F-actin bundling sites are conserved in proteins with villin type headpiece domains.

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1st Editorial Decision

April 10, 2020

RE: Manuscript #E20-02-0158

TITLE: F-actin bundling sites are conserved in proteins with villin type headpiece domains.

Dear Prof. Khurana:

Two experts in the field reviewed your manuscript. You will see that both reviewers found interesting your data on the F-actin bundling activity of villin type headpiece domains. However, you will see that they suggest major revisions that you should address in the revised version of your manuscript.

It is important to mention that these revisions will NOT require additional experiments.

I would like to invite you to revise your manuscript in response to these comments. You should in particular address the comment from reviewers #1 and 2 on the quantification of your cell-based assays in Figures 3 and 4.

Sincerely,

Laurent Blanchoin
Monitoring Editor
Molecular Biology of the Cell

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Dear Prof. Khurana,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org
Reviewer #1 (Remarks to the Author):

In this manuscript, George and coworkers utilize a combination of in vitro and cell-based assays to characterize the contributions of actin binding residues in the headpiece domains of villin and advillin to filament bundling. They identify four residues that are essential for bundling activity and confirm the importance of these residues to villin and advillin's roles in filopodial formation in HeLa cells. The authors also find that villin and advillin dimerize and propose that this self-association confers bundling activity to the proteins. The four residues characterized in this study are conserved in headpiece-containing proteins expressed in a broad range of organisms, suggesting a unified mechanism for actin binding and bundling by these proteins.

The authors nicely summarize previous work that has been undertaken to identify actin-binding residues in the headpiece domain. By coupling their co-sedimentation experiments with functional assays performed in cells, they are able to directly test the contributions of individual residues to the activity of villin and advillin. Overall, the experiments are well motivated, and the results are clearly described. I therefore support publication of this manuscript once a few points have been addressed (please see below).

(1) I suggest expanding on the description of the experiments performed with cross-linkers. For example, how were the three crosslinkers selected and how do they differ? Can the different crosslinking efficiencies observed for the three crosslinkers be interpreted or explained?

(2) On a related note, would it be possible to include a negative control for the crosslinking experiments? For example, an experiment performed with only the headpiece domain (or some other fragment of villin and advillin that is predicted not to self-associate) in the presence of the various crosslinkers?

(3) I suggest quantifying the cell assays shown in Figures 3 and 4. The Results section summarizes the qualitative differences in the number and lengths of filopodia/cell surface protrusions. These differences can be quantified to strengthen the conclusions that are drawn, as can the extent of villin or advillin localization along the lengths of the protrusions.

Reviewer #2 (Remarks to the Author):

In this article entitled "F-actin bundling sites are conserved in proteins with villin type headpiece domains" the authors studied the bundling activity of the actin-binding proteins villin/advillin which are involved in the actin organization of the brush border of intestinal and renal epithelial cells. Although the actin binding domains (gelsolin-like and head piece) of villin/advillin are well characterized at the structural level, the molecular determinants involved in the bundling activity were not fully understood before this study. To identify the bundling site the authors combined available structural data, mutagenesis, co-sedimentation assays and cell biology observations.

Major comments:

1) Among several observations, they determined that the amino acid K821, in the previously identified 821-KKEK-824 motif, is required for actin bundling by full-length villin. This observation seems to validate previous studies. The authors should clarify how new this result is for villin. Also, they should explain whether this conclusion is also valid for advillin without testing the corresponding KAAA mutant of advillin.

2) In Figures 1C and 1D, the use of GST-fused villin and advillin to study F-actin bundling is questionable. GST is a dimer which creates or promotes the bundling activity of actin-binding proteins. The authors should justify this choice and explain why they believe that it does not affect the results of their mutants.

3) In Figure 2A, the authors use chemical crosslinking to show that villin and advillin make dimers. They mention in the text that « For these studies, the GST tag in FL villin and advillin proteins was cleaved by thrombin digestion as described in the methods. ». In the gels of this Figure 2A, what is the band below the band indicated as the monomer ? This band was not observed in Figure 1. The authors should explain why they exclude that these two bands could be residual GST-fused villin/advillin and cleaved villin/advillin respectively. This would be a problem since GST-fused proteins dimerize through GST.

4) The following statement should be supported by further quantification : “HeLa cells expressing the villin mutants W815A and F827A did not display the typical long filopodia seen with FL villin but appeared to have much shorter and fewer cell surface protrusions (Fig 3B(b,e)).”

5) The authors claim that some of the villin and advillin mutants failed to co-localize with F-actin in protrusions. One can actually
see that the GFP-fused villin mutants are present in these protrusions. It is very possible that the ratio villin or advillin/actin is lower for the mutants. However, a quantification of the data is required to reach this conclusion.

Minor comments

1) In the abstract, the following sentence is a little bit ambiguous: “We used the villin NMR structure, mutational analysis, and functional assays to identify the actin bundling site in full-length human villin protein”. I would rather write: “We performed a mutational analysis based on the available NMR structure of villin...”. My first understanding was that the paper contains a new NMR structure, which is not the case.

2) In Figure 1A, it would be helpful to show the domain organisation of villin featuring the gelsolin-like core and the head piece.

3) In Figure 1C and 1D, I am very surprised to read in the legend that the cosedimentation assays contain only 0.76 µM. The respective intensity of the villin / advillin bands does not correspond well with the protein concentrations indicated in the legend.

Conclusion

This study provides some new information on the mechanism by which villin and advillin bundle actin filaments. However, to be published, a significant effort in the quantification of the data presented in figures 3 and 4 is required.
We thank the Monitoring Editor and the reviewers’ for a very thorough and thoughtful critique of our manuscript. We also thank the reviewers’ for highlighting the strengths of our study which includes, how it adds new information about the mechanism by which villin and advillin bundle actin filaments; and how it provides a unified mechanism for actin binding and bundling by villin, advillin and potentially other headpiece-containing proteins. In the revised manuscript, we have incorporated all the suggestions and recommendations made by the reviewers’ and the Monitoring Editor and we concur with them that this has greatly improved our manuscript. As instructed by the Managing Editor, these revisions do not include additional experiments. However, they include new quantification of data, which support our experimental findings. Our response to each of the reviewer’s comments is as below.

**Reviewer #1**

1. As suggested by the reviewer, we have expanded the description of the experiments using cross-linkers. This includes the rationale for the choice of cross-linkers, how the three cross-linkers differ from each other and a description of what our data with different cross-linkers tell us about the self-association properties of villin and advillin (pages 10-12). Using chemical cross-linkers of varying spacer lengths, we have previously reported that villin self-associates with most cross-linkers including DTNB (George et al 2007 J Biol Chem 282:26528-26541). This suggested to us that in villin dimers two thiol groups must come as close as 2Å (the length of a disulfide bond). In data shown here in Figure 3A, we demonstrate, as reported before, that under non-reducing conditions villin self-associates with the cleavable (EGS, DFDNB) and the non-cleavable (DSS) cross-linkers of varying lengths (EGS: 16.1Å, DSS: 11.4Å, and DFDNB 3.0 Å). Under similar conditions, we now
show that, recombinant advillin also self-associates with DSS and somewhat with DFDNB but does not self-associate in the presence of EGS. These data imply a defined molecular structure of the advillin dimer with a particular spacing of the reactive groups in the cross-linked protein (~11 Å but less than 16 Å). Under reducing conditions in MDCK cells, we have previously shown that villin self-associates well with DFDNB, DSS and EGS (George et al 2007 J Biol Chem 282:26528-26541). Similarly, in our manuscript here, we show that under reducing conditions the cleavable cross-linker EGS leads to villin self-association (Figure 3C). Under similar experimental conditions, we now show that, advillin in MDCK cells also self-associates in the presence of both the cleavable (DFDNB) and the non-cleavable (DSS) cross-linkers. Recombinant advillin does not self-associate in the presence of EGS hence, we elected not to study advillin self-association in cells treated with EGS. These findings in MDCK cells support our hypothesis that a defined molecular structure with a particular spacing of the reactive groups in cross-linked advillin protein is required (~11.4 Å but less than 16.1 Å). Nonetheless, we also note that while recombinant advillin does not self-associate well in the presence of DFDNB, in MDCK cells DFDNB treatment cross-links advillin protein just as well as EGS. One conceivable explanation for this is that, in cells advillin can associate with F-actin and this may generate a different molecular structure in which the inter-molecular spacing between reactive groups of ~3.0 Å, can be achieved. We have previously shown that villin self-association is regulated by F-actin (George et al., 2013). It is possible that a similar molecular mechanism regulates advillin dimerization in cells, where DFDNB works well to promote advillin self-association because of the presence of high F-actin levels, these are conditions that cannot be mimicked when DFDNB is used to crosslink recombinant advillin protein. Our data
2. In previous studies from our laboratory, we have demonstrated self-association of recombinant full-length villin, full-length villin endogenously expressed in Caco-2 cells and full-length villin exogenously expressed in MDCK cells (George et al 2007 J Biol Chem 282:26528-26541). In this previously published study, we employed multiple controls (both positive and negative) to demonstrate the specificity of the cross-linking assay. We used villin mutants that lack the dimerization site and the villin homolog gelsolin to demonstrate the lack of self-association compared to full-length villin protein. These findings also revealed that despite significant structural and functional homology, villin and gelsolin differ in their ability to self-associate (George et al 2007 J Biol Chem 282:26528-26541). In contrast, we determined that under similar conditions ezrin, a protein known to self-associate, formed dimers and oligomers (Berryman et al 1995 J Cell Biol 131: 1231-1242). Based on these previous findings we feel confident about the specificity of the cross-linking approach used here to demonstrate the dimerization of villin and advillin proteins (Figure 3A-B). This information has been included in the revised manuscript (pages 10-11).

3. As suggested by the reviewer data presented in Figures 4 and 5 (which were Figures 3 and 4 in the previous submission of this manuscript) are now quantified. These new data demonstrate quantitative differences in the length and number of filopodia formed in the
presence of full-length and mutant villin and advillin proteins (Supplementary Figures 1-2 (A-B); pages 13-14). Additionally, these data show reduced localization of mutant proteins to these cell surface structures (Supplementary Figures 1-2 (C); pages 13-14). The ratio of full-length and mutant proteins to F-actin distribution within these structures were measured to make this determination. Data shown in Figs 4A and 5A demonstrate comparable levels of protein expression for full-length and mutant villin and advillin proteins. These data reveal that the differences in the cell surface protrusions seen with mutant villin proteins are unlikely to be due to differences in mutant villin or advillin protein to F-actin ratios when compared with FL villin or advillin to F-actin ratios in transfected cells. Together, these mutants identify actin-binding residues in villin and advillin headpiece that are required for F-actin bundling and for the assembly of long cell surface protrusions in cells.

**Reviewer #2**

**Major comments:**

1. Using synthetic peptides and transfection of CV-1 cells to express wild-type and mutant villin proteins Friederich et al (Friederich et al 1992 Cell 70: 81-92) demonstrated in vitro that a conserved COOH terminal cluster of amino acids (821KKEK824) are crucial for villin’s F-actin binding function and for its function in promoting growth of microvilli/filopodia when over-expressed in CV-1 cells. In their study, they demonstrated that individually as well as a double amino acid substitution in the motif KKEK (i.e. KEEE) impaired the ability of villin to bind F-actin and to promote microvilli/filopodia growth in CV-1 cells. Based on that, the authors concluded that all four residues within
this headpiece motif $^{821}$KKEK$^{824}$ of villin are required for its F-actin binding function and for filopodia growth in CV-1 cells. The significance of these residues on F-actin binding was determined using synthetic peptides, while the significance of these residues on F-actin bundling by villin was determined by overexpressing the wild type and mutant proteins in CV-1 cells in order to detect changes in microvilli/filopodia growth. However, in a subsequent manuscript, Friederich et al reported that substitutions of both K822E and K824E (KEEE mutant) in a full-length recombinant protein, did not prevent F-actin binding or F-actin bundling by the mutant villin protein when compared to full-length recombinant villin protein (Friederich et al., 1999). Although no statistical analysis was performed, the authors reported a small (~30%) decrease in the in vitro F-actin binding affinity of this mutant (KEEE) compared to wild type villin protein. However, prior to these findings by Friederich and colleagues, Doering and Matsudaira had reported that replacement of at least one of these residues (K824) with cysteine had little effect on the in vitro F-actin binding function of the villin headpiece protein (Doering and Matsudaira, 1996). This would indicate that not all the residues within the KKEK motif are required for F-actin binding by the villin headpiece. More importantly, subsequent analysis of the villin headpiece protein by Rossenu et al showed that only K821 within the $^{821}$KKEK$^{824}$ motif is an F-actin binding residue (Rossenu et al 2003 J Biol Chem 278: 16642-16650). Even though Friederich et al used full-length wild type and mutant villin proteins; their findings were reported before the structural analysis of Rossenu et al. was available. To integrate the functional data from Louvard’s group with the structural data available from the isolated headpiece, we analyzed the last three residues in the $^{821}$KKEK$^{824}$ motif (i.e. $^{821}$KAAA$^{824}$) for their role in the actin bundling function of villin. Like Doering and
Matsudaira we find that K824 is not required for the F-actin bundling function of villin. Like Rossenue et al, we find that only the first lysine (K821) in the KKEK motif is required for the actin bundling function of villin (Fig 1E). The only notable difference between our study and those reported by Friederich et al is that we substituted all three amino acids within this motif with the hydrophobic amino acid alanine (KAAA) while Friederich et al substituted two lysines, K822 and K824 within this motif, with the charged amino acid glutamic acid (KEEE). As suggested by the reviewer, this discussion has been included in the revised manuscript (pages 5-7; 9-10). Furthermore, as shown in Figure 2 (which was Figure 5 in the previous submission of this manuscript), with the exception of the first lysine (K821) the remainder of the KKEK motif is not well conserved in either villin proteins from other species, advillin proteins from other species, or in other actin bundling proteins that contain a villin headpiece-like domain e.g. supervillin, dematin. Based on that, and our findings with the villin mutants, we hypothesize that other than K813, no residue within the \textsuperscript{813}KKEK\textsuperscript{816} motif of advillin is likely to be required for F-actin bundling by advillin. This would coincide with the existing structural data on the isolated advillin headpiece obtained by Vermeulen et al (Vermeulen et al 2004 Protein Sci 13: 1276-1287). As suggested by the reviewer, this discussion has been included in the revised manuscript (page 10).

2. We have previously demonstrated that GST protein by itself does not bundle F-actin (George et al 2007 J Biol Chem 282:26528-26541). These data are also provided in our manuscript in Figures 1C and 1E which show no effect of the GST control on F-actin bundling. Additionally, all mutants shown in Figure 1 contain a GST tag and despite that,
they fail to bundle F-actin (Figures 1B-E) further validating our findings that GST by itself has no effect on the F-actin bundling assay. This information has been included in the revised manuscript (pages 8-9).

3. The band below the band indicated as monomer in Figure 3A (which was Figure 2A in the previous submission of this manuscript) are degraded fractions of full-length recombinant villin protein. For these studies, we cleaved the GST tag from GST-villin protein and the cleaved protein was purified using Glutathione Sepharose. In this manner, only cleaved recombinant villin proteins that lacks a GST tag, were obtained. Any uncleaved GST-villin protein or degraded fractions of villin protein containing the GST tag were removed using Glutathione Sepharose. Please note, we do not see a protein band at the expected size of GST-tagged full-length villin/advillin proteins ~120 kDa (Figure 3A). Additionally, the DMSO controls, which also contain these degraded protein fractions, show no protein dimerization, thereby excluding the significance of these degraded protein fractions in villin/advillin self-association (Figure 3A). The degraded protein bands appear more prominent in Figure 3A because it is a Western Blot. In contrast, the protein degradation bands seen in Figure 1B (below 120 kDa) are less prominent because it is a GelCode Blue stained SDS-PAGE gel.

4. As recommended by the reviewer, data shown in Figures 4 and 5 (Figures 3 and 4 in the previous submission of this manuscript) have been quantified. These new data are provided in the revised manuscript in Supplementary Figures 1-2.
5. As suggested by the reviewer, we have quantified the ratio of villin and advillin proteins to F-actin within the cell surface protrusions. As shown in Supplementary Figures 1 and 2, the mutant villin proteins other than the villin mutant KAAA have a lower protein: F-actin ratio within these filopodia (Supplementary Figure 1). Likewise, the mutant advillin to F-actin ratio within the filopodia is significantly reduced compared to full-length advillin protein (Supplementary Figure 2). Data shown in Figures 4A and 5A demonstrate comparable levels of protein expression for full-length and mutant villin and advillin proteins. These data reveal that the differences in the cell surface protrusions seen with mutant villin or advillin proteins are unlikely to be due to differences in mutant villin or advillin protein to F-actin ratios when compared with full-length villin or advillin protein to F-actin ratios. Together, these mutants identify actin-binding residues in villin and advillin headpiece that are required for F-actin bundling and for the assembly of long cell surface protrusions in cells.

**Minor comments:**

6. In the revised manuscript, the abstract has been edited to reflect more clearly that we used existing NMR data on villin and advillin headpiece proteins for our studies. We regret this lack of clarity.

7. As suggested by the reviewer, Figure 1A has been edited and now shows the villin/advillin core domain in addition to the headpiece region.
8. The concentration of F-actin (0.76 μM) used for the assays shown in Figures 1C and 1D is correct. This data is similar to that reported by us previously (George et al 2007 J Biol Chem 282:26528-26541). We submit that the intensity of the bands in this figure reflect the gel-staining method employed for these studies namely, GelCode Blue staining. The water wash used to develop the GelCode Blue staining increases the staining sensitivity by providing a clear background to the gel and makes weakly stained protein bands more visible. It is possible that with similar amounts of protein, the intensity of the bands will differ significantly with other gel-staining reagents such as conventional Coomassie Blue staining.
RE: Manuscript #E20-02-0158R
TITLE: “F-actin bundling sites are conserved in proteins with villin type headpiece domains.”

Dear Prof. Khurana:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Laurent Blanchon
Monitoring Editor
Molecular Biology of the Cell

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Dear Prof. Khurana:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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Eric Baker
Journal Production Manager
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Reviewer #1 (Remarks to the Author):

The authors have addressed all of my concerns and I support publication of this manuscript.

Reviewer #2 (Remarks to the Author):

The authors have addressed my comments satisfactorily.