at 50 mM KCl. This should help to follow behaviour of pacsin2 in

the experiments where different salt concentrations were used. The range of the salt concentration to test was selected based on the following criteria: (i) 50 mM KCl (in standard, common 1xF-buffer) is, and was used to asses binding affinities of many actin binding proteins (and in this respect is sold by companies as Actin polymerization buffer; http://www.cytoskeleton.com/bsa02 and described in published methods (*e.g ; J Vis Exp. 2008 Mar 28;(13). pii: 690. doi: 10.3791/690; Actin co-sedimentation assay; for the analysis of protein binding to F-actin. Srivastava, and Barber*) allowing to compare obtained affinity with validated, canonical actin-binding proteins. (ii) The avoided the high salt concentrations (>250 mM KCl), in order not to interfere with the electrostatic nature of the pacsin2-F-actin interaction. Furthermore, high salt concentrations (500 – 600 mM KCl) are typically used to remove proteins (e.g. tropomyosin) from F-actin during its preparation (Spudich and Watt, 1971).

Text was amended on pages 6 and 7:

"To further investigate the nature of pascin2-F-actin binding and to ask whether other BAR domain containing proteins might interact with actin filaments, we assayed selected N-BAR (endophilin) and F-BAR (CIP4 and FCHO2) proteins for binding to F-actin. For endophilin we performed two types of experiments where, proteins (pacsin2 and endophilin) at constant concentration were incubated either with constant concentration of F-actin in presence of increasing concentration of salt (50 to 250 mM KCl), or with increasing concentration of F-actin at constant salt concentration (50 mM KCl) (Figure 2B-E). In the first experiment both proteins (pacsin2 and endophilin) co-sedimented with F-actin at 50 mM KCl (Figure 2B and Supplementary Figure S5). However, while there was only slight precipitation observed for pacsin2 when incubated in absence of F-actin, relatively high self-precipitation of endophilin was observed at the same conditions (Supplementary Figure S5). Increasing of the salt from 50 to 250 mM KCl led to reduction of the pacsin2-F-actin binding to approximately 40 % of binding at 250 mM KCl compared to the binding at 50 mM KCl that was considered as 100 % (Figure 2B and C). Thus binding of pascin2 to F-actin was found to be salt-dependent, indicating that pacsin2 most likely interacts with negatively charged actin filaments *via* its positively charge concave surface. In contrast to pacsin2, co-sedimentation of endophilin with F-actin was not significantly reduced in the presence of high salt concentrations (to 85 % at 250 mM KCl compared to 100 % binding at 50 mM KCl), showing salt-independent precipitation of endophilin as observed in the absence of F-actin at 50 mM KCL (Supplementary Figure S5) and therefore suggesting an unspecific interaction.

In the second experiment, where specificity of both, pacsin2- and endophilin-F-actin binding, was assessed by co-sedimentation assay with increasing amounts of F-actin, both proteins displayed precipitation in the absence of F-actin (Figure 2D and E). Increasing amounts of F-actin led to obvious enrichment of pacsin2 in the F-actin pellet fraction, while only slight enrichment of endophilin in the F-actin pellet fraction was observed at all actin concentrations tested (Figure 2D and E), indicating that co-sedimentation of endophilin is actin-independent. All together, our results suggested that endophilin does not interact with F-actin, what was further confirmed by our EM studies where no decoration of actin filaments with endophilin was observed, while clear binding of pacsin2tr with actin filaments could be seen (Supplementary Figure S6). In addition, we found that CIP4 and FCHO2, do not bind to F-actin in co-sedimentation and EM studies (Supplementary Results and Supplementary Figures

S7). Altogether our results suggest that pacsin2 binds specifically to F-actin, however, binding of BAR domain containing proteins to F-actin is not a common property of this protein superfamily. "

4) I would also like to see a "flotation" or reverse sedimentation experiment testing the idea that there is a competition between Pacsin binding to lipid vesicles versus F-actin. This is an easy experiment and a stringent way to demonstrate competitive lipid binding of Pacsin2 in the presence versus the absence of F-Actin.

We performed co-floatation experiments to address competitiveness of lipids and F-actin for binding to pacsin2. We observed that in the presence of liposomes, a significant amount of purified full-length pacsin2 was present in the lipid-containing fraction, whereas in the absence of liposomes all pacsin2 resided in the dense fractions. However, in the presence of F-actin, the amount of pacsin2 in lipid-containing fraction was slightly diminished suggesting that binding of pacsin2 to liposomes is partially competed in the presence of F-actin. These results are described in the main text (page 7) and illustrated by Supplementary Figure S8:

"To investigate competition in membrane and F-actin binding of pacsin2, a lipid co-flotation assay was performed. In the presence of liposomes, a significant amount of purified full-length pacsin2 was present in the lipid-containing fraction, whereas in the absence of liposomes all pacsin2 resided in the dense fractions. However, in the presence of F-actin, the amount of pacsin2 in lipid-containing fraction was slightly diminished. Thus, these data suggest that binding of pacsin2 to liposomes is competed in the presence of F-actin (Supplementary Figure S8).

5) Figure 3:

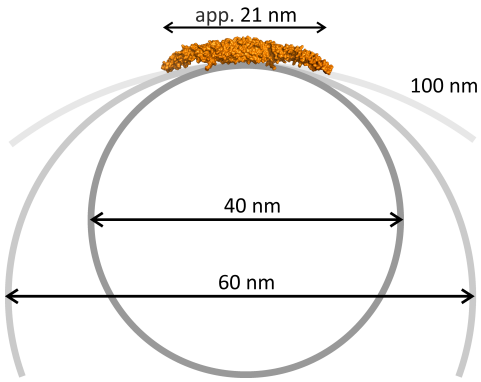
Overall the experiments shown here seem to be a distraction from the overall message of the paper and the figure seems out of place. It is very well established that Pacsin/Syndapin, like other BAR proteins, binds to membranes containing negatively charged lipid headgroups.

The aim of this experiment was to show the difference in binding to lipid membranes between the wild-type pacsin2 and various mutants, some of which were also used in F-actin binding assays. We feel it is important to include these results, since pacsin2 associates with membranes and F-actin by the same concave surface.

The experimental design, moreover, strikes me as problematic. While other groups have used SPR to study protein-membrane interactions, I have several concerns. First, we don't know what happens to the SUVs attached to the flowcell. Do they remain intact SUVs or do they collapse and fuse into something more like a supported bilayer?

For some of the lipid compositions and vesicular systems this is well established and liposomes do not fuse on the surface of the sensor chip to a continuous bilayer. See for example Cooper et al., Anal Biochem, 2000 and Anderluh et al., Anal Biochem, 2005.

If they remain SUVs, then we would not expect a gently curved F-BAR domain like Pacsin to bind efficiently. Several groups have noted that the gently curved F-BARs have curvature sensitivity in that they bind poorly to sonicated SUVs with a radius of curvature smaller than the intrinsic curvature of the F-BAR domain.



Pacsin2 intrinsic curvature corresponds to curvature of small unilamellar vesicles of approximately 60 nm in diameter. Our procedures for preparation of small unilamellar vesicles routinely results in vesicles of such diameter. Normally, F-BAR domains induce formation of wide tubules. However, for human pacsin 2, formation of low-diameter tubules and tubule constrictions was observed (Wang et al 2009). In the same study, human pacsin was shown to stabilize two classes of tubes with diameters of 53 ± 18 nm and 98 ± 34 nm.

Second, Pacsin polymerizes on membrane surfaces and this will lead to strong avidity effects, which are not discussed (but which make interpretation of apparent binding constants challenging if not meaningless). Third, the range of protein concentrations used in Figure 3 is reported in the methods but I would like to see the figure annotated with the molar concentrations for the traces.

We agree that the binding of pacsin2 to small unilamellar is complex event, which is actually reflected in the shapes of sensorgrams. For this reason we did not attempt to extract rate constants from sensorgrams, but employed equilibrium binding analysis, which allowed estimation of apparent equilibrium affinity constants. Such data analysis revealed differences in binding between different pacsin2 variants. The low amount of stably retained protein at the surface of vesicles after the dissociation phase indicates that no significant polymerization occurs at the surface of vesicles.

The concentration ranges used for titrations were the same for all proteins. The titration curves are now color-coded in order to make more clear which curve corresponds to which concentration.

Fourth, the observation of essentially no binding with the wedge loop mutants strikes me as difficult to understand since the concave face retains the generally basic charges that characterize other F-BAR domains and are sufficient for robust membrane binding.

It is known that wedge loop is essential for the membrane interactions of pacsins, see for example Wang et al., PNAS, 2009, in mutants in the wedge loop vesiculation was largely suppressed. In our case, 140 mM NaCl, which we used in the running buffer, perhaps masked the charges required for association between pacsin2 and small unilamellar vesicles.

6) Figure 5 and 7:

The authors state that panel A "it is clear that pacsin2tr is extensively bound to the actin filaments." I am afraid that this is not obvious to me at all from the data presented and it would be helpful if the authors made this claim more explicitly in terms of the data.

Perhaps this is not obvious to someone who has not worked on F-actin, but those of us who have worked on EM of F-actin for more than 30 years view such images as showing decoration. We have simply removed this sentence, as the resulting reconstruction shows the binding!

For the IHRSR structure, I think the analysis is intriguing but incomplete. The challenge of determining the helical symmetry that describes the bound Pacsin -- if there is a stereotyped symmetry -- is challenging but I believe not insurmountable with more data and further analysis. I would like to see 2D class averages from this dataset in order estimate the fraction of the F-actin that is decorated by Pacsin under these conditions versus undecorated. I would also like to see an asymmetric reconstruction generated, with no symmetry imposed whatsoever, of the decorated filament.

We appreciate the desire of the reviewer for us to extend the analysis. However, we believe that there is no evidence for any "stereotyped symmetry" or higher order symmetry (such as 1 pacsin per 3 actins, etc.) despite our having looked quite exhaustively. If such a symmetry existed, it must appear in power spectra, where one would see layer lines arising from a symmetry other than of F-actin. For example, when troponin is bound to a thin filament one can see that the actin helical symmetry is broken in such power spectra. The power spectrum on the left shows only the actin helical symmetry, although the peaks and relative intensities have been altered by the bound pacsin2. The reviewer must surely agree that if there were a higher-order symmetry (1 pacsin per 2 actins, 1 pacsin per 3 actins, etc.) this would appear in power spectra. We do not see what 2D class averages will reveal, since the main component of variation in trying to create such averages will simply be the azimuthal orientation of each segment. An actin filament is not like TMV or other structures where a pseudo-repeat is contained within all image segments. We are also unsure of what is meant by an asymmetric reconstruction. If one uses the IHRSR approach, an asymmetric reconstruction is generated each cycle, and then symmetrized. Is this the asymmetric reconstruction requested? If so, it is meaningless, as the images have all been aligned to a symmetrized volume generated in the previous cycle. Symmetrization is mainly a means to improve the SNR. In the limit of large N (where N is the number of segments) the asymmetric and symmetrized IHRSR reconstructions will be the same. Generating a truly asymmetric reconstruction of the decorated filament *ab initio* would either be impossible or be of such low resolution as to be meaningless. What size object should be chosen? Would this be 5 actins, 10 actins, 20 actins? We simply do not see the rationale given power spectra, which unequivocally show that the only symmetry present is that of F-actin.

Finally, in past publications these authors have shown how scanning transmission electron microscopy (STEM) should be used in challenging cases like this to determine precise mass-per-unit-length information in order to constrain symmetry assumptions or in this case to complement the biochemical estimates generated by co-sedimentation assays. This would be extremely valuable in this case.

The overall problem, which we acknowledge and address in the paper, is that during preparation for cryo-EM bound pacsin2 is simply falling off the filament. This is not unique for pacsin2, is not

unique for actin, and is not unique to us! Having stoichiometric data from STEM, where filaments are prepared by a very different technique (adsorption to a surface followed by freeze-drying) would therefore not elucidate anything except further highlight this artifact of cryo-EM preparation. When the DeRosier lab examined F-actin decorated by fimbrin ABD1 (Rost et al., 1998), they discussed this phenomenon: "In three-dimensional (3D) maps of decorated actin filaments, the features corresponding to the ABP often appear weak relative to actin. The cause for the weakening of the density corresponding to ABP is uncertain but loss of bound ABP during blotting and plunging of grids is one possible explanation." Consider the rigor decoration of F-actin by myosin S1. Every lab that has looked at this by cryo-EM finds some completely naked actin filaments, never seen by negative stain under the same conditions. And this is with an affinity of S1 for actin of ~ 10 nM, and working with a large excess of S1! If one looks at the recent acto-S1 paper (Behrmann et al., 2012), after they avoided selecting any naked actin filaments, always visible in the images (Stefan Raunser, personal communication), they reduced their data set from 60k segments to 35k after sorting for full decoration. And this was using biochemical conditions where we know in solution that more than 99.9% of the actins should be bound by a myosin head! If one looks at decorating microtubules with kinesin (Sindelar and Downing, 2007) it was stated "Maintaining the kinesin decoration on the microtubules during grid preparation for cryo-EM proved to be extremely difficult."

7) Figure 6:

This figure does not add any value to the paper. The arguments being made rely on comparing a TOCA-type F-BAR domain bound to a ~60+ nm membrane tubule to a wedge-loop "winged" type Pacsin F-BAR bound to an F-actin fiber. The comparison is especially strange regarding panel D, in which the view shown is orthogonal to the view that actually reveals the curvature of the F-BAR polymer. I recommend removal of this figure entirely.

As suggested by the reviewer, Figure 6 was removed and the following text below was removed from page 9:

"This can be seen (Figure 6) in a superposition of the membrane tubule on the pacsin2tr-F-actin reconstruction. This comparison provides the basis for modeling how pacsin2tr might actually be bound to F-actin."

8) Figure 8:

This is perhaps the most worrisome aspect of the paper. The authors show very convincingly that Pacsin2 has no effect on actin assembly or disassembly - which is hard to reconcile with their model of extensive binding and co-localization in vitro and in living cells. I would like to see the molar ratio of Pacsin2 relative to actin increased in both assays. If there is no effect whatsoever at 5-10x molar excess, then I would remain suspicious of their structural model. It is hard to imagine that a 2-start tip-to-tip polymer of F-BAR domains surrounding the filament, and all of the avidity effects such a model assumes, would have no measureable effects in this bulk assay.

As recommended by the reviewer, we tested the effect of higher molar excess of pacsin2 on both, polymerization and depolymerization of actin. We observed that pacsin2 is able to stabilize actin filaments in depolymerisation assay. We described these results in the amended text on page 11:

"Interestingly, when compared to a control experiment with actin alone, pacsin2tr did not display an effect on actin polymerization either at low (1:1) or at higher (1:5) actin to pascin2tr molar ratios (Figure 7B and Supplementary Figure S12). Similar results were

obtained when monitoring polymerization of pyrene-labelled actin with or without pacsin2tr. Furthermore, to investigate possible effects of pacsin2tr on actin nucleation, pacsin2tr was mixed with monomeric actin 5 minutes before inducing polymerization, followed by light scattering. However, similar to our previous observation, the kinetics of actin polymerization was indistinguishable from the control experiment in the absence of pacsin2."

Moreover, tropomyosin was shown to stabilize F-actin and to inhibit the actin severing activity of cofilin (Ono & Ono, 2002). In the polymerization assay, cofilin enhances the elongation rate of spontaneous actin polymerization by severing actin filaments and increasing the number of filaments ends (Carlier et al, 1997). As expected, when using cofilin in our assay, actin polymerization was accelerated and the kinetics reached a plateau at about 15 minutes (Figure 7B). However, pacsin2 did not inhibit this activity, suggesting that binding of pacsin2 to F-actin differs from that of tropomyosin. To further explore this phenomenon, we analyzed the effects of pacsin2tr on the *in vitro* depolymerization kinetics of pyrene-labeled F-actin diluted to a concentration below the critical concentration of the filament minus end in the absence or presence of cofilin (Figure 7C). As expected, fluorescence intensity decreased in function of time when pyrene-labeled F-actin was diluted in F-buffer (Figure 7C). The extent of depolymerization was similar when actin filaments were prepared by polymerization in the presences of pacsin2 at low (1:1 and 1:2) actin to pacsin2 molar ratios (Supplementary Figure S13). However, when F-actin was mixed with pacsin2 at higher (1:5 and 1:10, actin to pacsin2) molar ratios (Figure 7C), decrease in actin depolymerisation rate was observed, indicating that pacsin2 is able to increase stability of F-actin. When the F-actin, and F-actin-pacsin2 complexes were subjected to dilution-induced depolymerization in the presence of cofilin, hardly any difference in the depolymerization rates was observed (Figure 7C), suggesting that pacsin2 is not able to reduce the activity of cofilin in this assay. In conclusion, pacsin2 increases the stability of actin filaments upon binding but does not seem to have nucleation and/or severing activity itself nor does it seem to have an effect on the activity of cofilin. "

8) Minor concerns:

The manuscript has several typological errors, including inconsistent use of the convex versus concave descriptions of BAR domain surfaces.

We tried our best to spot and correct typological errors and eliminate inconsistent use of convex versus concave throughout the text.

Referee #2:

1) Fig.1 shows that Pacsin2 staining follows the F-actin staining. This does not necessarily imply a direct interaction. Have the authors used controls (i.e. staining with an unrelated IgG, secondary antibody only, etc) to rule out the possibility that the staining is unspecific. It would be nice to compare the staining for Pacsin2 "F-actin binding F-BAR protein" to the staining of a non-actin-binding F-BAR or BAR protein (e.g. endophilin and/or ectopic expression of a non-actin-binding mutant of Pacsin2).

Yes, we agree. The fact that staining of pacsin2 follows the F-actin staining does not imply a direct interaction. However, within the text we do not claim that results presented on figure 1 show direct interaction, but co-localization of pacsin2 with F-actin, which can be direct or indirect. To study direct interaction we performed co-sedimentation and EM studies.

To rule out non-specific binding of the secondary antibody, a control staining was carried out by omitting the primary antibody (MAbAF3) and also by replacing it with normal mouse serum, followed by the secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse IgG). In these control experiments no decoration of actin-rich cytoskeletal structures or cellular domains were seen. The actin-associated staining pattern seen in Figs A1 and A2 was also inhibited by pre-adsorption of MAbAF3 with purified pacsin 2.

We transiently expressed pacsin2-GFP under conditions that leads to overexpression of pacsin2. This, as known from other studies, brings about major tubulation in the membraneous structures and also changes in cytoskeletal structures. Due to these induced changes, we consider, in fact, that the demonstration of overlapping staining pattern in unperturbed, native cells is a much stronger indication, albeit suggestive, of physical interaction between pacsin2 and actin.

2) Fig.2B shows that Pacsin2 cosediments with F-actin. The authors clam that Pacsin2 is unique in this regard, since another BAR domain-containing protein (endophilin) does not bind. However, it is not possible to draw general conclusion based on this experiment, in particular since a high fraction of endophilin is found to sediment, suggesting that the protein is not stable (is it at all folded correctly?). The authors need to compare additional BAR domains in order to make general conclusions.

Please see reply to the point 2 of Reviewer 1

3) Fig.5A looks like a bad S1 filament decoration experiment. Is it really possible to draw any reliable conclusions from this material?

Yes, it is! We have worked on more than 30 F-actin binding proteins (and some of these are not published) and have never seen an instance where something that is not specifically bound to F-actin appears in a reconstruction as bound. We have seen the opposite on numerous occasions (Orlova et al., 1994) where most of the mass of something that appears to be decorating F-actin does not appear in the reconstruction. The high background in the image results from working with a large excess of pacsin2. Unlike single particle EM, where such a high background would be disastrous, the imposition of helical symmetry means that the background largely disappears. The Egelman lab currently has a filament structure with a stoichiometric background of GFP (1:1) due to the fact that the molecule of interest has a GFP tag. Despite this stoichiometric background, the molecule of interest is visualized at better than 4 Å resolution, yet the GFP is not seen at all in the reconstruction because it is disordered. Please see the comment below about BSA.

I would like to see a control experiment with BAR domains that do not bind to rule out the possibility that the decoration is an artifact.

We performed the control requested experiments with two F-BAR domain proteins (CIP4 and FCHO). We used both F-actin co-sedimentation assays as well as electron microscopy (negative stain). Both proteins did not associate with F-actin as shown on Supplementary Figure S7. In addition, we compared negatively stained actin filaments decorated with pacsin2 and those in presence of endophilin (under the same conditions), which does not bind to F-actin in co-sedimentation assays.

We have done a general control experiment a number of years ago, in response to such a request, where actin filaments were incubated with BSA. Micrographs showed filaments that actually look decorated, but a 3D reconstruction showed almost no additional mass bound to actin.

Supplementary Figure was added and text amended on page 7:

"In addition, we found that CIP4 and FCHO2, do not bind to F-actin in co-sedimentation and EM studies (Supplementary Results and Supplementary Figures S7). Altogether our results suggest that pacsin2 binds specifically to F-actin, however, binding of BAR domain containing proteins to F-actin is not a common property of this protein superfamily."

"In the second experiment, where specificity of both, pacsin2- and endophilin-F-actin binding, was assessed by co-sedimentation assay with increasing amounts of F-actin, both proteins displayed precipitation in the absence of F-actin (Figure 2D and E). Increasing amounts of F-actin led to obvious enrichment of pacsin2 in the F-actin pellet fraction, while only slight enrichment of endophilin in the F-actin pellet fraction was observed at all actin concentrations tested (Figure 2D and E), indicating that co-sedimentation of endophilin is actin-independent. All together, our results suggested that endophilin does not interact with F-actin, what was further confirmed by our EM studies where no decoration of actin filaments with endophilin was observed, while clear binding of pacsin2tr with actin filaments could be seen (Supplementary Figure S6). In addition, we found that CIP4 and FCHO2, do not bind to F-actin in co-sedimentation and EM studies (Supplementary Results and Supplementary Figures S7). Altogether our results suggest that pacsin2 binds specifically to F-actin, however, binding of BAR domain containing proteins to F-actin is not a common property of this protein superfamily."

We also refer to these experiments on page, citing again Supplementary Figure S6:

"Extensive binding of pacsin2tr to F-actin was observed by negative stain EM (Supplementary Figure S6A and B)."

The authors have used rabbit skeletal muscle α-actin (not skeletal actin, which they claim on p.19). However, α-actin is quite different from the β-actin/γ-actin which is found in non-muscle cells.

We used rabbit skeletal muscle α-actin in all our experiments. We made sure that this is now clear throughout the text: "rabbit skeletal muscle actin" is used.

Nevertheless, the sequence differences among different actin isoforms are quite small. Contrary to expectations, no one has yet found a protein that binds to one isoform of actin and not to another.

4) What are the structural evidences for the Pacsin2:F-actin interaction? The crystal structure of chicken Pacsin2 F-BAR domain appears OK, as far as I can tell but it is not novel enough to warrant publication in EMBO J. Regarding the modeling of the F-actin decoration I am not so convinced. I am surprised that it is possible to use the material in Fig.5A, since it looks a bit dirty to me.

This is discussed above in reply to Reviewer #1. We are actually working with a sample now on an unrelated project where the background is equally bad and we have better than 4 Å resolution. We are doing the *ab initio* chain tracing. This ability to use "dirty" samples arises from the simple fact that the imposition of helical symmetry gets rid of everything that is not rigidly bound to actin.

In addition, the stoichiometry of the interaction found by cryoEM and cosedimentation do not agree, which I think is a bit strange.

This is discussed above in reply to Reviewer #1.

The resolution of the reconstruction is rather low so how reliable is it?

The 12 Å resolution is actually higher than most published complexes of actin with actin-binding proteins! There are only a very few with higher resolution (Behrmann et al., 2012; Galkin et al., 2011; Sousa et al., 2013) and one at comparable resolution (Galkin et al., 2008).

I also find it a bit strange that Pacsin2 does not have any effect on actin assembly. Some of the authors have claimed previously that Pacsin2 (or Fap52) binds Filamin, is it possible that Filamin is needed for the interaction? The authors do not mention this article at all.

Indeed, pacsin2 was shown to interact with filamin A *via* its N-terminal portion (1-145), which comprises part of the tail, in particular with the filamin A segment encompassing Ig-like domains 14-16 and the calpain sensitive hinge I region. This binding might assists the interaction with F-actin, by co-localising the binding partners and increasing the local concentration, but we do not have any direct evidence for this.

6) What are the evidences for a shuttling between lipid membranes and actin filaments? This is really the key issue and the proofs for such a process must be solid. Unfortunately, I do not find any clear evidences for such a process in the current article. The mere fact that the same interaction motif, the F-BAR domain, can bind lipid membranes and F-actin does not imply that they do so in vivo. Does the Pacsin2 F-BAR domain prefer lipids to F-actin. Is it possible to compete out the F-actin binding with phospholipids or vice verse. It is not clear what would be the regulatory mechanism controlling the shuttling, the authors do not provide any data showing that Pacsin2 can in fact be transported from membranes to F-actin. The decoration experiment in Fig.5A is a bit artificial, since, inside cells, the actin filaments are unlikely to be naked, instead they are most likely associated with cross-linking proteins, side-binding proteins, NPFs, formins, etc. and Filamin. As far as I can judge, the shuttling hypothesis is an attractive, yet unproven, hypothesis. Unfortunately, I do not find any clear evidences for such a process in the current article. The mere fact that the same interaction motif, the F-BAR domain, can bind lipid membranes and F-actin does not imply that they do so in vivo.

Yes, but it does not rule it out either.

Does the Pacsin2 F-BAR domain prefer lipids to F-actin. Is it possible to compete out the F-actin binding with phospholipids or vice verse.

Pacsin2 seems to prefer lipids to F-actin, as we observed in co-flotation assay and in the fact there is much higher affinity of pacsin2 towards the lipids than towards the F-actin. In co-floatation assays

we used a phospholipids mix. The details of the co-floatation assay are reported page 7 (see above reply to Reveiwer #1) and Methods section and Supplementary Figure S8:

„Large unilamellar vesicles (LUVs) with molar lipid composition of POPC:POPE:POPS:PI(4,5)P2:Rhodamine PE = 50:15:20:10:5 (Avanti Polar Lipids) were prepared as previously described (Pykalainen et al, 2011). Purified full-length pacsin2 was incubated at the concentration of 1 µM with 100 µM LUVs in the presence and absence of 20 µM F-actin for 1 hour. The samples were centrifuged at 54 000 rpm with Beckman Optima using TLS-55 rotor (Beckman) for 30 min at 4°C. Each fraction was collected from top to bottom and equal amount of samples was run on SDS-PAGE followed by Coomassie staining. The protein amount of each band was quantified by densitometry analysis using ImageJ. The fluorescence intensity of the same fractions was measured at the wavelength of 583 nm using a LS 55 Fluorescence Spectrometer (Perkin-Elmer) to detect Rhodmaine-PE-labeled liposome fraction.“

It is not clear what would be the regulatory mechanism controlling the shuttling, the authors do not provide any data showing that Pacsin2 can in fact be transported from membranes to F-actin. As far as I can judge, the shuttling hypothesis is an attractive, yet unproven, hypothesis

We indeed do not know the regulatory mechanism behind, and here propose the shuffling hypothesis. All together our results suggest that shuttling between lipids and F-actin can happen. What is the situation inside of the cell is more difficult to imagine, taking into account complexity of all processes. However, pacsin2 binding to vesicles *in vivo* was confirmed and it is well established. It is not clear though how pacsin2 and other BAR domain proteins are released from the vesicles after endocytosis. Anyhow at one point pascin2 has to exist in lipid-free form inside the cell what gives it a chance to associate with F-actin. Further experiments need to be done to confirm this “attractive hypothesis”, as Reviewer #2 calls it.

Referee #3:

1) Page 6/Figure 2A: there are a few issues with this - from the co-sedimentation studies the authors determined a 3:1 ratio of actin (monomer):pacsin-2 (dimer). This is inconsistent with the model proposed in Figure 7 as well as the discussion where the authors say that, empirically, a 6:1 ratio is the true answer. This discrepancy needs to be resolved through additional experiments because otherwise one of the data streams (co-sedimentation or structural model) is pointless.

Discrepancy between ratios between pacsin2 and actin are due to use of different methods. The issue of F-actin decoration form EM studies was addressed in detail above; in brief, during preparation for cryo-EM bound pacsin2 is falling off the filament, which is neither unique for pacsin2, nor unique for actin. For details please see reply to Reviewer #1.

On the other hand there seems to be more pacsin2 pelleting with actin filaments in co-sedimentation assay. This is quite common for these methods and therefore the most probable binding stoichiometry is between observed ones. To reduce confusion we in the text removed discussion

about stoichiometry found by co-sedimentation assay. Stoichiometry used for the model generation needs to be mentioned, although we are aware that this is **not** the true answer.

Concerning Figure 2: the authors state that each experiment was carried out multiple times. If that is so, the data points should be represented with error bars. In addition, the large scatter observed for the delta1 truncation mutant is worrisome, and the assumption that the binding follows a regular binding curve seems arbitrary. In fact, a sigmoidal curve with a linear tail end seems to fit the data for the delta1 mutant just as well - meaning binding may be cooperative and become non-specific at some point. How would one know?

To address this point we repeated experiments for Figure 2 combining data with previous ones and calculated new KDs, which are not significantly different from original ones and added the error bars. The number of experiments (n=4) for each data point was added to the figure legend. Data for the delta1 truncation mutant (called in the amended text delta2) was removed, as the additional experiments could not clearly confirm specificity of the binding, as suggested by reviewer. Nevertheless, data obtained for delta2 mutant (in amended text delta1), which lacks the complete sequence encoding the wedge-loop clearly show that this part of the protein does not contribute to interaction with F-actin. The text was amended on page 6:

"Pacsin2tr was found to bind to F-actin with a dissociation constant of 1.92 ± 0.36 µM (Figure 2A). Since the wedge loop of pacsins was implicated in membrane binding (see below) and membrane tubulating activity, we tested whether this protruding structure is also involved in binding to the actin filaments. We constructed a wedge loop mutant, both in the full length (pacsin2-Δ1) and in the truncated pacsin2 variant (pacsin2tr-Δ1). In the mutant variant residues 121-125 were deleted and replaced by a single glycine so that the deletion results in the removal of the protrusion. The pacsin2tr-Δ1 mutant binds to F-actin with a similar dissociation constant (K_D of 2.73 ± 0.56 µM) as the wild-type variant (Figure 2A). This suggests that the wedge loop has no specific role in the interaction between pacsin2 and F-actin."

Caption of Figure 2A was changed accordingly:

"(A) Filamentous actin (6 µM concentration of the monomer) was incubated with various amounts of Strep-tagged pacsin2tr or pacsin2tr-Δ1, respectively. Upon ultracentrifugation, the amount of proteins in the pellet and supernatants was assessed by quantitative Western blotting using an anti-Strep antibody. The exponential binding curves fitted for each set of data points are shown. Data represent mean values (± S.E.) of four independent experiments."

3) Another questionable aspect is the quantitation of binding data from co-sedimentations that were done under low salt conditions (~50mM). The stated binding constants are not very likely to have much meaning. Fueling the same concern: the binding data shown in the supplemental material (tropomyosin + pacsin-2) were performed at a higher salt concentration (~100mM), where (surprisingly (?)) pacsin-2 binding seems to follow an almost linear (nonspecific?) behavior. The different binding assays should be uniform, including the SPR measurements. Using three different conditions for three different experiments that all try to characterize the same property (ligand binding) is messy, confusing, and almost certainly invalidates quantitative conclusions/comparisons. As is, these data

are weak and do not support the author's claims beyond the point of showing that some binding occurs.

Please see the reply to Referees #1 and #2. In brief, following suggestions of reviewers we firstly uniformed salt concentrations within a used assay, secondly, we performed pacsin2-F-actin co-sedimentations at different salt concentrations, and thirdly whenever we compared assays on different proteins, the experiments were performed under the same conditions. In addition to co-sedimentation assays at increasing salt concentrations (please see above) we performed also co-sedimentation assays of pacsin2 and endophilin with increasing amounts of F-actin. These assays together with EM studies and XL-MS data show specificity of the pacsin2 binding.

We therefore trust that data as presented now are clearer and still allows for better comparisons. Text was amended on pages 6 and 7, as detailed in replies for Reviewers #1 and #2.

4) Page6/Figure 2B: Testing an N-BAR protein is a great idea and makes the interesting point that not every BAR domain will bind F-actin. That said - an equally, if not more, interesting comparison would have been to use another F-BAR protein to see if discrimination happens among F-BAR domains. Based on the model presented in Figure 7, it seems possible that a significant number of F-BAR domains will engage F-actin, especially F-BAR domains from outside the pacsin subfamily because these domains lack the bends at the tips and hence conform more closely to the conformation that the authors impose on pacsin-2. If this were the case and were shown experimentally, it would greatly broaden the significance of the study because it would provide evidence for a generic and very elegant process that delivers F-BAR domains to membranes. Even if the result were negative, it would still be interesting because it would reveal an intriguing and very specific aspect of the pacsin-2 mechanistic cycle. The finding that N-BAR domains do not bind is interesting but not really all that surprising. Regardless - using endophilin as test case for the N-BAR domains was a poor choice because it spontaneously precipitates under the chosen experimental conditions, which makes the data look quite messy.

Following suggestions of reviewers we extended our study by testing two additional F-BAR domain proteins for binding to actin (CIP4 and FCHO). We used both F-actin co-sedimentation assays as well as electron microscopy (negative stain). Both displayed not binding. For details please see reply to Reviewers #1 and #2.

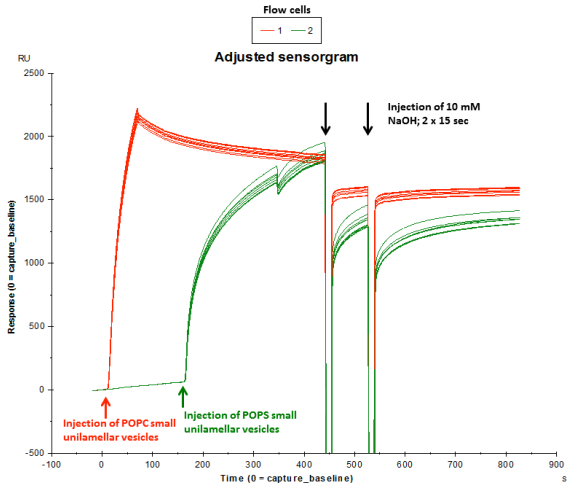
5) Page 7/Figure 3: The membrane binding experiments have a few issues as well. A first concern comes from the fact that curvature limited liposomes were used for the experiments. Behavior of pacsin-2 on those substrates will be significantly different from binding to substrates that could actually be remodeled. In support off this - observing "off rates" for pacsin-2 suggests that its interaction with the membrane is not related to what actually happens when it forms membrane associated scaffolds, which are very stable (=once assembled, they do not readily come apart = no, or extremely low off rates). Consequently - what do the measured binding affinities mean?

The curved liposomes should indeed be a good substrate for pacsin2 binding, see also the response to point 5 of the Referee #1. It is obvious that there is significant dissociation form the surface of the vesicles, as stated above, and we do not anticipate that these vesicles are the ideal substrate for

scaffold formation. However, the purpose of these experiments was to compare the interaction of the wild-type pacsin2 and various mutants with lipid membranes and this is indeed changed; we see significant differences between different protein constructs, see also below.

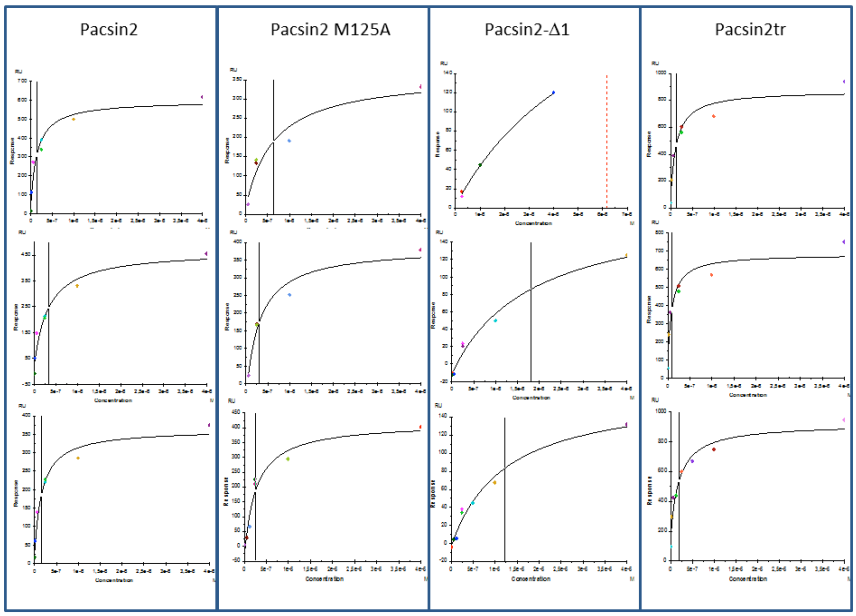
6) Secondly: stabilizing the sensor grids with 0.01M NaOH presumably works by removing excess alkyl baits from the chip surface. However, the same conditions may also result in partial hydrolysis of the lipids, giving rise to lysolipids, which may alter pacsin-2 binding. This would have to be experimentally investigated.

We never heard that this could be an issue and this procedure is actually a common practice, such treatment is even recommended by the supplier. It allows removal of loosely bound vesicles and not alkyl baits that are covalently attached to the dextran layers of the sensor chip. Routinely people use 2 injections for 60 sec of 100 mM NaOH, while in our case we used 2 injections for 15 sec of 10 mM NaOH. Such treatment, together with removal of vesicles by octylglucopyranoside at the end of experiment, allowed reproducible deposition of small unilamellar vesicles on the surface of the sensor chip (see figure below that shows deposition of small unilamellar vesicles on the surface of the sensor chip for one titration experiment). We agree that the role of lipid membrane composition on Pacsin2 binding would need to be investigated, but we feel that this is out of the scope of this paper.



7) Third, the authors say that each titration was done three times - showing the error bars on the "RU vs concentration plots" therefore seems a sensible thing to do when presenting the data. The reason for bringing it up: errors are quite large at 50% of the mean in all but one case. The one exception has an error so large that it makes the average number meaningless. Given these issues, the overall purpose of these experiments is not entirely clear.

In Figure 3 we show a single binding experiment for each protein construct: the raw sensorgrams, together with the equilibrium binding responses and fitted binding isotherm. For each protein we performed three independent titrations and in the text we report averages of these independent K_D estimations. This was already described in the first version of the manuscriupt in the Materials and Methods section. For the referees inspection we here include all experiments that were used for K_D estimations (see the figure below). As mentioned above, the purpose of these experiments was to compare the binding of the wild-type pacsin2 and its variants, which is clearly different: binding of pacsin2 M125A and pacsin 2-Δ1 is significantly different from binding of pacsin2 or pacsin2tr.



8) Page 7: a crystal structure of pacsin-2 BAR domain is reported, based on previous work by Toro et al. 2004. These efforts were superseded by a murine pacsin-2 structure (PDBID 3LLL from Plomann et al. 2010) with the same space group and a very similar unit cell. Assuming the same packing, the only significant difference of the structure reported here is the higher resolution since mouse and chicken pacsin-2 BAR domains are almost identical. This could be made clearer but regardless, the "hinge" model, with flexibility of the ends of the domains due to Pro144 and Pro220, was previously proposed by Plomann et al. 2010, raising the question what is gained by presenting a "sequel" in this paper - it increases the length, but does not add anything new to it. Maybe this aspect could be moved entirely into the supplemental information).

Following Reviewer's advice we removed Figure 5A, but kept Figure 5B, which illustrates structural plasticity of pacsin family, which we think is important for the mechanism and generation of EM model. We would please like to leave it at the discretion of the editor whether to remove this figure as well. We are quoting Plomann et al 2010 here: "A comparison of the currently available pacsin structures with FAP52 (= OUR chicken pacsin2), human pacsin 1, and possibly other F-BAR proteins would further illuminate the role of rigid-body movements in the distal parts of F-BAR domain dimers and their role in membrane-curvature sensing."

7) Page 8/Figure 5: the authors state that "it is clear that pacsin-2tr is extensively bound to actin filaments". How is this clear? The micrograph shown in Fig 5A is not particularly good, and suggests that there is a huge excess of free pacsin-2 that is not bound.

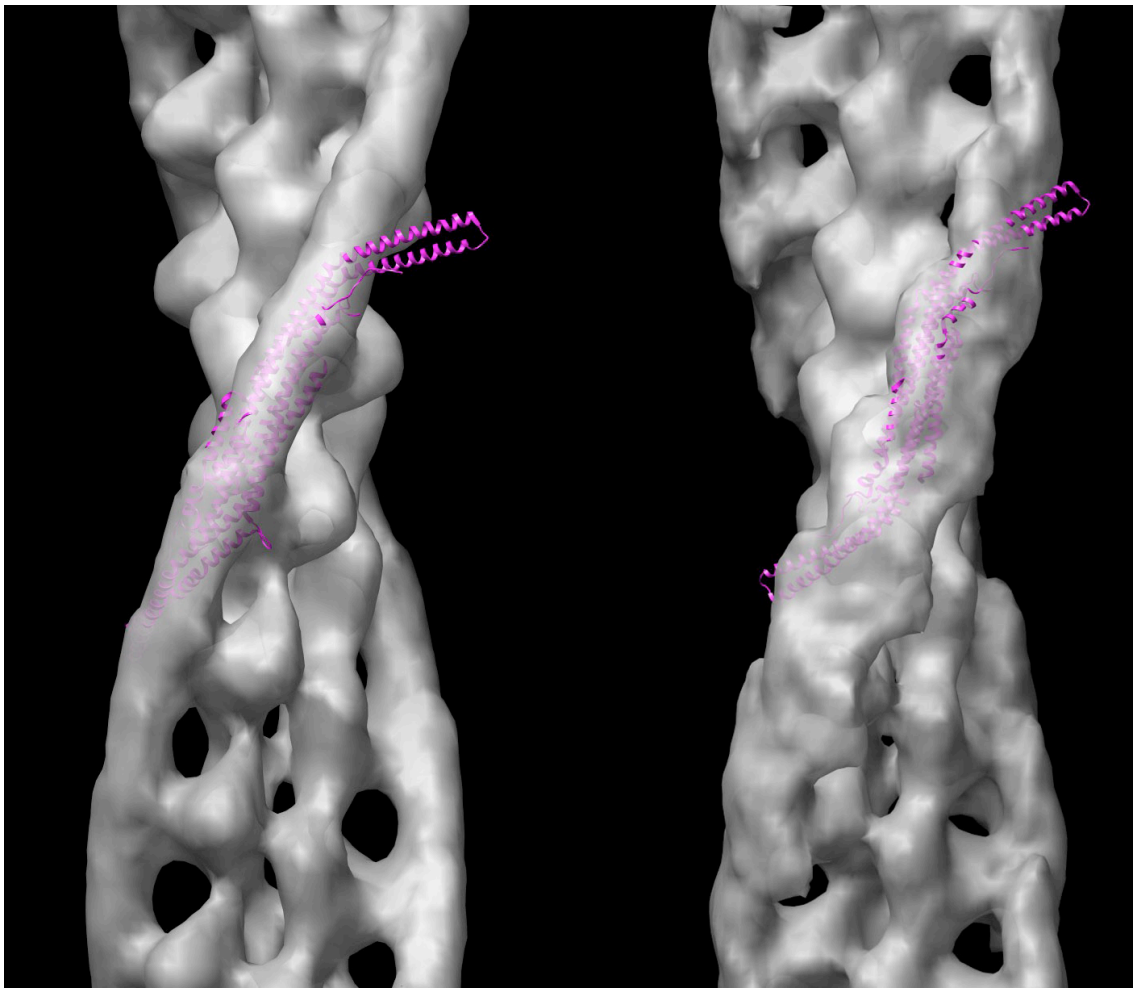
This is addressed above, please see reply to Reviewer #1, point 6.

8) A side-by-side comparison of a micrograph showing plain actin filaments with those decorated by pacsin-2 may help to appreciate the author's statement.

Done. Figure 5A was added, showing naked actin filaments.

9) Concerning the technical details of the reconstruction: what would justify rejection of ~80% of the data? Using ~5,600 out of ~32,000 segments may show what the authors want to see, but it also says very bluntly that the interactions are sporadic at best.

This all gets to the problem of loss of decoration during cryo-EM preparation. The global reconstruction, not excluding any segments, looks very similar (with just slightly less mass due to the pacsin) so it is not as if our selection process has changed the overall conclusion in any way. Filaments were imaged by negative stain, and then sorted against two references: one was naked actin, the other was an actin filament with density running where the pacsin is bound. The reconstruction from the filaments selected as naked is shown below on the left, and from those selected as decorated is shown on the right. The dimer from the FAP52 crystal structure is shown for purposes of scale. Two points are clear. One, the decoration in negative stain is significantly heavier than in cryo-EM. And two, even segments selected as naked have a significant amount of pacsin bound. So binding is not sporadic.



10) This is relevant for the functional model that is presented later because it casts doubts that key aspects of this model hold up under physiological conditions. Concerning the resolution estimate: how was this obtained?

The best metric for resolution comes from reality, not any internal test of consistency (such as the FSC). We know the structure of F-actin. We can filter this structure to various resolutions, and the best match to the actual reconstruction is at about 12 Å. We think that the FSC measure of resolution can be extremely misleading. Consider a very recent paper on the MAVS filament (Xu et al., 2014) where a resolution of 9.6 Å was obtained by the FSC criterion. We actually know that the entire structure is a complete artifact, due to the imposition of the wrong helical symmetry. The reason we know this is that we have a reconstruction of the same filament at ~ 3.6 Å, where the resolution is supported by the match to an atomic model.

11) Concerning data collection: a wide range of defocus values was used for recording the data (1.9-4.1micron). This begs the question how the image data were treated for the effects of the Contrast Transfer Function (which is not described anywhere).

We have expanded the Materials and Methods to explain how the CTF was treated. Text was added on page 24:

"Images were multiplied by the theoretical CTF function, which is the simple application of a Weiner filter in the limit of very low SNR. After the reconstruction was generated it was corrected for the fact that the images were multiplied by the CTF twice (once by the microscope, once by us) by dividing by the sum of the squared CTFs."

Similarly, the filaments look quite flexible. Were the data corrected for out-of-plane tilt? If so, it should be stated.

We did not correct for out-of-plane tilt, since such corrections are only needed at higher resolution. The filaments are no more flexible than pure F-actin (which we have now reconstructed at better than 5 Å resolution, and where out-of-plane tilt correction is essential). The characteristic amount of out-of-plane tilt that we see for pure F-actin is on the order of 6°. It might be expected that this amount of tilt is limiting our current resolution, but having a higher resolution reconstruction with actin's symmetry imposed on the pacsin will not provide any benefits.

12) Page 8&9/Figure 6: The authors state that the curvature of pacsin-2 filaments on actin is quite similar to that of membrane-bound F-BAR domains. This point is questionable because the overlay shown in Figure 6D demonstrates quite clearly that the actin-bound pacsin-2 filaments do not align with those on the membrane-bound scaffold. In fact, if the section of the pacsin-2 filament that is shown represents a single pacsin-2 dimer, then the interactions are very different from what was observed in the membrane-bound form. For instance, the pacsin-2 filament "skips" an entire "row" of the membrane-bound " CIP4 filament" and forms a 20-degree angle with respect to the line cutting across the membrane tubule. This skew angle is twice of that observed in the narrow membrane tube shown in Frost et al (Fig 5A). Notably, the rigid body fit of F-BAR dimers to that tube appears "strained" and suggests that the structure is approaching the maximum curvature that F-BAR domains of CIP4 can support on bilayers (in fact, the lower end of tube diameters that can be formed (Fig 2B in Frost et al) coincides with the diameter of the narrow tube that is shown in Fig 5A). Doubling this angle implies a local curvature that is much higher than what CIP4 F-BAR domains can support. Thus, it seems that the mode of interaction between pacsin-2 and actin filaments is quite different from what is observed for other F-BAR domains on membranes. Further

support for that view comes from EM-images of membrane-bound pacsin1 (Wang et al, 2009, Fig 1), which suggests that the "tilde" shape of pacsins causes them to form "stacked rings" on membranes rather than elongated helical filaments.

Following suggestions of reviewers we have removed this figure.

13) Page 9/Figure 7: In this paragraph the authors make a phenomenological argument, leading to the conclusion that a ratio of 6 actin momomers/pacsin-2 dimer is "about right". The authors support this idea with a speculative model, which they present in Figure 7. The concern here is that the authors ignore the actual molecular shape of pacsin-2 (as shown in the crystal structure) and, for convenience, "straighten" the molecule (presumably to fit their general vision of how this assembly "should" look like). Given that this straightening does not seem to happen in the context of bona-fide membrane-bound scaffolds (at least not those where molecular details were discernable, Wang et al 2009), what makes the authors think that it would happen through the very loose interaction with the actin-filaments? The argument that other F-BAR domains are not twisted and that pacsin-2 may just pivot around the conserved prolines that are the hallmark of this subfamily seems quite arbitrary. Where would the bending energy come from? Have the authors considered that the shape differences between different F-BAR subfamilies may actually be functionally relevant? Furthermore, the authors justify the modeling by stating that they cannot retrieve the proper structure of the actin-bound pacsin-2 because its substoichiometric relation with regard to actin distorts the reconstruction when enforcing the helical symmetry of the actin filament. While it is true that imposing the helical symmetry of actin will average out the details for the pacsin-2 filaments - who says that only the actin symmetry has to be imposed? Given the nature of the sample, why did the authors not segment the raw reconstruction and search separately for the helical symmetries of the two components?

This is discussed above. Analysis of power spectra show that there is absolutely no evidence for such a lower symmetry. We have discussed how powerful such information from power spectra can be with regard to a controversy about the ParM filament (Galkin et al., 2012). Wang et al 2009 reported EM micrographs of negatively stained LMVs, which do not allow to discern the molecular the shape of pacsin2 on vesicles. Even though we are symmetrizing the pacsin density, it is more consistent with the straightened model, as are the XL-MS crosslinks.

With regards to the bending energy, similarly to what has been proposed for pacsin interaction with membranes (Wang et al 2009), the energy can be provided by the electrostatic nature of the interaction between F-actin and pacsin. A smaller interaction interface between pacsin and F-actin compared to the membranes is also reflected in higher K_D.

There is structural and computational evidence that pacsins display structural plasticity (Plomann, 2010, Yo, 2013), suggesting that their structure might be best represented as a conformational ensemble. As the reviewer we also strongly believe that the shape differences between different F-BAR subfamilies may actually be functionally relevant. In order to highlight this, we inserted this text on page 13:

"Specific structural differences together with structural plasticity renders F-BAR domains capable of performing diverse functions and adopting variable curvatures, which might be required for binding to differently curved membrane structures as well as to actin filaments."

14) Implementing this in IHRSR should be quite possible since the user has to specify a cutoff radius that determines what part of the reconstruction will be used to search for helical symmetry. Done this way, separate reconstructions could be calculated for the actin and pacsin-2 parts before merging them into a composite reconstruction that properly reflects the helical symmetry of each component (similar things have been done in other and far more complicated cases).

We do not think that this is possible, and actually do not know of any such implementation. We would love to see a reference. The problem is that the reconstruction is generated by aligning segments to projects of a helically-symmetrized volume. But given no evidence for any alternate symmetry, finding one would almost by definition be artifactual.

15) As is, the authors use IHRSR as a "turnkey" technology, trying to cut corners along the way. This does not seem acceptable.

We simply do not understand the argument. We have published > 100 papers using the IHRSR method or helping others to use it, following our development of the method. We feel, for reasons discussed above, that we simply cannot extract more information. If the reviewer would like to suggest concrete approaches, we would be happy to consider them.

16) If for some reason a thorough reconstruction proves to be impossible, then the least one can ask for is a significant mutational analysis of the model presented in Figure 7 (just using the wedge mutants as backing argument is insufficient since the authors have no way of telling how these deletion mutants locally distort the structure of the BAR core). In its current state, and keeping in mind the comments made in the previous paragraph, the author's model is a colorful fantasy - and possibly not much more than that.

Reply in order to validate the F-actin:pacsin2 interface, we performed crosslinking experiments and MS analyses of crosslinked products. These results confirmed, that pacsin faces F-actin with its concave site, and allowed to place “in register” pacsin dimers on actin filaments. Text was added on page 10, Figures 6B and C, Supplementary Results, Supplementary Table S1 and S2, Supplementary Figure S11 were added.

„In order to identify residues that are proximal in the pacsin2/F-actin complex, we performed cross-linking experiments followed by mass spectrometric analysis. For cross-linking experiments, we used the zero-length cross-linker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), which links salt bridged residues. A band of about 90 kDa appeared on SDS-PAGE, indicating the formation of specific cross-links between one actin subunit (42 kDa) and one pacsin2 subunit (52 kDa) (Supplementary Figure S9). To identify the sites of intermolecular cross-links, this 90 kDa band was excised from SDS-PAGE and analyzed by high resolution LC-MS/MS after trypsin treatment. A representative MS/MS spectrum of a specific cross-link product is depicted in Supplementary Figure S10, which allowed the identification of a linkage between Lys62 of pacsin2 and Asp25 of actin. In total 9 cross-links were identified for the pacsin2/F-actin complex (Supplementary Table S1). The major cross-linking products involve the adjacent aspartic acid residues (24 and 25) and glutamic acid residues (99 and 100) on actin subunits, and two clusters of lysine residues (53, 64 and 101; 143, 147 and 150), on the concave surface of the pascin2 BAR domain (Figure 6B and C). The first lysine cluster is located in the pacsin2 dimerization domain and the second is close to the proline residue 144 that introduces the specific kink in the F-BAR of

pacsin2, causing diverse orientations of the tails in the pacsin protein family (Figure 4 and 6B). These actin and pacsin2 residues were mapped on the model obtained by three-dimensional EM reconstruction, showing that, assuming a modest structural plasticity, they can fall within the distance range of salt bridged residues residing on different polypeptide chains in known structures of protein complexes (Supplementary Results, Supplementary Table S1 and S2, Supplementary Figure S11). Moreover, the distances between the two lysine clusters in the F-BAR domain of pacsin (47 – 68 Å) correspond well with the distance between the clusters identified on actin protomers (58 Å). Thus, the cross-linking data clearly support the current pacsin2/F-actin model obtained by three-dimensional reconstruction and help to map proximal binding sites between pacsin2 and actin (Figure 6C). The identification of these cross-links agrees with the electrostatic nature of the pacsin2-F-actin interaction, already observed in co-sedimentation assays."

17) Page 9/last paragraph: while the motivation for looking at effects of pacsin-2 binding on actin dynamics appears reasonable, a more thorough justification that considers actual cellular concentrations of the various interaction partners may change the perspective on the usefulness of these experiments.

While actin is the most abundant protein in the eukaryotic cells, while the abundance of pacsins is not really documented, their effects on actin dynamics in a living cell is naturally difficult to extrapolate from in vitro data. Our data here "only" show that despite a similar mode of binding to F-actin, tropomyosin has an effect on actin dynamics while pacsin under the same experimental conditions does display this capacity.

18) Page 11/Figure 9: the authors speculate that local induction of actin polymerization by pacsin-2 may serve a self-regulating purpose to recruit pacsin-2 that is released from the membrane. At first this seems perfectly reasonable until one does the math. Doing the latter suggests that binding affinities do not really mean much in the context of crowded scaffolds since one of the binding partners is present in large molar excess. Because of that, most of such processes are kinetically controlled. Where affinity comes back into play is in the specifics of the model that the authors propose because in this case the component that "shuttles" (pacsin-2 in this case) will partition between two possible targets. If one were to take the authors data at face value, it would mean that the membrane always wins because the "binding affinities" are about an order of magnitude higher. Given the 6:1 ratio the authors suggest for the actin:pacsin-2 interaction, it should be a fun exercise to calculate whether one could possibly cram enough actin next to the membrane such as to cause a significant relocation of pacsin-2 from the membrane to actin. In other words: this part of the mechanistic model shown in Fig 9 seems unlikely to play a role. In this context it also is worthwhile to point out that the fluorescence microscopy does not really help to address this issue because it lacks the resolution to show that any of the pacsin-2 in the vicinity of membranes is actually bound to actin. Given these unresolved issues, this leaves the "delivery" aspect of the model, which this reviewer thinks is GREAT!

We are well aware that detailed studies will be required in the future to corroborate or dismiss any of the proposed models. However, with respect to the seemingly large difference in affinities of

pacsin2 between membranes and actin filaments (when taking our data at face value that make the shuttling model seemingly improbable) one has to recall the multitude of additional processes that take place in vivo e.g. at membrane sites where an endocytic process is just going on. Lipases (e.g. PLC which degrades negatively charged PIP2 lipids) are active components in such a process and can rapidly alter the electrostatic nature of a local membrane surface. Pacsins may thereby be released from such membrane sites and then the "low" affinity of pacsin for F-actin may be sufficient for its sequestration by the surrounding cytoskeleton. Accordingly, we specified the respective model by replacing two sentences in the Discussion on page 23 by:

"**Conceivably, phopsholipid modifying enzymes (e.g. phospholipase C) may regulate membrane association of pacsin2 by locally altering the electrostatic nature of the membrane and the nearby actin cytoskeleton may sequester the released pacsin2 thereby providing a storage system close to sites of high membrane dynamics until pacsin's membrane activity is again required**."

Minor concerns/editorial comments:

Page 3: "tip to tip oligomerization of the F- and N-BAR ...." This is factually incorrect. Both F- and N-BAR scaffolds have (extensive) lateral interactions between BAR-dimers. In F-BAR these interactions are directly between the coiled-coil cores, in N-BAR the lateral interactions involve the H0-helices.

We thank reviewer to point this mistake out. The text was amended on page 3:

"Tip-to-tip and/or lateral interactions of the BAR domain dimer units result in long filaments that wind around membrane tubules in a spiral-like form that promote further tubule formation (Frost et al, 2008; Mim et al, 2012; Shimada et al, 2007)."

Page 4 (second paragraph): need to insert an "in" into the first sentence

Done.

Page 4 (last paragraph): "... whereby the convex site of the F-BAR ...."; should read concave

Done.

Page 6: "In order to quest" is not an English phrase

Done – replaced by:

"… in order to address the question"

Page 6: "unspecificity" is not an English word

Done. Entire section was re-written.

Figure 3 has two sets of curves for Pacsin2-delta1. Is the second one supposed to be Pacsin2-delta2? There also should be error bars for the binding curves.

We corrected the name on the Figure. For the error bars please see the answer above.

Figure 5A has no scale bar

Done, scale bars were inserted in Figures 5A and B.

Figure 8B has no error bars

Figure B shows one representative experiment, such presentation of this data is common in the literature, please see for example: Rybakova I N , and Ervasti J M J. Biol. Chem. 2005;280:23018-23023. For clarity we introduced this text on the caption of Figure 7B:

"The results of a typical experiment are shown."

Page 13: what do the authors mean by "rigid cast" when talking about N-BAR? These domains form quite flexible scaffolds. Moreover, N-BARs are perfectly capable of accommodating different curvatures (Mim et al 2012), just like F-BARs. Thus, it is unclear what the authors are talking about here. Similarly the discussion about what bends membrane and what senses curvature is very messy. Both F- and N-BARs can do both, and the degree to which each property contributes to function in vivo is very much an unresolved issue.

Following the right observation of the Reviewer the sentence on the "rigid cast" was modified in order to make it clear that we are referring here to the BAR domain structure in in solution, and not in lipid bound form. The paragraph was amended as follows:

"Furthermore, N-BAR domains tend to display less structural plasticity in solution, as shown in a small angle X-ray study of the endophilin N-BAR domain (Wang et al, 2008) and molecular dynamics simulations of amphiphysin (Blood & Voth, 2006). F-BAR domains display structural plasticity, rooted in the flexibility of the tails, resulting in being capable of adopting diverse curvatures. This has been seen in structural studies of human pacsin1 and 2 and murine pacsin2 (Plomann et al, 2010; Wang et al, 2009), of FCHO2 F-BAR domain (Henne et al, 2007), of F-BAR domains of FBP17 and CIP4 (Frost et al, 2008) and in a recent molecular dynamics simulation study of ECF F-BAR domain (Yu & Schulten, 2013)."

Page 19: typo, should read "octylglucopyranoside". How much buffer was used to wash out the detergent? Any residual detergent left will change the properties of the next lipid coating of the chip.

We corrected the text. We have washed the sensor chip with the stabilisation routine and additional washings, in each cycle the sensor chip was washed by the running buffer for at least five minutes (at flow rate 10 µl/min). In our experience this is enough to remove residual detergents from the flow cells and microfluidic system and allowed reproducible deposition of small unilamellar vesicles, see the figure to comment 6 above, and reproducible binding experiments.

Supplementary Info: There is no overlay in Supplementary Figure 1, and the description of what was done is not clear.

Overlay was added to Supplementary Figure 1.

2nd Editorial Decision - The EMBO Journal

Thank you for your point-by-point response provided upfront. I have consulted referee #3 for arbitrating advice on it. I have by now also received input from this referee on your revised manuscript and you can find both the report and the advice below.
As you will see, referee #3 and referee #1, both EM experts, are not convinced that your structural data sufficiently support your conclusions. They provide arguments from different angles, but both

think that the structural model and the stoichiometry provided are rather speculative and should be removed from the manuscript. This, however, reduces the advance of the manuscript and I am very sorry to say that we therefore cannot offer publication here.

That being said, we still recognize the potential of your work and I realize that it might be too challenging to ultimately proof your proposed mechanism in a timely manner. I would therefore like to propose a transfer of your manuscript to our sister journal EMBO Reports, which publishes conceptually novel findings, without necessarily the kind of mechanistic detail for which we are looking at The EMBO Journal. I have already discussed with Dr. Barbara Pauly from EMBO Reports, and she and her colleagues agreed to publish your manuscript after removal of the model (Figure 6) and all remarks regarding the proposed stoichiometry and binding affinities. A shortening or reorganization to meet the EMBO Reports standard format, and amendments to address the following concerns still raised by referees #1 and #3 would be required for a successful transfer:
Referee #1: please address/discuss points 2-4
Referee #3: please address/discuss the issues raised in points 4, 5 and 7.

I am very sorry to disappoint you on this occasion and I hope you will view the possibility of a transfer with no further peer-review favorably. If this is the case, please use the link below to transfer the manuscript directly.

REFEREE REPORTS:

Referee #1:

Summary:
The regulatory mechanisms that govern the activity of BAR domain proteins remain poorly understood. This is especially true with regard to the interface between cellular membranes and the actin cytoskeleton -- critical interfaces where BAR domain proteins often function. The revised manuscript strengthens the claim that the F-BAR protein Pacsin2/Syndapin binds F-actin directly via the same surface that engages target membranes. The discovery and characterization of this interaction will have far-reaching implications for our understanding of membrane remodeling processes. The revised report has improved substantially in certain respects and I would like to see these observations make into print for the broader community. However, technical and conceptual problems preclude me from recommending it for publication in its current form by EMBOJ.

Major Points:
1) The 3D cryoEM reconstruction.

1a) The author's make salient points in their rebuttal letter concerning different approaches to 3D reconstruction, the challenges presented by samples that are destabilized by cryoEM grid preparation, and the impact of fixation by negative stains or in preparation for STEM. Nevertheless, the major conceptual points of my critique (and the critique of reviewer 3) were brushed away rather than dealing with the central problems:

1b) The destabilization of the complex during cryoEM grid preparation is all the more reason to determine the stoichiometry through a mass-per-unit length STEM study using fixed samples.

1c) Imposing the symmetry of actin within the IHRSR algorithm is obscuring the structure of interest. Symmetrization as implemented within IHRSR improves SNR for actin, but clearly degrades the resolution and distorts the structure of Pacsin. I strongly disagree with the authors that an asymmetric reconstruction of this complex is meaningless. If Pacsin binds to F-actin via defined interactions (as the authors propose), then the structure of this minimal unit can and should be solved without iterative symmetrization of the volume based on the structure of F-actin. I hope the authors will consider approaching this as an asymmetric single particle project. I am confident that modern classification algorithms in 2D and in 3D will sort out segments of the filaments that are decorated by Pacsin. Reconstructing these coherent segments, without helical averaging, should reveal the structure of Pacsin bound to F-actin.

1d) Short of re-solving the structure as single particle project as outlined above, I would like to see

their crystal structure fit into the pacsin2 density in figure 5C. As it stands, I can't see whether/how the overall shape of the "winged" pacsin molecule can be accommodated by their density.

1e) The caption for Fig. 6A states "The yellow surface at the bottom is a three-dimensional reconstruction of the atomic model shown, after imposing the actin helical symmetry and filtering to 12 Å resolution" Is the yellow density in 6A therefore a calculated density from the atomic coordinates of the hypothetical model illustrated in 6C? If so, I think the speculative nature of this density has to be make absolutely clear. I find this modeling effort troubling, as it appears the hypothetical structure in Fig 6 is quite different from the volume shown in 5C.

2) Distinguishing between self-assembly versus F-actin binding by centrifugation.

2a) When I compare Figures 2D-E and Fig S5, there appears to be inconsistency in the amount of Pacsin2 that pellets in the absence of actin. There is a very faint band in Fig S5, a more impressive band in Fig. 2D. More frustrating, there is no quantification of the amount of Pacsin2 that pellets in the absence of Actin in Fig 2E.

2b) The data in Fig. S7 clearly show a clear actin-dependent pelleting of FCHO2 -- despite the caption title and the main text stating otherwise.

3) X-linking and mass spectrometry

3a) This appears to be a nice addition to the paper. However, in my laboratory this approach is prone to false positives as well as false negatives that are challenging to rule out. To strengthen our confidence in these new results, the authors could report on the identification of "positive control" hybrid peptides that arise from the expected crosslinks based on the known actin-actin and pacsin-pacsin structures.

4) Other comments

Lines 152-154: The authors state that both full-length pacsin2 and a C-terminally truncated version bind to actin and refer readers to Figure 2, but the data for full-length pacsin2 is not shown.

Referee #2:

In my view, the authors have responded well to all the questions. I have no further comments.

Referee #3:

1/ The authors did address a good number of the original concerns that were related to binding studies/biochemical assays - this is a strong aspect of the revision
2/ The authors added crosslinkling and mass-spectrometry data to strengthen their model - this is a strong aspect of the revision, and certainly helps

3/ I commented on the use of curvature limited vesicles for measuring membrane association. The authors decided to ignore this, and basically make an argument that in lay language sounds like the following: ""well - curvature does not matter to us. We just happen to see an effect on these substrates, so we use them to be able to say "something"". Personally, this does not meet my own standards and I find the authors stance very "sloppy" in the sense that they produce numbers for the sake of producing numbers without considering the relevance of those numbers. But, I could live with those results - they don't do any fundamental damage, they just waste space.
4/ I commented on technical issues with using SPR. Specifically, I was concerned about the possibility that washing the SPR chips with NaOH to dislodge unbound vesicles may result in the unwanted formation of lysolipids due to spontaneous hydrolysis of phospholipids. The authors chose to ignore this concern as well. They hide by pointing out that this is what everybody does and that this protocol is following the manufacturers instructions. Fair enough. Personally though: I do not really care what the manufacturer says or what other people are doing because basic organic

chemistry dictates that ester bonds found in phospholipids will instantaneously and irreversibly succumb to hydrolysis when exposed to a solution of pH 12 or 13. How to resolve this: well, if standards in the "fields" the authors refer to are that poor, I guess that one will have to live with it then. As for me, if I were to read this after publication, I would simply ignore these results because they likely do not mean anything that one would need to be aware of/concerned with.
5/ Related to the previous point: the authors use pure POPS vesicles. This is a very artificial choice because it has nothing to do with physiological conditions. Therefore, the binding affinities they measure and present most likely do not mean anything at all. Again - it's one of these cases where experiments are done just for the sake of it. The results that fall out of these measurements do not provide any useful insights for understanding how any of this works in vivo. Moreover, the differences in binding affinities for the various mutants are, in most cases, not statistically significant. If the authors insist on keeping these types of results - I would recommend that they reduce this to a single sentence stating that curvature limited, pure POPS vesicles bound the various pacsin2 variants with submicromolar to low micro molar affinities. What I do not get: why did they not use a natural lipid mix or "plasma membrane mimetic" - suitable lipid compositions are well known. While this still would be somewhat artificial (because we do not know how locally lipid compositions may deviate from bulk plasma membrane), the numbers would be more realistic. Why would this be important? To me this would matter because their final model proposes a dynamic partitioning of pacsin2 between the membrane and actin. Thermodynamic affinities for engaging either one of these substrates are not likely to be meaningful (because of the large local concentrations of the substrates) - however, the affinities give some sense of the "on" and "off" rates for binding, and it is those parameter that are driving these things in vivo.
6/ The authors take the "tilde" shaped pacsin structure and straighten it out to fit their reconstruction. I commented on this, but the authors don't care. They just refer to observed flexibility in the tails and make a hand waving argument that energetically this straightening could be paid for through electrostatic interactions. Well - I am not convinced it could because under physiological conditions, there is a lot of electrostatic screening (because of the intracellular ion concentrations), and because of the fact that the pacsin-actin interaction is so low affinity that it falls apart upon freezing (= binding cannot hold more than a few kcal/mol = probably not enough to straighten a proline kink). If the authors want to insist on the modeling they do - well, that is their prerogative. But in this case, they should back it up with explicit calculations showing that the binding energy can offset the bending energy of the tail regions.
7/ I pointed out that the F-actin presents a very different curvature/substrate for pacsin binding than what is observed in the membrane bound scaffolds of the F-BAR protein CIP4. The authors responded by removing the ill-conceived figure they originally used to support their point. However, in the revised text - the erroneous claim is still there (line 272-273). Seems to me, the authors really do not understand what they are talking about.
8/ I had a lot of issues with the F-actin:pacsin2 binding stoichiometry because the authors got conflicting answers using different approaches. In their original rebuttal they concede that that this is true. However, in the revised version they still keep pushing for a 6:1 stoichiometry (lines 274-283) because based on their reconstruction that "looks about right". Well, personally I am not impressed by their argument. They have fallen in love with their model, and they keep pushing it no matter what. The point is, the stoichiometry really does not matter at all - so why mentioning it at all. As is, the authors have no experimental evidence in support of what they put forward. All they can say is the "pacsin binds F-actin" - anything beyond that is fiction.

Arbitrating comments:
I side with the authors when it comes to the "asymmetric single particle" reconstruction. To me it is not immediately clear how one would do that without using a priori knowledge about how this complex is supposed to look like. This is because in a true single particle reconstruction, the "particles" must have random orientations to sample all possible views that are needed for an undistorted reconstruction. In the case of the Factin-pacsin complex, I cannot see how one could accomplish this without invoking some sort of helical/rotational symmetry. Specifically - the sample is a filament that is always seen from the side. As the authors point out correctly: boxing independent segments and treat them as truly independent single particles will not get you an answer because no matter how you do this, the only difference in "view" is in the azimuthal angle. For a complete data set you'd need "end on views" (like looking at the filament along its long axis) - and those views you cannot get. So this part of the argument comes out in favor of what the authors say. The only way that I can think of how this could reasonably be approached as a "single particle" project is to collect dual-axis tomograms, next extract subvolumes which are then treated as single

particles. There are recent examples where this approach has yielded resolutions far better than what is presented here. HOWEVER, this approach will not work (in all likelihood) because of the enormous background of unbound pacsin2 (in the original rebuttal the authors make a big fuss about the fact that invoking helical symmetry is the ONLY reason why they can reconstruct this at all without being "killed" by the messy background). That said, what I do not really understand is why the authors did not even try the simpler thing I suggested: segment out different regions from the non-symmetrized reconstruction and search for any potential helical symmetry of the pacsin2 component. Granted: this only has a chance of working after the VERY FIRST round of reconstruction because during this first cycle the authors claim to have used a plain cylinder as a model. This model is "featureless" and therefore the reconstruction will not be biased by the actin filament structure. Once the parameter search locks in on the actin helix, and once that helical symmetry has been incorporated into the model, any regularity in the pacsin is irreversibly lost. Now, I can understand why the authors may have ignored that suggestion because they show in their first rebuttal a power spectrum of the sample and comment that this does not show any signs of anything but an actin helical component. Fair enough - this may be true. But if it were, the authors also put themselves into a "deadend". If there is indeed NO regularity in the F-actin:pacsin2 interaction then that is EXPLICITLY saying that there is NO stoichiometric relation between these two molecules at all = their claim of a 6:1 stoichometry is complete bogus and all remarks related to "stoichiometry" AND the model shown in Fig 6 SHOULD/MUST BE deleted because there is ABSOLUTELY NO experimental evidence for it (also see point 6 in my comments above). Doing so would also take care of the reviewer's request for having STEM experiments done. In other words: the data put forward by the authors can be reduced to the conclusion: "F-actin binds pacsin2". It's the prerogative of the authors to show what is shown in Fig 5 to support this at a structural level. The authors even may isolate one pacsin2 molecules and show how it interacts with the actin filament + discuss their cross linking/mass spec data in this context. However, any molecular interpretations along what is shown in Fig 6 should not be attempted because based on what is presented, this interpretation is mostly fiction/wishful thinking. By imposing the F-actin symmetry, the authors have averaged away any structurally relevant detail of the pacsin2. Interpreting their reconstruction in terms of actual pacsin2 structure, and proposing that actin binding causes straightening of the pacsin2 tails is, to say it politely, very reaching (see point 4 in my remarks above). The big question is: if the authors were to eliminate Fig 6 and all the discussion associated with it, along with all discussion about "stoichiometry" and "binding affinities" - is there still enough left to warrant publication in EMBOJ? I think so because the additional biochemistry and cross linking/mass spec + the basic reconstruction (Fig 5) make an important contribution to this field. However, I think that in its "shortened" form (that sticks with the data and eliminates all speculation) this manuscript may be more suited as a Report and that report should be published without further delay.

Transfer to EMBO reports - authors' response to original referees' comments 21 July 2014

Following the suggestion from the EMBOJ editor, we are submitting here the manuscript, originally submitted to EMBOJ:

"Direct Interaction of Actin Filaments with F-BAR Protein Pacsin2"

We amended the manuscript following the suggestions of the EMBOJ editor:

Referee #1: please address/discuss points 2-4

Referee #3: please address/discuss the issues raised in points 4, 5 and 7.

We furthermore considerably shortened the manuscript from 90.000 to 40.700 characters. To do so we shortened introduction, results and discussion parts, reduced the figures from 8 to 4 and

moved a considerable amount of Methods and materials to the Supplementary Section. Supplementary Section figures were also reduced form 16 to 12.

We hope the manuscript in this form will find your approval.

Point-by-point reply to reviewers' suggestions:

Referee #1:

2) Distinguishing between self-assembly versus F-actin binding by centrifugation.

2a) When I compare Figures 2D-E and Fig S5, there appears to be inconsistency in the amount of Pacsin2 that pellets in the absence of actin. There is a very faint band in Fig S5, a more impressive band in Fig. 2D. More frustrating, there is no quantification of the amount of Pacsin2 that pellets in the absence of Actin in Fig 2E.

We see some fluctuation in the precipitation of pacsin2 in individual co-sedimentation assays, as clearly observed and demonstrated in the figures and recognized by reviewer. With this experiment we addressed the response of the protein to increased concentrations of actin and salt. In each of these experiments, pacsin2 and endophilin were exposed to the same conditions and each experiment was repeated several times. From both types of experiments it is clear that, while pacsin2 responded to the increasing salt and actin concentrations (although with some amount of protein found in the controls (pellet) fraction without F-actin), this was not the case for endophilin, which responded only marginally. Thus self-assembly as mentioned by the reviewer, if it were to take place in the case of endophilin, would not be affected by salt, or would not contribute to the binding to F-actin. In case of pacsin2 self-assembly might be possible, however, we never observed higher oligomers formation for pascin2 when subjected to size exclusion chromatography. Secondly, the amounts of protein that were found to co-sediment with F-actin were always several times higher compared to the amounts of protein pelleting in the absence of F-actin (control experiments), clearly indicating actin driven/dependent pelleting of pacsin2.
Regarding the Figure 2D-E, the amount of both pacsin2 and endopholin that pellets in the absence of actin was quantified previously and subtracted from the amounts of protein binding in the presence of actin. This was done to allow better comparison, in other words to remove noise from the data, as both proteins pellet alone to the different extent (please see figure below). In addition, this is clearly stated in the figure legend for Figure 1C as: Data are presented as amount of protein bound to F-actin, from which amount of protein found in the pellet fraction without F-actin has been subtracted. For your consideration and for comparison we attach a graph where subtractions of protein found in the pellet when incubated without F-actin were not done:

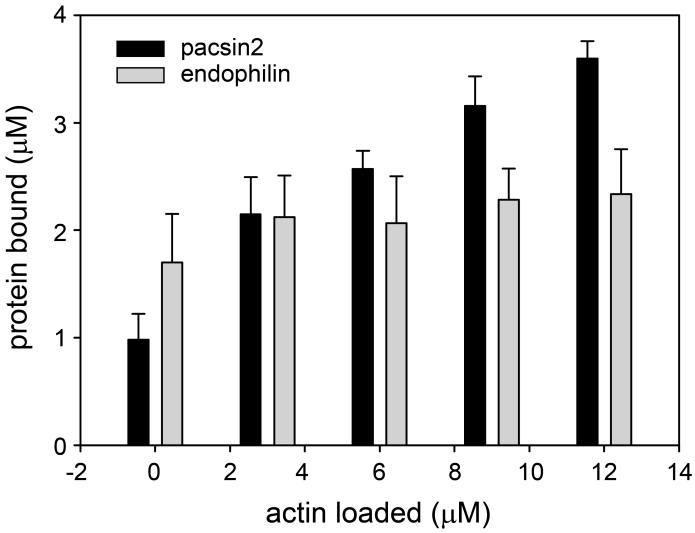


Figure: Pacsin2 and endophilin A1 co-sediments experiments
Densitometric quantitation of gel bands, obtained in experiments of Figure 1C. Here the amount of pacsin2 and endophilin that pellet in the absence of actin was not subtracted, compared to Figure 1C. Mean values (± S.E.) of three independent experiments are shown.

2b) The data in Fig. S7 clearly show a clear actin-dependent pelleting of FCHO2 -- despite the caption title and the main text stating otherwise.

We agree with the reviewer to the extent that FCHO2 shows clear actin-dependent pelleting, and therefore we subjected the protein to the EM studies. These studies, however, revealed the formation of aggregates surrounding naked actin filaments, and no association with F-actin. Formation of soluble aggregates of FCHO2 explains why in the co-sedimentation assay the protein does not pellet in the absence of actin, whereas it associates and pellets in an unspecific way when actin is present. To improve the clarity of the text we amended the main text on page 5:

In addition, we found un-specific or no binding of FCHO2 and CIP4, respectively, to the F-actin by using of co-sedimentation and EM studies (Supplementary Fig S3 and S4).
Accordingly we modified the figure legend of Figure S3 to:
Figure S3: Unspecific association of FCHO2 with actin filaments.
(A) FCHO2 was analyzed in co-sedimentation assay after incubation with or without preassembled actin filaments at final concentration as shown on the figure. Actin filaments and proteins bound were sedimented by centrifugation, and equal amounts of supernatant (s) and pellet (p) fractions were subjected to SDS-PAGE; separated proteins were visualized by Coomassie Brilliant Blue staining. (B) Electron micrograph of negatively stained F-actin (2 μM) incubated with FCHO2 (10 μM) for 20 minutes show no association of FCHO2 with actin filaments. Thus association with F-actin, as observed in co-sedimentation assay, was not confirmed by EM, where the protein was found to form aggregates surrounding naked actin filaments. The scale bar represents 100 nm.

3) X-linking and mass spectrometry

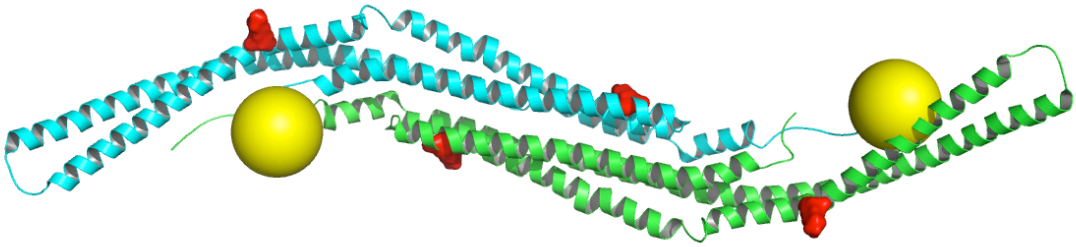
3a) This appears to be a nice addition to the paper. However, in my laboratory this approach is prone to false positives as well as false negatives that are challenging to rule out. To strengthen our confidence in these new results, the authors could report on the

identification of "positive control" hybrid peptides that arise from the expected crosslinks based on the known actin-actin and pacsin-pacsin structures.

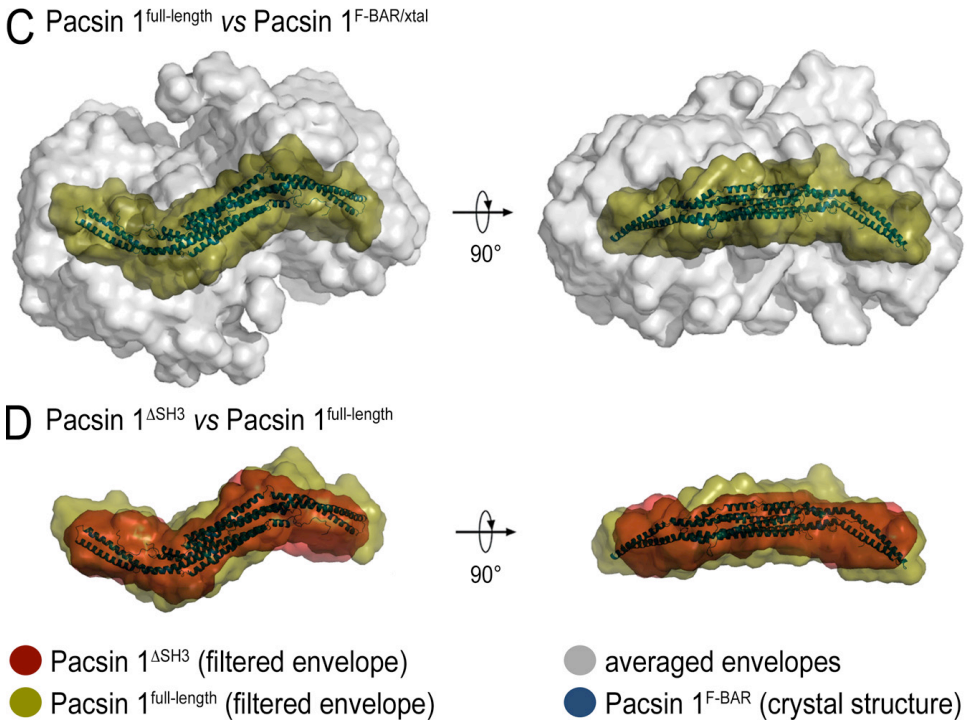
Following suggestion fo the reviewer we inserted information on internal peptides within pacsin molecules in the pacsin:actin complex, determined in the band corresponding to the molecular weight of one pacsin and one actin molecule in Supplementary Table S2:

Protein, residue	**Protein, residue**	**Highest score**
pacsin2, K67	pacsin2, E424	9.372
pacsin2, K146	pacsin2, E406	7.642

For both internal cross-links, one residue maps to the F-BAR domain, while the second maps to the SH3 domain, which is at the C-terminus (Figure, SH3 domain yellow ball, K67, K146 red). The SH3 domain is unfortunately not present in our crystal structure nor in other structures of pacsins. The SAXS analysis of full-length pacsin suggests that the SH3 domains may be linked flexibly to a rigid F-BAR domain dimer in solution (Figure adapted from Wang et al, PNAS 2009), because their position relative to the F-BAR domain could not be determined unambiguously (Wang et al, PNAS 2009).



They furthermore showed that pacsin's membrane deformation activity was significantly impaired by SH3 domain, suggesting an auto-inhibitory role of these units in pacsin, which in turn suggest proximity of SH3 domain to the concave, membrane binding surface.



4) Other comments

Lines 152-154: The authors state that both full-length pacsin2 and a C-terminally truncated version bind to actin and refer readers to Figure 2, but the data for full-length pacsin2 is not shown.

The Reviewer #1 must have overlooked that in former Figure 2 (now Figure 1) interactions of both pacsin2tr (shown in Figure 2A) and pacsin2 full-length (in detail shown in Figure 2B) with actin ***are shown****. This is stated in the legend of Figure 1 as well. In addition, interaction of full-length pacsin2 with F-actin can be also seen in Supplementary Figure S1. To improve the text, we modified the main text on page 4 as follows:*

Both full-length pacsin2 as well as the C-terminally truncated pacsin2 variant pacsin2tr (residues 1-324) that mainly comprises the F-BAR domain were found to directly interact with F-actin (Fig 1A and B, Supplementary Fig S1).

Referee #3:

4/ I commented on technical issues with using SPR. Specifically, I was concerned about the possibility that washing the SPR chips with NaOH to dislodge unbound vesicles may result in the unwanted formation of lysolipids due to spontaneous hydrolysis of phospholipids. The authors chose to ignore this concern as well. They hide by pointing out that this is what everybody does and that this protocol is following the manufacturers instructions. Fair enough. Personally though: I do not really care what the manufacturer says or what other people are doing because basic organic chemistry dictates that ester bonds found in phospholipids will instantaneously and irreversibly succumb to hydrolysis when exposed to a solution of pH 12 or 13. How to resolve this: well, if standards in the "fields" the authors refer to are that poor, I guess that one will have to live with it then. As for me, if I were

to read this after publication, I would simply ignore these results because they likely do not mean anything that one would need to be aware of/concerned with.

We respect reviewer's view on the issue. We could perform confirmatory experiments that lipids in liposomal membrane are not excessively damaged during the preparation of suitable surface for molecular interaction analysis. However, these will take additional time and in our opinion it will not change conclusions made in the paper. Such treatment is used repeatedly with many researchers (Cooper M, 2000, Anal Biochem, 277:196-205; Critchley P, 2004, Biochem and Biophy Res Commun 313:559-567; Shen K, 2008, Biochemistry 47(34):8855-8865; HelmHolz H, 2010, Biochim et Biophy Acta 1798:1944-1952; Stahelin RV, 2013, MBoC, 24:883-886) in the field and the data obtained in such way are qualitatively and quantitatively similar to other independent approaches.

5/ Related to the previous point: the authors use pure POPS vesicles. This is a very artificial choice because it has nothing to do with physiological conditions. Therefore, the binding affinities they measure and present most likely do not mean anything at all. Again - it's one of these cases where experiments are done just for the sake of it. The results that fall out of these measurements do not provide any useful insights for understanding how any of this works in vivo. Moreover, the differences in binding affinities for the various mutants are, in most cases, not statistically significant. If the authors insist on keeping these types of results - I would recommend that they reduce this to a single sentence stating that curvature limited, pure POPS vesicles bound the various pacsin2 variants with submicromolar to low micro molar affinities. What I do not get: why did they not use a natural lipid mix or "plasma membrane mimetic" - suitable lipid compositions are well known. While this still would be somewhat artificial (because we do not know how locally lipid compositions may deviate from bulk plasma membrane), the numbers would be more realistic. Why would this be important? To me this would matter because their final model proposes a dynamic partitioning of pacsin2 between the membrane and actin. Thermodynamic affinities for engaging either one of these substrates are not likely to be meaningful (because of the large local concentrations of the substrates) - however, the affinities give some sense of the "on" and "off" rates for binding, and it is those parameter that are driving these things in vivo.

We would like to thank reviewer for his opinion. It was not our intention to mimic physiological conditions with our choice of lipid membranes. The purpose of SPR experiments was to compare membrane interactions of the wild-type pacsin2 and its mutants. Our SPR results do not allow determination of on and off rates and we stated this clearly in the previous version of the manuscript. We chose this particular membrane system (small unilamellar vesicles composed of negatively charged lipids) in order to achieve significant interaction of pacsin2 and to easily detect differences between the WT and mutants. Other model membrane systems would also be, as reviewer correctly noted, somehow artificial, since it is impossible to know at the moment what are the best conditions for Pacsin2-membrane interactions with regards to lipid composition and curvature of the membrane. We feel that this is beyond the scope of the current paper and is a matter for future research. We would like to stress again that the purpose of SPR experiments was to get an idea what is the surface that Pacsin2 uses for the membrane interactions. Perhaps the intention of SPR experiments was not stated clearly in the previous version of the manuscript and in the revised version we make sure to state this clearly. Furthermore, as proposed in-vivo model on a

dynamic partitioning of pacsin2 between the membrane and actin was removed from the text, as suggested by Reviewer #3, we believe that the results generated on an indeed artificial system do not compromise the main conclusions of the manuscript.

7/ I pointed out that the F-actin presents a very different curvature/substrate for pacsin binding than what is observed in the membrane bound scaffolds of the F-BAR protein CIP4. The authors responded by removing the ill-conceived figure they originally used to support their point. However, in the revised text - the erroneous claim is still there (line 272-273). Seems to me, the authors really do not understand what they are talking about.

We apologise for this, this text together with the entire section on stoichiometry and on the molecular model was removed from the main text, as suggested by the reviewer #3.

1st Editorial Decision – EMBO reports 29 July 2014

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees that were asked to assess the revised version now support its publication in our journal.

Before the manuscript can be officially accepted, please attend to the remaining, formal issues:

1. Referee 1 still asks for some minor clarifications and textual changes to be incorporated (please see his/her report below). With regard to this reviewer's comment on the data using POPS-containg SUVs, we feel that you can keep them in the manuscript if you wish.

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

You will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFREE REPORTS:

Referee #1:

This report is a drastically shortened version of a previous manuscript, describing the interaction of the F-BAR domain protein pacsin 2 with actin. In response to previously raised concerns, the authors have removed speculations that were not supported by data.

The finding that pacsin binds actin is important, adding another dimension of complexity to the membrane interface. The observation that other F-BAR/N-BAR domain proteins (CIP4, FCHO2, endophilin were tested) fail to bind actin provides additional significance and sets the stage for future studies that may shed light on the physiological role of actin:pacsin2 binding. The basic conclusion that pacsin2 binds to actin is well supported by data, including a 3D-reconstruction from

TEM images of unstained samples. That said, the authors chose to keep some of the weaker data as well. While evidently those experiments are doable, they do not add value to the manuscript because they are far removed from anything that could have a physiological meaning. However, the importance of the basic finding that pacsin2 can associate with actin is sufficient to warrant publication.

Specific comments are as follows:

1/ Abstract: what do the authors mean by "mechanical force needed for membrane remodeling is provided by the actin cytoskeleton"? Most BAR domain proteins can induce tubulation/curvature on their own.

2/ Line 69/70: sentence makes no sense.

3/ Line 135: related to experiments investigating the potential impact of pacsin 2 binding on actin dynamics: what is the concentration of pacsin 2 in vivo? Based on that and considering cellular concentrations of actin, could an actin:pacsin ratio of 1:10 ever occur in a cell? If not, what is the point of these experiments? It seems that the authors are trying too hard to find some complicated meaning in their basic observation that pacsin 2 binds actin.

4/ Line 148-162: as pointed out in previous reviews, investigating binding of pacsin 2 constructs to pure POPS SUVs is a meaningless exercise. There is nothing to be learned from these data. Too sad the authors chose to keep this part of the manuscript.

5/ Line 175: "...crystallized pacsin 2" - the related pdb entry (4BNE) is of the F-BAR domain only.

Referee #2:

I reviewed this manuscript extensively for another journal (the EMBO journal). In its current form, the manuscript has satisfied all of my technical and interpretative concerns. I believe their discovery will be broad interest and should be published in its current form.

1st Revision - authors' response 10 August 2014

Point-by-point reply to reviewers' suggestions:

Referee #1:

1/ Abstract: what do the authors mean by "mechanical force needed for membrane remodeling is provided by the actin cytoskeleton"? Most BAR domain proteins can induce tubulation/curvature on their own.

Reviewer #1 is right, due to shortening of the abstract this sentence is not correct in the context of the text. The first two sentences of the abstract were replaced by:

"Two major mechanisms have emerged as major regulators of membrane shape: BAR domain-containing proteins, which induce invaginations and protrusions, and nuclear promoting factors, which cause generation of branched actin filaments that exert mechanical forces on membranes."

2/ Line 69/70: sentence makes no sense.

Reviewer #1 is right, there was a grammatical mistake, the sentence was corrected:

"Liposome binding studies with pacsin 1 revealed that the tubulation-inducing activity of the F-BAR domain alone is diminished in the full-length protein due to the autoinhibition by the C-terminal SH3 domain and the preceding linker (19, 21)."

3/ Line 135: related to experiments investigating the potential impact of pacsin 2 binding on actin dynamics: what is the concentration of pacsin 2 in vivo? Based on that and considering cellular concentrations of actin, could an actin:pacsin ratio of 1:10 ever occur in a cell? If not, what is the point of these experiments? It seems that the authors are trying too hard to find some complicated meaning in their basic observation that pacsin 2 binds actin.

Given that pacsin2 binds actin filaments in a tropomyosin-like fashion it came natural to us to investigate, whether pacsin2 also has an effect on F-actin depolymerisation, as does tropomyosin.

The concentrations of pacsin 2 in the cell are unfortunately not known, but it is hard to imagine that bulk pacsin 2 concentrations exceed those of actin, as the latter is one of the most abundant proteins in a eukaryotic cell. These biochemical experiments were performed in a test tube with purified components, where the law of mass action holds for the bulk concentrations of reactants. The reviewer is of course right if one considers the law of mass action in under bulk conditions. Local concentrations in cellular compartments might though be very different, in particular when it comes to membrane and cytoskeletal structures where local concentrations of components can be much higher. We designed this experiment based on study of effects of tropomyosin on decrease in actin depolymerisation (Biochemistry. 1989, 28(21):8501-6), where molar ratios of F-actin and tropomyosin were also about 1:10, albeit absolute concentrations used were lower, indicating a weaker interaction in case of pacsin2.

4/ Line 148-162: as pointed out in previous reviews, investigating binding of pacsin 2 constructs to pure POPS SUVs is a meaningless exercise. There is nothing to be learned from these data. Too sad the authors chose to keep this part of the manuscript.

Given that the editors allow us to choose, we opt for keeping these results in the manuscript. There is a vast and respectable body of literature, which we provided in the previous reply that supports these experiments, too sad that the reviewer did not take this information into her/his consideration.

5/ Line 175: "...crystallized pacsin 2" - the related pdb entry (4BNE) is of the F-BAR domain only.

We thank the reviewer for this, we corrected the sentence as follows:

„To map/fit pacsin2 into the additional electron density, we crystallized F-BAR domain of pacsin2 (28) and solved its structure to 2.57 Å, with final refinement statistics of R_{work} = 0.184 and R_{free} = 0.222 (Supplementary Fig S7, Supplementary Table S1).”

2nd Editorial Decision – EMBO reports 11 August 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.

