THE
EMBO
JOURNAL

Manuscript EMBO-2010-76724

How a Single Residue in Individual β-Thymosin/WH2 Domains Controls their Functions in Actin Assembly

Dominique Didry, Francois-Xavier Cantrelle, Clotilde Husson, Pierre Roblin, Anna M Eswara Moorthy, Javier Perez, Christophe Le Clainche, Maud Hertzog, Eric Guittet, Marie-France Carlier, Carine van Heijenoort and Louis Renault

Corresponding author: Louis renault, CNRS

Review timeline:

Submission date:	09 December 2010
Editorial Decision:	25 January 2011
Revision received:	31 May 2011
Editorial Decision:	21 June 2011
Revision received:	21 October 2011
Additional Correspondence:	14 November 2011
Accepted:	18 November 2011

Transaction Report:

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

1st Editorial Decision 25 January 2011

Many thanks for your message and clarification about the relationship between your current study and the results in Hertzog et al. I have now had the chance to discuss the matter with my colleagues, including our Chief Editor Bernd Pulverer. We find your arguments reasonable, and given the overall interest expressed by the referees, we would like to invite you to submit a revised version of the manuscript - addressing all the points raised by the referees.

The controls highlighted by referees 1 and 3 are critical, as you recognise, and I would also encourage you to follow the suggestion of referee 3 point 4: to widen the relevance to other WH2 domains. As this referee states, additional point mutations would not be required, but analysis of the wild type domains would be very valuable - assuming this is feasible within the timeframe of the revision. I would also stress the necessity to clarify the text to make it explicit how these results relate to your previous work, and what the major advance is here. While the referees have not seen the response you sent me last week, potential publication of your manuscript will be contingent upon their being satisfied with your explanations on this front.

I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and it sounds as though you may be able to complete the necessary experiments well within this time. As a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Also, if - for any reason - you do foresee a problem in meeting the three month deadline, please let me know and we may be able to grant an extension.

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

With best wishes,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The WH2 repeat is a small actin interacting motif found in numerous monomer sequestering and filament assembly proteins. Despite the different activities, individual core WH2 motifs, comprised of a short N-terminal amphipathic helix and the WH2 signature sequence LKKT/V, were shown to interact similarly with the barbed end of the actin monomer by binding with their helix into a pocket between subdomains 1 and 3 of actin and then extending towards subdomain 2. While the small peptide TB4 containing one WH2 motif sequesters actin, the first domain of Ciboulout also containing one WH2 motif (Cib/D1) does not sequester actin, but instead promotes filament assembly in a profilin-like fashion. In this present study by Didry et al. the authors analyzed the properties of TB4/CidD1 chimeras by a combinatory approach including actin assembly assays, structure analyses and mutagenesis to identify critical elements specifying function of WH2 motifs. With Lys14, located within the short linker connecting the helix and the LKKT/V consensus sequence in TB4, they claim to have discovered the key residue controlling function of short WH2 domains in actin assembly. The crystal structure of actin-TB4 revealed that Lys14 of TB4 can establish a strong salt bridge to Glu-334 of actin, while in the corresponding position of Cib/D1, Gln27 can form only weaker hydrogen bonds to Ala144 and Glu334. This together with NMR and actin assembly assays performed at low and high salt led the authors to conclude that the third residue of the FxxxK linker controls the ionic strength dependence of the affinity for actin, and hence their different function in assembly. Along this line they identify a charged residue (Arg-54) in WIP containing an extended WH2 domain. Mutation of this residue to Glu abolishes the salt bridge and converts WIP from a sequestering protein into a profilin-like assembly factor. The manuscript is well written, the topic is of great interest to the field and I do not have a substantial criticism on the methodology of this study. However, I am confused as the authors have already claimed to have identified key residues in entirely different positions of TB4 and Cib/D1 responsible for sequestration or actin assembly in previous work (Hertzog et al., 2004, Cell). Using a similar approach, in that study they identified Thr20-Glu21 and Glu35-Gln36 in TB4, both of which are located C-terminal of the LKKT/V consensus sequence. This discrepancy needs be resolved and appropriately discussed before this paper can be considered for publication.

Issues to be addressed:

1) Important control experiments are missing. What is the activity of a TB4 mutant containing Gln instead of Lys in the third position of the FxxxK linker? And conversely, what is the activity of a Cib/D1 mutant containing Lys instead of Gln in the third position of the linker?

2) What is the functional consequence of mutations of Thr20-Glu21 and Glu35-Gln36 of TB4 into Ala as found in Cib/D1, and/or of changes of the corresponding Ala residues of Cib/D1 into those of TB4?

3) The shown alignment of extended WH2 domains in Figure 1 bottom is somewhat misleading and incomplete. The shown sequence of N-WASP encompasses the second WH2 motif and its C-terminal extension. The C-terminal extension of the first WH2 domain actually contains the Arg residue as shown for MIM and WIP (see Hertzog et al., 2004). Additionally, this Arg or a conserved Lys are also present in actobindin D1. Does a conversion in the central position of the linker in N-WASP or actobindin D1 (both Gly) to a Lys residue result in a switch of function and then allow sequestration of monomer?

Referee #2

This report is an impressively detailed and thorough examination of the interaction of WH2 domain sequences with actin, and goes a long way toward solving the paradox that closely-related WH2 sequences can either promote or inhibit actin polymerization. The combination of biochemical, hydrodynamic, and structural data provide a strong mechanistic basis for the observed biological activities. Overall, the presentation is clear and logical, both text and figures.

I would suggest a few points to clarify:

page 8, second paragraph, line 9, "amphipathic" is mis-spelled;

page 9, end of top paragraph, "comforted"?? Did they mean "confirmed"?

page 10, and again on page 18, "challenge"? investigate, test?

Several figures (4, S7, S8) show multiple experimental traces, presumably from multiple individual runs of the same experiment, but there is no explanation of the considerable variation between individual traces, or why it would not be appropriate to average the data. The authors should discuss this issue, at least briefly.

Referee #3

This manuscript continues from previous work of the same group, analysing the molecular details of β-thymosin/WH2 motifs and their interactions with actin. These actin monomer binding domains are conserved elements found in numerous actin regulatory proteins, yet they fulfil often strikingly different roles (actin nucleation, elongation, severing, and actin monomer sequestration or 'funnelling'). Despite the general importance of these domains for many different phases in actin dynamics, the molecular basis for their different functional effects has not been well understood. The emerging view is that differences are specified by the modular arrangement of the domains and/or differences in their amino acid composition.

In the current paper, the authors perform detailed biochemical and structural analyses of Tβ4 and Ciboulot, and chimeras of these proteins. Surprisingly, and in stark contrast to their earlier models, they find that the monomer-sequestering activity of Tβ4 is not derived from the additional C-terminal helix lacking in non-sequestering WH2 domains, but instead from the specific amino acid sequences of the linker regions connecting the actin binding segments of the WH2. Based on this information, they conclude that the effect of a specific WH2 motif on actin dynamics can be extrapolated from its amino acid composition rather than the presence/absence of the additional C-terminal helix as they previously suggested. Overall, the experimental design and the data are of high quality, but I have two major reservations about this work:

(1) I am deeply concerned that the data here directly contradict previously published data from the same group (in quite prominent journals), in which they showed that two amino acid residues located in the pointed end-binding C-terminal helix of thymosin β4 were found to be essential for sequestering. Instead, now they conclude that the C-terminal helix is not required for sequestering, and that only the amino acid composition of the linker is critical for sequestering. This is troubling, and has to be resolved and explained. And if the authors are to take the stand that the previous work was incorrect, I think it is necessary that the previous papers be retracted before the current paper is published.

(2) In several instances, there are key experiments missing needed to support the conclusions they are making (see below).

Specific suggestions:

1. It would be informative to examine the rates of nucleotide exchange on actin monomers bound to WH2 motifs/chimeras, since these parameters might give additional insight into the interaction of the peptides with the pointed end of the monomer.

2. A chimera should be made of Thymosin β4, in which the linker is replaced by that of CibD1. This is a key experiment to verify that indeed the linker, and not the C-terminal helix of Thymosin β4, is responsible for sequestering activity.

3. Some parts of the manuscript are quite complex and confusing how they were written. The authors should do a thorough editing with help from colleagues outside of their lab to clarify the text for non-specialists. Also, the authors should consider moving some paragraphs, e.g. on page 9, to the methods section.

4. To elevate the conclusions of the paper, one should go further, and not only analyze the WH2 motif from WIP, but also those from dictyostelium VASP, VopF-D3 and VopL-D3, as mentioned in the discussion on page 17, and compare their sequestering activities side-by-side with WH2 motifs which they predict not to sequester, e.g. VopL-D2 and VopF-D2. For this, they should perform steady state F-actin measurements and determine the KDs of the peptides for actin. This could be done quickly, and additional point mutations would not be required.

1st Revision - authors' response 31 May 2011

Referee 1:
Issues to be addressed:

1) Important control experiments are missing. What is the activity of a TB4 mutant containing Gln instead of Lys in the third position of the FxxxK linker? And conversely, what is the activity of a Cib/D1 mutant containing Lys instead of Gln in the third position of the linker?

We have performed these controls. Data are shown in Figure S4. The mutation Q27K in CibD1 causes a mild (2-fold) increase in its affinity for G-actin, yet is sufficient to switch the profilin-like activity of wt CibD1 into a sequestering activity in steady-state measurements of actin assembly, comforted by barbed end growth assays showing a total inhibition of actin assembly at barbed ends by CibD1-Q27K (Table 1). The mutation K14Q in Tb4 leads to a large (17-fold) decrease of its affinity for G-actin. In steady state measurements, the sequestering behaviors of Tb4 K14Q at pointed and barbed ends correspond to a Kd of ~35 and 58 mM, respectively , suggesting that the mutated Tß4 has gained some ability to support barbed end assembly. Consistently, barbed end growth was not totally inhibited at saturation by the peptide and a value of k_+ of 0.7 $\mu M^{-1}.s^{-1}$ was derived for the association rate constant of Tß4-K14Q:actin to the barbed ends (Table 1). However the very low affinity and low value of k+ make it difficult to assess whether the sequestering function has been almost abolished or converted into a detectable profilin function. Our data suggest that the K14Q mutation in Tß4 greatly weakens the affinity of Tß4 because it is adjacent to the N-terminal α-helix which itself binds weakly to the barbed face of G-actin due to its short size (residues 5-11) (Fig.1A, Table 1). These explanations have been included with Figure S4 in sup. Mat. and in the results page 7 line 32.

2) What is the functional consequence of mutations of Thr20-Glu21 and Glu35-Gln36 of TB4 into Ala as found in Cib/D1, and/or of changes of the corresponding Ala residues of Cib/D1 into those of TB4?

We had previously shown that mutations of Thr20-Glu21 and Glu35Gln36 of TB4 into Ala/Ser as found in Cib/D1 converted Tß4 into a functional homolog of profilin (Hertzog et al., 2004). We have added new data (Figure S11) showing that when the same mutations are introduced in chimera 2, which comprises the long N-terminal α-helix of CibD1 followed by Tß4 sequence and behaves as

© European Molecular Biology Organization

a high affinity sequesterer, they fail to convert chimera 2 into a functional homolog of profilin. Thus, mutations/sequence divergence in the central and C-terminal region of ßT/WH2 domains (the conclusion is shown to apply to both Tß4, CibD1 and WIP) are less efficient to control the function than mutations affecting the strength of strong electrostatic bonds close to the central LKKT/V motif. These points are raised in the additional data (Figure S11) and are commented in the Discussion page 14 line 20.

3) The shown alignment of extended WH2 domains in Figure 1 bottom is somewhat misleading and incomplete. The shown sequence of N-WASP encompasses the second WH2 motif and its C-terminal extension. The C-terminal extension of the first WH2 domain actually contains the Arg residue as shown for MIM and WIP (see Hertzog et al., 2004). Additionally, this Arg or a conserved Lys are also present in actobindin D1. Does a conversion in the central position of the linker in N-WASP or actobindin D1 (both Gly) to a Lys residue result in a switch of function and then allow sequestration of monomer?

The sequence alignment of ßT/WH2 domains in figure 1A now includes the first WH2 domain of N-WASP. This domain contains an Arg residue at a position homolog of R749 in MIM and R54 in WIP, followed, like in MIM, by a short C-terminal region that does not extend to nor can cap the pointed face of actin. Consistently neither the first WH2 of N-WASP nor MIM sequester G-actin.

The linker of N-WASP or actobindin D1, like in most WH2 domains, is two-amino acids shorter than in ßT domains and thus adopts a straight coil conformation between the N-terminal helix and the central LKKT motif. Known WH2:G-actin structures show that the side chains of the residues of such a short linker are too far from conserved Glu/Asp of G-actin to form a salt bridge like in Tß4.

Referee 2:

I would suggest a few points to clarify:
page 8, second paragraph, line 9, "amphipathic" is mis-spelled;
page 9, end of top paragraph, "comforted"?? Did they mean "confirmed"?
page 10, and again on page 18, "challenge"? investigate, test?

Several figures (4, S7, S8) show multiple experimental traces, presumably from multiple individual runs of the same experiment, but there is no explanation of the considerable variation between individual traces, or why it would not be appropriate to average the data. The authors should discuss this issue, at least briefly.

We have corrected misspelling and inappropriate terms on page 8, 9, 10.
The legends of the figures (4 + S7 and S8 now corresponding to S9 and S10, respectively) that show multiple experimental traces have been clarified. We in particular specified that each experimental trace corresponds to the variations of the intensity of NMR HSQC ^{1}H-^{15}N cross-peaks corresponding to a specific N-terminal or C-terminal residue of chimera 1 or 2 in the unbound or actin-bound state as a function of KCl concentration. The descriptions of the experiments have been extended in the supplementary materials.

Referee 3:

major reservations about this work:

(1) I am deeply concerned that the data here directly contradict previously published data from the same group (in quite prominent journals), in which they showed that two amino acid residues located in the pointed end-binding C-terminal helix of thymosin β4 were found to be essential for sequestering. Instead, now they conclude that the C-terminal helix is not required for sequestering, and that only the amino acid composition of the linker is critical for sequestering. This is troubling, and has to be resolved and explained. And if the authors are to take the stand that the previous work was incorrect, I think it is necessary that the previous papers be retracted before the current paper is published.

This point is similar to issue 2 of referee 1 (see our answer above). We emphasize that our present data do not contradict but confirm and expand the initial main points made by Hertzog et al. (2004). We do confirm that the control of the dynamics of interaction of only the central and C-terminal regions of ßT/WH2 domains is key in the functional regulation of these peptides. We make a step forward in understanding the structural basis for this control, as follows. In previous views, the central and C-terminal regions of ßT and WH2 domains were thought to be regulated by making with the pointed face of actin either stable interactions in sequestering β-thymosin domains (Domanski et al, 2004; Irobi et al, 2004) or more or less loose interactions in assembly promoting ßT/WH2 domains (Aguda et al, 2006; Chereau et al, 2005; Ducka et al, 2010; Irobi et al, 2004; Lee et al, 2007; Hertzog et al, 2004; Rebowski et al., 2010). The present structural and functional analyses in physiological versus low ionic strength conditions provide additional insight into the structural features that generate remarkable subtleties in functional regulation of these peptides: The size of the N-terminal amphipathic helix plays an important role in defining the weight that mutations in the central and C-terminal regions have on the function in actin assembly. Weakly binding short size N-terminal helices allow these mutations to control the function and counteract the effect of the electrostatic interactions of the linker region. In contrast, the longer more strongly interacting N-terminal helix of CibD1 does not allow the mutations in the central/C-terminal regions to effectively abolish the sequestering function (Compare chimeras 2 and 4 in Figure S11). In turn, replacing the NQD sequence of the linker of CibD1 by the corresponding DKS sequence of Tß4 or the point mutation Q27K at the critical third position of its linker is sufficient for the central/C-terminal region of CibD1 to more stably interact with actin subdomains 2 and 4, converting D1 into a sequesterer.
These clarifications have been added with Figure S11 in Supplementary Material and in the Discussion page 14 line 20.

(2) In several instances, there are key experiments missing needed to support the conclusions they are making (see below).

Specific suggestions:

1. It would be informative to examine the rates of nucleotide exchange on actin monomers bound to WH2 motifs/chimeras, since these parameters might give additional insight into the interaction of the peptides with the pointed end of the monomer.

Profilin-like and sequestering ßT/WH2 peptides both slow down nucleotide exchange on actin monomers with quantitative differences that do not correlate with their different functions in actin assembly nor with significant sequence truncations. For example the profilin-like MIM WH2, which lacks a C-terminal α-helix, inhibits nucleotide exchange to the same extent as Tß4 and a truncated version of Tß4: Tß4 residues 2 to 33, which lacks the C-terminal α-helix, inhibits nucleotide exchange similarly to full-length Tß4 after accounting for the 4-fold-lower actin-binding affinity of this truncated peptide (Chereau D. et al., Proc Natl Acad Sci U S A. 2005 Nov 15;102(46):16644-9). Similarly, the profilin function is conserved in plant profilins which do not increase the rates of nucleotide exchange on actin, further suggesting that there is no correlation between the profilin function in the barbed end assembly and the rates of nucleotide exchange on actin (Perelroizen I et al., J Biol Chem. 1996 May 24;271(21):12302-9). Examining the rates of nucleotide exchange on G-actin thus does not allow to distinguish between profilin-like and sequestering activities or between different C-terminal interactions of ßT/WH2 peptidic chains.

2. A chimera should be made of Thymosin β4, in which the linker is replaced by that of CibD1. This is a key experiment to verify that indeed the linker, and not the C-terminal helix of Thymosin β4, is responsible for sequestering activity.

This comment is similar to point 1) of referee 1 (new Figure S4 in sup. Mat. commented in the results page 7 line 32). The analysis of Tβ4 with the point mutation K14Q at the third position of the FxxxK linker, causing disruption of the salt bridge between K14 and E334 of actin. The very large decrease in affinity of the mutated Tß4 makes it difficult to assess whether the sequestering function has been almost abolished or converted into a profilin function. However both the reciprocal experiment (conversion of CibD1 into a sequesterer by mutation Q26K, the C-terminal helix being unchanged) and the opposite functions of chimeras 1 and 2 which harbor the same C-

terminal helix as Tß4 strongly argue that the C-terminal helix does not determine the function. Additionally, the sequence of WIP WH2 is not predicted to contain a C-terminal helix and is poorly homologous to Tß4, yet it displays a sequestering activity which is even more efficient than by Tß4.

Thus a long C-terminal region in ßT/WH2 peptides is strictly required for inducing sequestration by capping interactions with G-actin pointed face but it can accommodate quite different sequences, including for example Tb4, Ciboulot D1 (chimera 2, 3, 4 and CibD1-Q27K), or WIP WH2 C-terminal sequences.

3. Some parts of the manuscript are quite complex and confusing how they were written. The authors should do a thorough editing with help from colleagues outside of their lab to clarify the text for non-specialists. Also, the authors should consider moving some paragraphs, e.g. on page 9, to the methods section.

We have clarified the manuscript as suggested.

4. To elevate the conclusions of the paper, one should go further, and not only analyze the WH2 motif from WIP, but also those from dictyostelium VASP, VopF-D3and VopL-D3, as mentioned in the discussion on page 17, and compare their sequestering activities side-by-side with WH2 motifs which they predict not to sequester, e.g. VopL-D2 and VopF-D2. For this, they should perform steady state F-actin measurements and determine the KDs of the peptides for actin. This could be done quickly, and additional point mutations would not be required.

This is indeed a very good suggestion and we do proceed in such exciting directions to derive a large scale comparison of these and other WH2 domains. This effort is required, and data are being collected, to get a comprehensive view of the function of all sequence elements of ßT/WH2 domains, but we think this complete analysis is beyond the scope of the present study. The following data support however the relevance of our conclusions in other ßT and WH2 domains and have been added: in ddVASP, a short truncated version from its WH2 (containing a FxKxx linker adapted for a salt bridge, Fig. 7) binds better G-actin than its human counterpart (containing no such linker, Fig. 7) and this higher affinity plays a role in promoting fast filament elongation by ddVASP (Breitsprecher D., et al., EMBO J. 2011 Feb 2;30(3):456-67). Most importantly the experiments and conclusions on Tß4, CibD1 ßT and on WIP WH2 domain validate the presented structural mechanisms in the two subfamilies (ßT and WH2) of intrinsically disordered actin-binding peptides. The results presented here constitute a solid basis to investigate the functions of ßT and WH2 domains inserted in many other modular proteins using limited site-directed mutagenesis or chimeric proteins. These explanations have been included in the discussion in the last paragraph from page 15 line 21 to page 16 line 30.

2nd Editorial Decision 21 June 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-76724. It has now been seen again by referees 1 and 3, whose comments are enclosed below. As you will see, referee 1 is now satisfied with the revision, but referee 3 still raises a number of issues with the revised version. His/her first major point can be dealt with by appropriate discussion, but the second concern relates to experimental data requested in the first round of review and not provided in the revision.

The referee suggested that you test additional WH2 domains directly in your assays to provide better evidence that the nature of the linker region is indeed predictive of the sequestering vs. assembly-promoting activity of these domains. You argue in your point-by-point response that this lies beyond the scope of the current manuscript, but I have to say that I agree with the referee that such analysis would be important to demonstrate that your model is generalisable. You state in the discussion that you have preliminary data that VopF3 does indeed have sequestering activity, as predicted, and so it would minimally be essential only to test one additional - predicted assembly-promoting - WH2 domain to satisfy the referees' request. Of course, if you do have data on more domains, then this would further strengthen your argument, but at a minimum, I do have to insist that you include the data on VopF3 and on one more WH2 domain. Given that these experiments should be straightforward, we can permit an exceptional second round of revision to allow you to incorporate these data, but I would stress that a positive outcome here will be contingent upon the addition of these data - to the satisfaction of the referee.

I also have a few points from the editorial side:
- Please can you make sure you include the PDB accession codes in the revised version of your manuscript? We require the data to be deposited at the time of acceptance.
- Several of the presented panels appear to show data generated from a single experiment. I assume that these are representative of a number of independent replicates, but this needs to be stated in the figure legend, and the 'n' number given. If possible, it would of course be better to show averaged data and error bars from several replicates. Similarly, you show error bars in figure 4, but do not mention in the legend what these represent.
- Please can you include an 'Author Contributions' statement (below the acknowledgments)?

I look forward to receiving your revised manuscript, and please don't hesitate to get in touch if you have any questions or comments about the revision.

Best wishes,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The authors have improved the study by the addition of new experiments and controls, and have satisfactorily addressed most of my concerns raised in the original manuscript. I have no further objections and support publication in the EMBO Journal.

Referee #3

The response by the authors was insufficient. They replied well to my concern about some of the apparent contradictions between their current study and Hertzog et al., 2004. It is now more clear from the manuscript that both the N-terminal pointed end-binding region and C-terminal helix and linker region contribute to WH2 activity. However, they have refused to address another key discrepancy - in Hertzog et al., 2004, it was stated that "The strength of interaction of their (the peptide´s) C-terminal region with actin solely determines whether they prevent or allow the pointed end of the actin molecule to associate with the barbed end of a filament". By comparing the sequences of the C-termini of different WH2 motifs (Figure 6B in Hertzog et al., 2004), the authors predicted sequestering or assembly-promoting functions of distinct WH2 motifs. However, by looking at the data from the present manuscript I find that chimera 1 displays an assembly promoting activity - contrary to the proposed model back in 2004, which predicted a sequestering activity. Chimera 3 however displays sequestering activity when assembly promoting activity was predicted. Thus, the predictions made in Hertzog et al., 2004 (Figure 6B) are not consistent with the data in the current study for one WH2 domain. I appreciate that predictions based on models and their assumptions can be wrong, but it should be clearly stated in the text.

More importantly, this example illustrates the importance of direct tests being performed to validate such predictions - and to avoid similar future mistakes. The authors need to experimentally test at least two more WH2 motifs side-by-side in their own hands (e.g. the previously mentioned WH2 motifs of VASP, VopF or VopL peptides, or Tetrathymosin D3 and D4) rather than simply predicting their function. This will quickly reveal whether or not the model is correct for other WH2 motifs - which would be key for publication in EMBOJ. This is also very important for the scientific community, since WH2 motifs are ubiquitous actin binding motifs present in a large variety of proteins, and many research groups (without the tools to perform such rigorous analyses as presented here) rely on predictions from studies such as this one to draw conclusions on protein function.

In the absence of this test, this paper describes a single phenomenon rather than a general mechanism. There is a critical distinction. I do not see why the authors have been reluctant to

perform a few additional routine steady-state experiments (as shown e.g. in Supplementary Figure 4) with two more WH2 motifs in order to confirm the proposed model. Clearly, this is not beyond the scope of the paper, and if they are proposing a general mechanism of WH2 domain binding, the analysis needs to be extended to multiple WH2 domains.

2nd Revision - authors' response 21 October 2011

Detailed response to reviewer 3 comments:

The response by the authors was insufficient. They replied well to my concern about some of the apparent contradictions between their current study and Hertzog et al., 2004. It is now more clear from the manuscript that both the N-terminal pointed end-binding region and C-terminal helix and linker region contribute to WH2 activity. However, they have refused to address another key discrepancy – in Hertzog et al., 2004, it was stated that "The strength of interaction of their (the peptide's) C-terminal region with actin solely determines whether they prevent or allow the pointed end of the actin molecule to associate with the barbed end of a filament". By comparing the sequences of the C-termini of different WH2 motifs (Figure 6B in Hertzog et al., 2004), the authors predicted sequestering or assembly-promoting functions of distinct WH2 motifs. However, by looking at the data from the present manuscript I find that chimera 1 displays an assembly promoting activity - contrary to the proposed model back in 2004, which predicted a sequestering activity. Chimera 3 however displays sequestering activity when assembly promoting activity was predicted. Thus, the predictions made in Hertzog et al., 2004 (Figure 6B) are not consistent with the data in the current study for one WH2 domain. I appreciate that predictions based on models and their assumptions can be wrong, but it should be clearly stated in the text.

Our studies previously published in Herzog et al. in 2004 focused on the role of the C-terminal segment of tβ domains, and clearly showed that, in the case of tβ4, charged residues in the central and C-terminal segment of the protein indeed determined the sequestering or elongating function of the domain. This enabled us to correctly predict at that time the function of other domains, as shown in the Figure 6B of the paper.

We agree that the results obtained in Hertzog et al. did not allow one to predict correctly the function of the two chimeric proteins CH1 and CH3. This result actually motivated the study proposed here, since it appeared from the analysis of chimeras 1, 2 and 3, that for these peptides that have long N-terminal helices, the problem was more complex. We inserted the fact that our first results in Hertzog et al. did not allow one to predict correctly the function of the chimeras in the discussion of the paper.

One of the main idea of the Hertzog et al. paper, that is the control of the sequestering versus elongating function through the level of locking of the pointed end of actin by the C-terminal fragment of the domains is actually confirmed by the present study. We show here that this lock is not only controlled by the direct interaction of the C-terminal fragment, but also by the global interaction strength, and, more strikingly, by an electrostatic interaction lower down the sequence. The fact that the formation, or not, of a salt bridge, located before the C-terminal end, controls the dynamics and thus the level of protection of the pointed end of actin, allowing or not the elongation at the barbed end of the filament is clearly new. More generally, our results put forward the general idea that some specific elements distributed along the whole primary sequence jointly contribute to the function of the intrinsically disordered domain. We have clarified the text accordingly and added further explanations especially in the discussion page 15-line 24.

More importantly, this example illustrates the importance of direct tests being performed to validate such predictions - and to avoid similar future mistakes. The authors need to experimentally test at least two more WH2 motifs side-by-side in their own hands (e.g. the previously mentioned WH2 motifs of VASP, VopF or VopL peptides, or Tetrathymosin D3 and D4) rather than simply predicting their function. This will quickly reveal whether or not the model is correct for other WH2 motifs - which would be key for publication in EMBOJ. This is also very important for the scientific community, since WH2 motifs are ubiquitous actin binding motifs present in a large variety of proteins, and many research groups (without the tools to perform such rigorous analyses as presented here) rely on predictions from studies such as this one to draw conclusions on protein

function.
We have added in the results page 11 line 28 the new data on Tetrathymosin D3, D4 and D1 with the new figures 5A,B and Sup. Fig.12. The supplementary analysis is in agreement with our view on the strong impact of a FxKxx linker on the function of different ßT sequences. Here is the analysis added page 11, line 28: The *C. elegans* Tetrathymosinß (TT) protein displays both G-actin sequestering and filament binding capacity via its 4 ßT repeats (Van Troys et al, 2004). Only the fourth ßT domain of Tetrathymosinß (TTD4) contains a FxKxx linker appropriate for a sequestering function (Figure 7). To see if a FxKxx linker could further control the function with other ßT sequences than Tß4 and CibD1-3, we analyzed TTD4 with TTD1 and TTD3. The latters do not display the FxKxx linker signature and the three ßT correspond in TT to the three most divergent ßT sequences from Tß4. In line with our view on the strong impact of the linker on the function of different ßT sequences, TTD4 displayed a poor ability to support barbed end assembly despite its truncated Cterminal region compared to Tß4 sequence. At physiological ionic strength, all three ßT bound weakly G-actin, which made difficult to assess their respective functions in Fbuffer except for TTD4 (Figure S12). Their differences in actin assembly were thus highlighted at 25mM KCl where the affinities of the three ßT for G-actin are increased. TTD3 affinity remained however too low to impact significantly actin assembly as isolated domain. At low ionic strength, TTD1 and TTD4 totally inhibited pointed end growth like Tß4 or CibD1 (Figure 5A). Barbed end growth was only modestly inhibited at saturation with TTD1, consistent with a profilin-like function, and a value of k+ of 4 µM-1.s-1 was derived for the association rate of TTD1:actin to the barbed end (Figure 5B). In contrast, the behavior of TTD4 was consistent with a sequestering activity and a total inhibition. At last we have added in the discussion page 17, line 17 the new reference (Yu et al., (2011) Nat Struct Mol Biol. 18(9):1068-74) which establishes the sequestering behavior of VopL third WH2 domain by observing its inhibitory effect on the nucleation activity of VopL C-terminal dimerization domain.

Additional Correspondence 14 November 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-76724R1. It has now been seen again by referee 3, whose comments are enclosed below. As you will see, he/she still has some reservations about how your data fit with earlier work, but despite this still finds the study to be a valuable contribution and recommends publication. I am therefore pleased to tell you that we will be able to accept your manuscript to be published in EMBOJ.

However, before we can accept the paper, I do just have a couple of issues from the editorial side - regarding statistics on your data:
- Figures 1C, 2E and 5 all appear to show the results of a single experiment, rather than averages of replicates. Is this the case? It needs to be clearly stated in the figure legends that these data panels show a single representative experiment, and how many times the experiment was repeated.
- In figure 3E, you show error bars, but do not state what these represent.
- In figure 4, you need to give the 'n' number.

If you can make these various clarifications in the figure legend, and then send the manuscript text back by email, we should then be able to accept the paper without further delay.

REFEREE REPORT

Referee 3:

The revised manuscript adequately addresses most of my previous concerns. I still have a major concern about the present work contradicting previous work from the same lab. However, there is a wealth of detailed structure/function data provided, which will be useful on many levels to the field, and thus deserves to be published. This is despite the fact that the paper leaves me more confused than ever about what is the true relationship between different WH2 domains' interactions with actin and their effects on actin dynamics.